Part One
Fundamentals of Chemokines and Chemokine Receptors

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

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Structural Aspects of Chemokines and their Interactions with Receptors and Glycosaminoglycans

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1.1
Introduction

Chemokines are a large subfamily of cytokines (~50 in humans) that can be distinguished from other cytokines due to several features. They share a common biological activity, which is the control of the directional migration of leukocytes, hence their name, *chemoattractant cytokines*. They are all small proteins (approx. 8 kDa) that are highly basic, with two exceptions (MIP-1α, MIP-1β). Also, they have a highly conserved monomeric fold, constrained by 1–3 disulfides which are formed from a conserved pattern of cysteine residues (the majority of chemokines have four cysteines). The pattern of cysteine residues is used as the basis of their division into subclasses and for their nomenclature. The first class, referred to as CXC or α-chemokines, have a single residue between the first N-terminal Cys residues, whereas in the CC class, or β-chemokines, these two Cys residues are adjacent. While most chemokines have two disulfides, the CC subclass also has three members that contain three. Subsequent to the CC and CXC families, two additional subclasses were identified, the CX_3C subclass [1, 2], which has three amino acids separating the N-terminal Cys pair, and the C subclass, which has a single disulfide.

The first chemokine, PF-4, was identified in 1977 [3] but it was not for almost a decade that other members of the family started to emerge, with the discovery of the proinflammatory chemokines: IP-10 was identified in 1985 as a protein showing homology to PF4 [4], while IL-8 and the MIP-1 proteins were isolated in the late 1980s as active protein from tissues or culture supernatants. The neutrophil chemoattractant, IL-8, was purified from culture supernatant of stimulated blood monocytes [5] and the monocyte chemoattractants MIP-1α and MIP-1β were purified from LPS-stimulated mouse macrophages [6]. The primary amino acid sequence of these chemokines rapidly led to the identification of the highly conserved four-cysteine motif described above and also allowed their classification into the two principal subclasses. The number of chemokines then grew rapidly

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through homology cloning using the conserved motifs, but the real explosion in the identification of members came from EST database searches [7]. Initially, chemokines were given names usually associated with their activity; for example, the MIP-1 proteins were discovered as "*m*acrophage *i*nflammatory *p*roteins". Similarly, PF-4 (platelet factor IV) was a factor produced from platelets. However, since members of the family were often identified concomitantly by different laboratories resulting in different names, a systemic nomenclature was introduced in 2000 in order to introduce harmonization [8]. In this nomenclature, the ligands are named according to subclass (CC, CXC, C, CX3C) followed by L for ligand and a number. Under this nomenclature IL-8 became CXCL8 while MIP-1α became CCL3. This nomenclature was created for human chemokines based on their genomic localization, but was rapidly "pirated" for the mouse chemokines, since even prestigious journals insisted that the new nomenclature be applied to the mouse chemokines! Interestingly certain chemokines are not found in both the human and mouse systems. For instance CXCL8 does not exist in the mouse, and the equivalent of several mouse chemokines such as lungkine and MCP-5 (CXCL15 and CCL12, respectively), have not been identified in humans (as shown in Table 1.1), which shows the old and new nomenclatures for human chemokines. In the rest of the chapter, we refer to chemokines by their new nomenclature.

Initial support for the division of chemokines into the α (CXC) and β (CC) subclasses was not only structural, but also based on biological activity as it described leukocyte specificity. The discovery that chemokine receptors were seven transmembrane spanning G protein-coupled receptors (GPCR) in the early 1990s [9, 10] was extremely important for the pharmaceutical industry as it presented a novel target class in the GPCR family which represent up to 60% of the targets of marketed medicines. The initial hope was that individual leukocyte populations would express a single chemokine receptor, which held firm until the cloning of the third CXC receptor, CXCR3 [11]. Until this point, the CXC chemokines were thought to be responsible principally for neutrophil recruitment and were therefore implicated in acute inflammation, while CC chemokines recruited other leukocyte types and were thus involved in chronic inflammation. However CXCR3 is mainly expressed on activated T cells, and its ligands were initially identified as IFNγ inducible polypeptides and are therefore pivotal in chronic inflammatory disorders. The subsequent identification of CXCR4 and CXCR5, as well as several CC chemokine receptors and their respective ligands, then introduced yet another concept in chemokine biology – that chemokines could be further subdivided into two broad classes on the basis of: (i) those that are inducible and therefore involved in inflammation and (ii) those that are constitutively expressed and are involved in leukocyte homing.

This chapter concentrates on the structure of chemokines and their receptors and how these aspects may be related to their biology. Understanding the relationship between the structure and function of chemokines has lead to ideas of how chemokines can be modified to produce analogs that are useful for modifying disease, in animal models and perhaps in man in the future.

Table 1.1 The old names and the new systematic nomenclature of the human chemokines are listed side by side and grouped into their respective CXC, XC, CX3C and CC families. For example, I-309 (old) is now referred to as CCL1 (new). Mouse chemokines for which no human homologs has been identified are shown in parentheses.

Old name	Systematic nomenclature	Old name	Systematic nomenclature
Gro-α	CXCL1	I-309	CCL1
Gro-β	CXCL2	MCP-1/MCAF	CCL2
Gro-γ	CXCL3	MIP-1α	CCL3
PF4	CXCL4	LD78	CCL3L1
ENA-79	CXCL5	MIP-1β	CCL4
GPC-2	CXCL6	RANTES	CCL5
NAP-2	CXCL7	(C10, MRP-1)	(CCL6)
IL-8	CXCL8	MCP-3	CCL7
Mig	CXCL9	MCP-2	CCL8
IP-10	CXCL10	(MRP-2, CCF18, MIP-1γ)	(CCL9,CCL10)
I-TAC	CXCL11	Eotaxin	CCL11
SDF-1α/β/δ/γ/ε/φ	CXCL12	(MCP-5)	(CCL12)
BCA/BLC	CXCL13	MCP-4	CCL13
BRAK/bolekine	CXCL14	HCC-1	CCL14
(m Lungkine)	(CXCL15)	HCC-2/Lkn/MIP-1δ	CCL15
	CXCL16	HCC-4/Lec	CCL16
		TARC	CCL17
Lymphotactin-1	XCL1	DC-CK1/PARC	CCL18
Lymphotactin-2/SCIM-1β	XCL2	MIP-3b/ELC/Exodus-3	CCL19
		MIP-3a/LARC/Exodus-1	CCL20
Fractalkine/neurotactin	CX3CL1	6Ckine/SLC/Exodus-2	CCL21
		MDC/STCP-1	CCL22
		MPIF-1/Ckbeta-8	CCL23
		MPIF-2/Eotaxin-2	CCL24
		TECK	CCL25
		Eotaxin-3	CCL26
		CTACK/ILC	CCL27
		MEC	CCL28

1.2 Receptor–Ligand Interactions

The classification of chemokine receptors is based on the ligands they bind, in other words CXC receptors bind CXC ligands, CC receptors bind CC ligands and so on, as shown in Figure 1.1. Chemokine receptors have been identified that bind chemokines but do not signal. One of these, the Duffy antigen receptor for chemokines (DARC) is a promiscuous chemokine receptor expressed on erythrocytes that binds both CC and CXC ligands [12]. In contrast, the decoy receptor D6 only binds CC chemokines [13]. Thus, with some exceptions like DARC, the chemokine system is specific with respect to the binding pattern, in that chemokines in each class do not

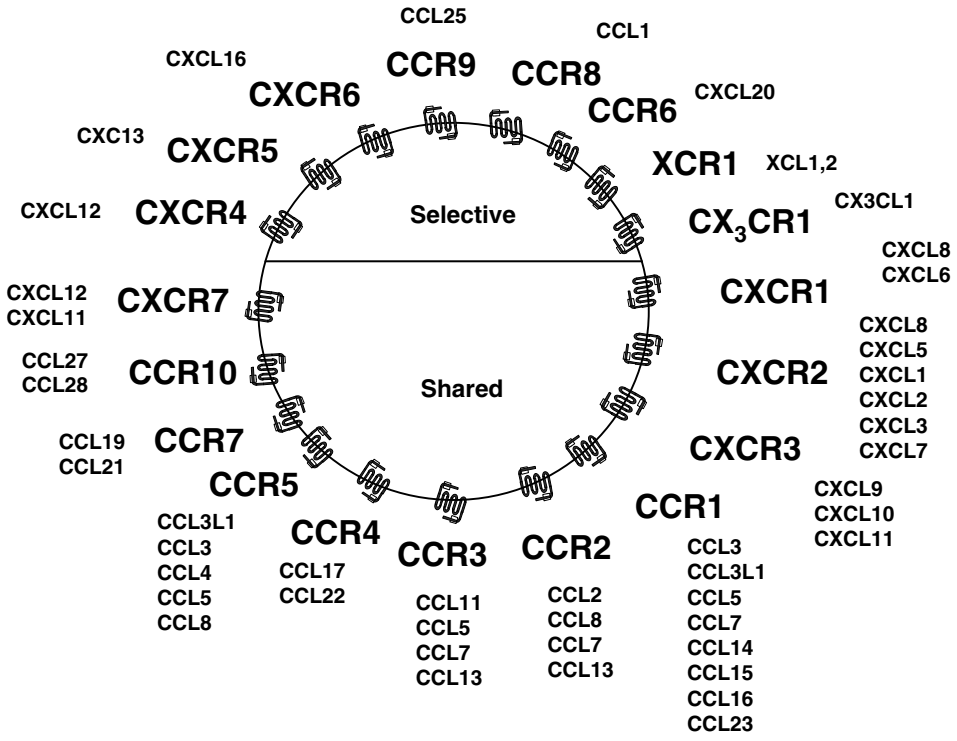

Figure 1.1 This diagram shows the pairing of chemokine receptors with their respective ligands. For example, CCL2, CCL8, CCL7 and CCL13 are all ligands of the receptor, CCR2. Some receptors like CXCR4 are much more selective and have a single ligand.

bind to receptors of another class. However, binding across classes has been demonstrated with antagonists. Thus CXCL9, CXCL10 and CXCL11, the agonists of CXCR3, also bind to CCR3 as antagonists and inhibit Th2 cell migration [14]. Interestingly a chimera consisting of the first eight residues of CCL11 (and the remainder consisting of CXCL11) bound CCR3 more strongly than the parental chemokines. CCR3 can also be antagonized by CCL18, despite the fact that the receptor for CCL18 remains unidentified to date [15]. Viruses have also adopted deviations from classical chemokine receptor pharmacology. For example the virally encoded chemokine receptor, US28, does not demonstrate reciprocal heterologous competition like most chemokines/receptors. Instead, one of its ligands, CX3CL1, cannot be competed by certain CC chemokines, whereas these CC chemokines are all displaced by CX3CL1 [16]. We believe that the study of the virally encoded members of the chemokine system will teach us a great deal about the intricacies involved in chemokine/receptor interactions, since viruses have produced chemokine ligands, such as vMIP-II, that can bind across chemokine receptor subclasses.

Beyond the basic rule of subclass selectivity (with the exceptions noted), the binding patterns of the chemokine system is far from simple! First, the assignment of

receptor-ligand pairs arises from *in vitro* assays, and one should be aware that the situation *in vivo* may be different due to factors that cannot be captured *in vitro*. Second, the majority of receptor/ligand interactions are not specific in that several receptors bind more than one ligand – in fact only about one-third are specific single ligand binders to date. Third, the reason that this statement is qualified by "to date," is that as the identification of new ligands continued, absolute specificity has tended to disappear, although the question remains as to whether there are additional ligands to be identified. CXCR1 was classified as a specific receptor for CXCL8 for seven years, until CXCL6 was identified as a ligand [17]. As an extreme example, CCR1 binds at least eight ligands. The situation is further complicated by the fact that certain chemokines are ligands of more than one receptor, which is best exemplified by CCL5 which binds to CCR1, CCR3 and CCR5.

However, the biology that has emerged over the past decade or so has identified a broad definition which supports the classification of selective versus shared receptors. The selective receptors have been shown to generally correspond to those which are constitutively expressed and are involved in development and homeostasis. In contrast, the shared receptors are those which are inducible and associated with inflammatory disease [18]. The fact that the shared receptors are the "villains" in disease makes the task of understanding how to target them a challenge, particularly if one is interested in using neutralizing antibodies against the ligands. Intuitively one would suggest that a small molecule inhibitor of the receptor would be the chosen strategy, or alternatively a neutralizing receptor antibody, but neither of these strategies is that simple. Therefore neutralization of a prominent ligand could be a successful strategy – one could suggest CCL2 for CCR2 or CXCL10 for CXCR3? However, it is not always easy to establish which ligand is the most potent and has the highest affinity for a certain receptor. This is well illustrated by CCR5, and a comparison of the rank order of the published potencies of its ligands. Using a calcium mobilization assay, the rank order potency of ligands on CCR5 expressed in CHO cells was reported as CCL5 > CCL4 > CCL3 [19] whereas in RBL cells stably transfected with CCR5, the rank order was CCL5 > CCL4 = CCL3 [20] and in HEK293/CCR5 transfectants the order was different again, with CCL3 > CCL5 > CCL4 [21]. However in the third example, the form of CCL3 used was the allelic variant, CCL3L1 (LD78β instead of LD78α), which has a Pro instead of a Ser residue at position 2 and two S/G switches (Figure 1.2a). Thus although CCL3 is often described as being a ligand for CCR5, its affinity is approximately 100 nM, whereas CCL3L1 has an affinity of 1 nM.

Another complexity arises from the fact that although certain chemokines can bind to several different receptors, the induced biological activity may differ significantly and can even vary depending on the cell type on which the receptor is expressed. CCL5 induced downregulation of three of its receptors and the ensuing recycling illustrates this phenomenon nicely. On incubation with CCL5 *in vitro*, the surface expression is reduced by approximately 80% in each case of CCR1 and CCR5 from the surface of PBMC [22, 23] and CCR3 from eosinophils [22, 24]. However on removal of the chemokine from the culture medium, very different patterns of receptor recycling are observed. In the case of CCR5, receptor density returns to that

(a)

```
                *         20         *         40         *         60         *
CCL3    : ASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA : 70
CCL3L1  : APLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPSVIFLTKRCRQVCADPSEEWVQKYVSDLELSA : 70
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(b)

```
                  *         20         *         40         *         60         *
SDF1_alpha   : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_beta    : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_gamma   : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_delta   : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_epsilon : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_theta   : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70

                        80         *       100         *       120         *       140
SDF1_alpha   : CIDPKLKWIQEYLEKALNK---------------------------------------------------- :  89
SDF1_beta    : CIDPKLKWIQEYLEKALNKRFKM------------------------------------------------ :  93
SDF1_gamma   : CIDPKLKWIQEYLEKALNKGRREEKVGKKEKIGKKKRQKKRKAAQKRKN--------------------- : 119
SDF1_delta   : CIDPKLKWIQEYLEKALNNLISAAPAGKRVIAGARALHPSPPRACPTARALCEIRLWPPPEWSWPSPGDV : 140
SDF1_epsilon : CIDPKLKWIQEYLEKALNNC------------------------------------------------- :  90
SDF1_theta   : CIDPKLKWIQEYLEKALNKIWLYGNAETSR---------------------------------------- : 100
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Figure 1.2 Alignment of the allelic CCL3 variants (a) and the splice variants of CXCL12 (b).

observed initially [23]. With CCR3, only 70–80% of the initial receptor density is observed, but with CCR1, no recycling is observed [22]. While the CCR3 receptors that do not recycle have been shown to traffic to the lysosomal compartment where they are degraded, the fate of CCR1 remains to be established. Therefore the apparent redundancy of a chemokine binding to more than one receptor may not be as redundant as meets the eye.

An additional layer of complexity has been found for the chemokine CXCL12, where six splice variants have been identified (Figure 1.2b [25]. The main difference is the extended C-termini of the δ and γ isoforms. The γ isoform has an extremely large number of basic residues resulting in a significantly increased affinity for GAGs [26, 27]. Beyond this observation little is known about the biological relevance of these iosforms, and the vast majority of data concerns CXCL12α. Similarly, the vast majority of data for CCL3 is for the LD78α allele and not the high affinity ligand, CCL3L1 described above.

1.3 Ligand Structure

1.3.1 Tertiary and Quaternary Structures

As described above, chemokines are ∼70–125 amino acid proteins that usually contain two disulfide bonds, with the exception of a few that have a single or three disulfides (e.g., XCL1 and CCL21, respectively). The presence of the disulfides enables primary sequences of low homology to adopt similar tertiary folds that

would likely be stable in the absence of the covalent structural constraints. CX3CL1 and CXCL16 are unique among the chemokines in that they contain a chemokine domain fused to a long extracellular mucin-like stalk, a single transmembrane helix and a short cytoplasmic domain; thus in contrast to most chemokines that are soluble secreted proteins, these chemokines are tethered to cell surfaces or can be proteolytically cleaved into a soluble form [2, 28]. Sequence similarity of the chemokine domain can vary from 20% to 80–90%. Nevertheless, chemokines adopt a remarkably conserved tertiary structure consisting of a disordered N-terminal region that is always important for signaling, a disordered "N-loop" ending in a 3_{10} helix, a three-stranded antiparallel beta-sheet and a C-terminal alpha-helix that packs against the sheet [29] (Figure 1.3a). Some chemokines like CCL27, JE and XCL1 have domains that extend beyond the C-terminal α-helix, and like the N-termini, these domains are disordered. In mouse JE the extension is 49 amino acids longer than the human homolog and it is glycosylated in mammalian cells. However, the functional roles of these extended regions are unclear. For example, an 8.5-kDa truncated form of JE missing most of the extra C-terminal residues is completely functional in migration assays of mouse and human monocytes *in vitro* [30]. However, whether it is fully functional *in vivo* remains to be determined.

While the tertiary structures are similar among all chemokines characterized to date, and some are monomeric in solution, many chemokines oligomerize, forming

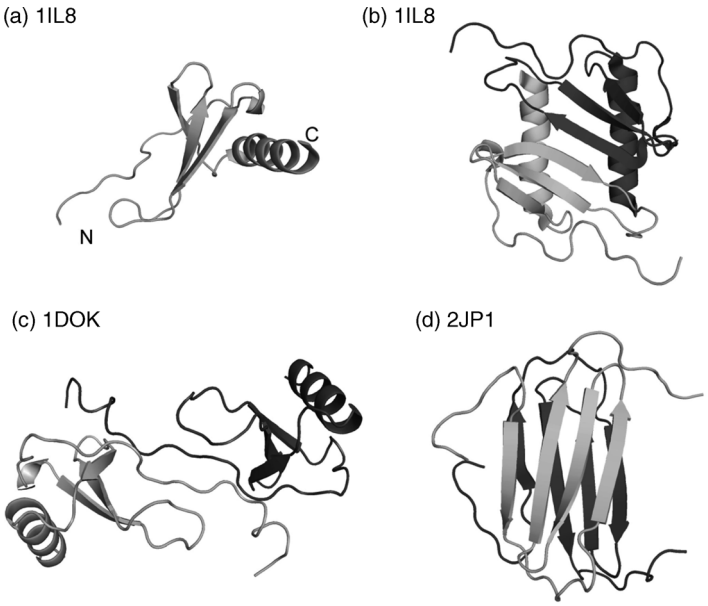

Figure 1.3 Ribbon diagrams of chemokines. (a) Monomer structure of CXCL8. (b) Dimer structure of CXCL8. (c) Dimer structure of CCL2. (d) Noncanonical structure of XCL1 stabilized by low salt and high temperature. PDB IDs are indicated next to each figure. The figures were generated in PyMol (DeLano Scientific).

dimers, tetramers and higher order oligomers. As described below, emerging data suggests that these different oligomeric forms are functionally significant and thus could add a great deal of biological diversity to an otherwise common structural fold. The formation of the oligomeric structures is not an all or nothing situation, however, and the propensity for oligomerization varies significantly from those that form stable multimers in solution under physiological conditions, to those that have weaker tendencies to oligomerize and can be readily shifted between oligomeric states by solution conditions (chemokine concentration, pH, salt, buffer), or by interactions with other molecular entities such as glycosaminoglycans (GAGs). For example, CCL7 is a monomeric ligand of CCR2, whereas another CCR2 ligand, CCL2, dimerizes in solution but can be shifted into a monomeric or tetrameric form by adjusting solution conditions or by interaction with GAGs [31, 32]. Likewise, CXCL12 exists in a monomer–dimer equilibrium and can be shifted towards the dimer by GAGs, phosphate ions and sulfate ions [33]. CXCL4 is a stable tetramer in solution [34], while CCL5, CCL3 and CCL4 form higher order oligomers under relatively normal physiological conditions [35], but can be destabilized into dimers by low pH and high salt (e.g., CCL5) concentrations [36]. Indeed, when one reads papers that report the oligomerization states of chemokines it is important to note the solution conditions, as many chemokine structures have been solved at low pH to disfavor oligomerization. By contrast, the observation of tetramers in crystal structures may be facilitated by favorable packing interactions in the crystal. However, independent of these biophysical studies, there is significant *in vitro* and *in vivo* biochemical evidence for the function of chemokine oligomers, and it is clear that one such role involves interactions with GAGs (see below).

Two common dimer motifs are generally associated with CC and CXC chemokines. CXC dimers, formed by the prototypical chemokine CXCL8, interact through amino acids in the first strand of the beta-sheet to form a dimer with an overall six-strand beta-sheet platform topped by two alpha-helices (Figure 1.3b) [29]. By contrast, most CC chemokines that dimerize (e.g., CCL2, CCL8) do so through residues near the N-termini forming a much more elongated structure than the CXC dimers (Figure 1.3c) [37]. However, at least one CC chemokine CCL20 has been reported to form a CXC-like dimer, calling into question the strict assignment of CC and CXC dimer motifs to ligands from the respective CC and CXC families [38]. CX3CL1 was solved as a monomer by NMR [39], although it showed a tendency to dimerize (Mizoue and Handel, unpublished data) and crystallized as a tetramer with the main dimeric substructure reminiscent of the CC-dimer motif [40]. XCL1 is the most unique chemokine and forms two entirely different structures that interconvert rapidly (100 ms); one structure is a canonical monomeric chemokine fold, which is stabilized by low temperature and high salt conditions (10 °C, 200 mM NaCl). The other structure is favored by low salt and high temperature conditions (0 M NaCl, 40 °C), and is a four-stranded antiparallel beta-sheet that self-associates as a novel head to tail dimer (Figure 1.3d) [41]. Interestingly, only the canonical chemokine fold acts as a receptor agonist and binds weakly to GAGs, while the novel dimer binds strongly to GAGs, but does not effectively activate the receptor XCR1.

CXCL4, the first chemokine structure to be solved, forms a stable tetramer in solution. Interestingly, a positively charged ring of lysine and arginine side chains encircles the PF-4 tetramer sphere, presenting a continuous binding site for heparin [34]. Recently, it was shown that the presence of unfractionated heparin, stabilizes these tetramers effectively into strings of tetramers and, notably, these are forms that are recognized by heparin-induced thrombocytopenia antibodies [42]. Although CCL2 forms predominantly dimers in solution, the addition of heparin octasaccharides causes it to form tetramers. High protein concentration and the presence of phosphate shift the equilibrium toward larger multimers as well (Handel, unpublished data). Interestingly, despite solution studies that show a bias toward dimers, crystallization of CCL2 trapped a tetrameric form similar to the CXCL4 tetramer. Like CXCL4, the CCL2 tetramer has an elongated ring of basic residues which have been shown to be important for heparin binding (Figure 1.4a) [32]. Furthermore, both of these structures contain CXC and CC-like dimers as substructures, and like the CCL20 dimer, suggest that CC and CXC dimers can be formed from chemokines from different families. CXCL10 was solved as a typical monomer in solution [43] but crystallized in three different tetramer forms; one resembles the CXCL4 and CCL2 tetramers while the other two are unique extended 12-stranded beta sheet structures, all with CXC dimers as substructures [44] (Figure 1.4a, c). Mouse IP-10 also crystallized as yet a fourth type of tetramer with heparin binding sites localized to the interfaces of each of the dimers, suggesting stabilization of the tetramer by heparin as observed for CCL2 [45]. The tetramer in the crystal structure of CX3CL1 is also entirely unique and mediated by water molecules

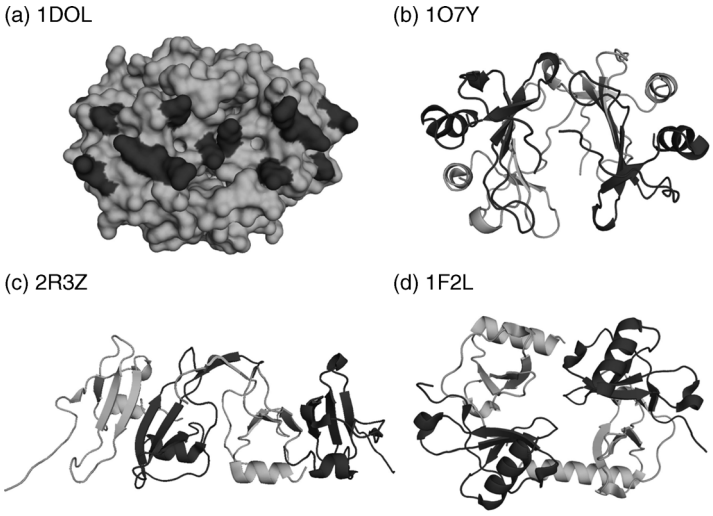

Figure 1.4 Structures of chemokine tetramers. (a) Tetramer structure of human CCL2 with GAG-binding eptiopes highlighted in dark gray. (b) Tetramer structure of human CXCL10, M form. (c) Tetramer structure of mouse CXCL10. (d) Tetramer structure of human CX3CL1. PDB IDs are indicated next to each figure. The figures were generated in PyMol (DeLano Scientific).

thus explaining the tendency to remain monomeric in solution, at least in the absence of GAGs (Figure 1.4d) [40]. In principle, different oligomeric forms like the four CXCL10 tetramers could represent structures capable of discriminating between different types of GAGs, but further study is necessary to determine if this is the case.

1.3.2 Functional Role of Tertiary and Quaternary Structures

As alluded to above, several studies suggest a functional role for chemokine oligomers, however monomeric forms of chemokines are sufficient for binding receptors to induce cell migration and cellular activation *in vitro*. Rajarathnam and coworkers demonstrated this finding by chemically synthesizing a variant of CXCL8 in which the amide nitrogen of leucine-25 was methylated to selectivity block formation of hydrogen bonds between monomers and thereby prevent dimerization [46]. Similarly a synthetic N-methylated variant of CCL5 was made for the same purpose [47]. In a different approach, mutants of CCL2 and CCL4 containing Pro to Ala substitutions in the N-terminal region were engineered to prohibit dimerization [48, 49]. In all cases, the variants were significantly or completely impaired in their ability to dimerize yet they showed full wild-type affinity and activity when tested in *in vitro* receptor binding assays and functional assays of receptor activation including *trans*-filter cell migration. From these data, it was concluded that the monomeric forms of chemokines are sufficient to activate the receptor for these biological responses.

Nevertheless, the functional importance of oligomerization was revealed with subsequent *in vivo* studies using an intra-peritoneal recruitment assay and the monomeric variants of CCL2, CCL5 and CCL4. In contrast to their ability to recruit cells *in vitro*, these mutants were incapable of causing cell migration into the peritoneal cavity *in vivo* [47]. While the mechanism is not entirely clear, biophysical and biochemical studies have associated oligomerization with binding to glycosaminoglycans, which are also required for *in vivo* but not *in vitro* cell migration [32, 50] (see below). These results also demonstrate the conflicting results that one can observe between *in vitro* and *in vivo* studies because of the complex nature of *in vivo* cell migration which involves interactions of migrating cells with other cells (e.g., endothelial cells), interactions between adhesion proteins on opposing cell surfaces, transcytosis of chemokines across endothelial cells, and other phenomena that are poorly recapitulated with simple *in vitro trans*-filter migration assays.

Although less well studied, chemokine oligomerization also appears to be important for cellular activation and related signaling processes. For example, while CCL5 monomers are capable of inducing cell migration, only wild-type CCL5 which forms large oligomers, but not an E66S mutant which is dimeric, was capable of activating the protein tyrosine kinase pathway in T cells leading to cell activation and associated events [51]. Similarly, the E66S mutant failed to induce T cell apoptosis [52]. As discussed below, obligate monomeric variants have been shown to have anti-inflammatory properties.

1.3.3
Hetero-Oligomerization

Chemokines also form hetero-oligomeric complexes. CCL3/CCL4 heterodimers were first shown to be secreted by human monocytes and peripheral blood lymphocytes by immunoprecipitation and immunoblot [53]. Subsequently, CXCL4/CXCL8, CXCL4/CCL5, CCL2/CCL8 and CCL21/CXCL13 were also shown to heterodimerize by co-immunoprecipitation and/or NMR [54–58]. Functional consequences have been associated with the ability of the requisite heterodimerizing chemokines to cause synergistic or additional responses not seen with either chemokine alone. For example, the presence of angiogenic CXCL8 with anti-angiogenic CXCL4, which form CXC heterodimers, increases the anti-proliferative activity of PF-4. Similarly, CCL5 and CXCL4, which form CC heterodimers, synergistically enhanced proinflammatory interactions, such as monocyte recruitment. Furthermore, peptides that inhibit heterodimerization not only attenuated monocyte recruitment, but were able to reduce the progression of diet-induced atherosclerosis in mice, a disease with a significant monocyte/inflammatory cell component [58]. However, the CCL5/CXCL4 study is the only example where there is strong direct physical evidence that the associated dimers are actually responsible for the observed functional effects rather than just the mere presence of the two chemokines in solution that happen to be able to associate. If further studies provide evidence that heterodimers are truly important for signaling, interfering with this interaction could prove to be a viable therapeutic strategy.

1.4
Receptor Structure

Chemokine receptors belong to the Class A family of G protein-coupled receptors (GPCRs), whose prototypic member is rhodopsin, a GPCR involved in light perception. Like other GPCRs, chemokine receptors are characterized by seven transmembrane helices connected by extracellular and intracellular loops of varying lengths, as well as extracellular N-terminal and intracellular C-terminal domains which show the most sequence diversity. A conserved structural feature of chemokine receptors includes the presence of a disulfide bond between the extracellular side of transmembrane segment 3 (TMS 3) and extracellular loop 2 (ECL 2); it is thought to have a role in structure/folding as well as functionally coupling changes in the conformation of ECL 2 upon ligand binding with TMS 3, a helix known to be critical to receptor activation in bovine rhodopsin and the β2 adrenergic receptor [59–61]. In chemokine receptors, there is also a disulfide between the N-terminus and ECL 3 (Figure 1.5a). Since the receptor N-terminus is involved in chemokine binding (see below) and since TMS 6 and 7 are connected by ECL 3 and are thought to be important for the activation switch, this disulfide may also couple ligand binding on the extracellular side to activation on the intracellular side through these helices. Other important motifs include the DRY motif at the base of TMS 3 just prior to ICL 2

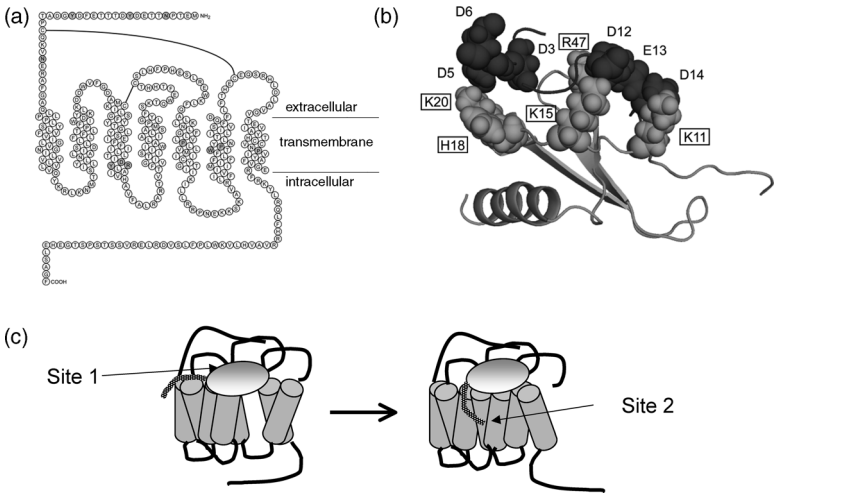

Figure 1.5 (a) Snake-like diagram of CCR1 as an example of the topology and post-translational modifications observed in chemokine receptors. The two conserved disulfides are connected by dark lines. Potential N-linked glycosylations sites and tyrosine sulfation sites in the N-terminal domain are shown as shaded circles labeled N and Y, respectively. The DRY box, required for G protein coupling is highlighted on the intracellular side of transmembrane 3. Prolines and the Tryptophan rotamer toggle, thought to be important for receptor activation, are shaded in helices 5, 6 and 7. Many chemokine receptors have cysteines in their C-terminal domain which are modified by palmitoylation. CCR1 does not get palmitoylated but CCR5 has such sites for example. (b) Monomer structure of CXCL8 (light gray) complexed with a modified peptide derived from the N-terminus of the receptor CXCR1 (PDB ID 1ILQ). The peptide binds across the surface in an extended fashion, binding in a cleft formed by a loop and beta-hairpin. Some of the basic residues on the ligand (in boxes) and acidic residues on the peptide (not boxed) are labeled and may confer specificity although most intermolecular contacts are hydrophobic. Figures were generated in PyMol (DeLano Scientific). (c) Cartoon illustration of the two site model of receptor activation. In this model, the main body of the chemokine (round oval) docks with the receptor N-terminus and extracellular domains (dark lines). The N-terminus of the ligand (stippled line) than interacts with the receptor helical bundle to induce the requisite conformational changes required for receptor activation.

which is required for G protein coupling, several conserved prolines that are thought to be important for conformational changes by introducing weak pivot points in helices, and a Trp that is thought to act as a "rotamer toggle" activation switch by rotamer interconversion [59, 61]. Other patterns include sequence motifs in the C-termini which are involved in receptor internalization often through binding of beta-arrestins and in intracellular trafficking patterns that dictate whether the receptor recycles to the cell surface or gets targeted for degradation. Chemokine receptors also frequently contain palmitoylation sites on cysteines in their C-termini, which tether the flexible C-termini to the membrane. The extracellular N-terminal domains which are relatively short and thought to be fairly flexible, at least in the absence of bound ligand, frequently contain putative N-linked glycosylation sites, as well as tryosine

sulfation motifs which are characterized by tyrosines flanked by acidic residues. While the functional roles of tyrosine sulfation and glycosylation have not been fully elucidated, tyrosine sulfation has been shown to modulate the affinity of certain receptors for their ligands [62]. Figure 1.5a shows a snake-like diagram of the chemokine receptor CCR1, illustrating some of the motifs described above.

No structures of chemokine receptors have been determined. However, on the basis of mutagenesis studies, it is clear that the N-termini of chemokine receptors are involved in ligand binding. Capitalizing on this observation, several studies have been done in which peptides from the N-termini of chemokine receptors have been synthesized and utilized in binding and structural studies with ligands [63–65]. These studies generally suggest that the receptor N-terminus binds to chemokines in a relatively extended conformation, with acidic residues from the receptor interacting with basic residues on the ligand (Figure 1.5b). Receptor peptides that are tryosine sulfated typically bind with higher (micromolar) affinity compared to nonsulfated peptides, confirming the importance of this post-translational modification. While some studies suggest that the peptides bind preferentially to the monomeric form of chemokines, consistent with the concept that monomeric forms are sufficient for activating the receptor, in the case of SDF-1/CXCL12 and a sulfated peptide from CXCR4, a 2:2 complex was favored [63]. However, one must keep in mind that these studies are taken out of context of the full receptor and the results may be influenced by the lack of other relevant interactions. In an attempt to recapitulate additional interactions between ligands and receptors, chimeric soluble proteins with the receptor N-termini and extracellular loops were made by attaching these elements to a soluble scaffold (the B1 domain of protein G) [66]. Importantly, the binding affinities of the soluble receptor mimics were highly correlated with the apparent affinities of the native receptor, demonstrating that the chimeras could capture some of the relevant interactions. However, it is obvious that to truly understand how chemokines bind and activate their receptors, that structures with full length receptors will be needed. Fortunately, progress in the determination of GPCR structures has exploded since 2007, and thus one can hope that chemokine receptors will yield to structure determination as well [67]. Until then, the two-site model of receptor activation continues to be the prevailing model (Figure 1.5c).

The two-site model is similar to that proposed for the chemoattractant protein C5a and suggests that the main body of the ligand first interacts with extracellular domain (s) of the receptor. Based on the NMR studies described above, the N-terminus of chemokine receptors would then feature prominently in interactions with the main body of the ligand, although other ECLs are also known to contribute. This interaction then positions the chemokine such that the flexible N-terminal region is able to interact with a second site, possibly in the receptor helical bundle similar to the binding site of small molecule ligands in the β2 adrenergic receptor. These interactions together induce the requisite conformational changes in the receptor, thereby triggering signal transduction. In contrast to the chemokine systems where the flexible N-terminal region is known to play the role of the signaling trigger, it is the flexible C-terminal domain in the case of C5a as well as another chemoattractant called chemerin [68].

1.4.1
Modifications to the N-Termini of Chemokines and their Effect on Receptor Activation

There is substantial evidence for the triggering role of the N-termini of chemokines, in support of the two-site model (Figure 1.5c). The importance of the N-termini of chemokines in receptor activation was first demonstrated with bacterially expressed [69] or chemical synthesized mutants of CXCL8 [70]. Both approaches demonstrated that the three-amino-acid "ELR" motif preceding the CXC sequence in the N-termini of CXCL8 was required for optimal binding and signaling. The ELR motif is found on all neutrophil attracting CXC chemokines, but is absent on members such as CXCL4 and CXCL10 that lack this activity. Interestingly, when the ELR motif was introduced into CXCL4, it was able to attract neutrophils [71]. By contrast, removal of the first five amino acid residues from CXCL8, which retained R6 of the ELR motif, produced a protein that was able to bind to its receptor with reasonably high affinity but could not induce signaling [72]. Furthermore this analog could antagonize the actions of CXCL8 highlighting the importance of the N-terminus in activation through site 2, while not affecting site 1. Other modifications to the N-termini of several chemokines further strengthened this argument, and their antagonist properties are discussed below, since they retain biding to site 1 but do not trigger signaling through site 2.

In additional to artificial mutations, natural modifications of the N-terminal domains have been described for many chemokines [73]. Several classes of enzymes have been shown to be responsible for N-terminal truncations including matrix metalloproteases (MMPs), DPIV/CD26, leukocyte elastase and cathepsins to name a few. These enzymatically cleaved forms usually are completely or partially inactivated with respect to signaling relative to the wild type, but sometimes they show increased activity and often they retain high affinity binding. Sometimes there is an effective switch in receptor specificity. For instance, (3–68)-CCL5, a cleavage product of CCL5 produced by CD26, has impaired signaling activity on monocytes through loss of affinity for CCR1, the principal receptor on circulating monocytes, and antagonizes monocyte chemotaxis. However its affinity for CCR5 is enhanced, and it is more potent in inhibiting HIV infectivity [74]. When modified CCL5 variants which have anti-inflammatory properties are administered to mice, their processing in the circulation can be directly demonstrated using surface enhanced laser desorption ionization (SELDI) – a protein chip technology coupled to a MALDI spectrometer. Using this technique, the GAG-binding mutant, ^{44}AANA^{47}-CCL5, was shown to be rapidly oxidized, cleaved to the 3–68 form and then further truncated to produce the 4–68 form [75].

As mentioned above, there are also numerous reports of N-terminal truncated variants that have increased activity relative to their wild-type counterparts: truncation of CXCL1 and CXCL3 resulted in chemokines with enhanced abilities to induce calcium signaling and neutrophil chemotaxis [76]. Furthermore, (5–73)-CXCL2 is ten times more potent than the full length chemokine [77]. Perhaps the most impressive gain of function is for the low affinity CCR1 ligands, CCL15 and CCL23 which have an extended N-terminus of 16–20 amino acids and a third disulfide bridge; processing of

their extended N-terminal by elastase, cathepsin-G or chymase, or synovial fluid, results in up to a 1000-fold increase of affinity for CCR1, rendering them more potent than CCL5 and CCL3 [78].

Conversely many chemokines are rendered inactive by N-terminal processing. For example, MMP cleavage of CCL2 to produce (5–76)-CCL2 [79] and DPPIV/CD26 cleavage of CCL11 to (3–74)-CC11 [80] abrogates activity, and the physiological relevance of the latter result was confirmed by enhanced eosinophil mobilization induced by the administration of CCL11 into DPPIV-deficient mice.

Chemokine processing is not limited to the N-terminus. In addition to CD26 and MMP N-terminally truncated versions of CXCL9, CXCL10 and CXCL11, truncation is also observed at the C-terminus. The signaling properties of (1–77)-CXCL10 and (1–73)-CXCL10 are very similar, whereas processing of the C-terminus of CXCL9 diminishes signaling [81]. In addition, heparin binding of CXCL11 is significantly reduced in the 1–58 cleaved form compared to the full-length 1–73 protein.

1.5
Glycosaminoglycan Binding Sites

Several years ago the immobilization of CXCL8 on endothelial surfaces first demonstrated the importance of chemokine/glycosaminoglycan (GAG) interactions [82]. Subsequently selectivity in this interaction was demonstrated using electrophoresis of chemokines in the presence of different heparin fragments [83] and a solid phase heparin binding assay [84]. Interestingly, GAGs have been shown to both potentiate and inhibit chemokine activities. The potentiation of chemokine activity by GAGs was reported *in vitro* using CHO cells that are deficient in cell surface GAGs where it was demonstrated that while biological activities such as receptor binding and activation is independent of the presence of GAGs, the expression of cell surface GAGs helped to sequestrate the chemokines [85]. Using chemokine mutants with abrogated GAG binding capacity, the chemokine/GAG interaction has been shown to be essential for the ability of chemokines to recruit cells *in vivo*, while this interaction is not required in the commonly used *in vitro trans*-filter chemotaxis assay [47]. Other examples of biological relevance include the fact that CCL5 secreted from the alpha granules of platelets is found in large GAG associated complexes, and the GAG association is required for the anti-HIV inhibitory properties of this chemokine [86]. CCL5 has also been shown to enhance infection of HIV-1 in macrophages [87], as well as other viral infections *in vitro*, albeit at micromolar concentrations, which is attributed in part to the oligomerization property of this chemokine [88, 89]. As discussed below, this property is mediated by the binding of CCL5 to GAGs.

GAG binding sites are generally identified by alanine scanning mutagenesis studies of the chemokine of interest. However, biochemical methods can be used to predict the regions that are involved, which can be subsequently confirmed by restricted mutagenesis. An example of such a method involves the use of protein bound to heparin beads, which is subsequently covalently stabilized by chemical

crosslinking. Following proteolytic digestion, peptides that remain bound to heparin, presumably GAG-binding epitopes, are identified by N-terminal sequencing [90]. In a second related approach, samples of chemokine/heparin complexes or chemokine alone are submitted to tryptic digestion and mass spectrometry identification; peptide fragments that are present only in the heparin containing samples correspond to fragments protected by heparin and therefore GAG-binding sites.

The relative of affinities of chemokines or chemokine mutants for GAGs has been measured by several different methods. These include heparin sepharose chromatogaphy [91–93], electophoresis in the presence of different GAG species [83], solution binding assays with radiolabeled heparin [94], binding of radiolabelled chemokines to heparin immobilized on beads [84, 93] and an ELISA-type assay of chemokine binding to heparin immobilized on specialized plates (Epranex plates, Plasso Technologies Ltd.) [95, 96]. The most common procedure is the analysis of the NaCl concentration required to elute the chemokine from heparin sepharose columns. In these analyses, certain mutants are no longer able to bind to the column, as was observed for MIP-1α [92] and MIP-1β, where the binding was abrogated in the presence of physiological concentrations of NaCl [91]. However, in the case of chemokines that bind much more strongly to heparin, such as CXCL8, CXCL12 and CCL5, mutation of the heparin binding sites does not totally abolish the capacity to bind to heparin, indicating either that other specific sites exist, or that there is a certain amount of nonspecific electrostatic interaction. For example, analyses of the CCL5 triple ^{44}RKNR^{47} alanine mutant indicate the former. The residual 20% binding capacity that this mutant retained for heparin, had the same affinity for heparin as wild-type CCL5, but lost selectivity for the four GAG families, heparin, heparin sulfate, dermatan sulfate and chondroitin sulfate [93]. Recently, a binding assay using Epranex plates has been frequently used. This assay was used to compare the GAG binding affinities of citrullinated CXCL8 to those of the wild type [96]. It was also used to demonstrate that eotaxin selectively binds to heparin, but not to heparan sulfate or a range of other GAGs [95].

Receptor binding regions and GAG binding regions delineated by mutagenesis studies for several chemokines initially led to the idea that receptor and GAG binding domains were spatially separated in that receptor binding was governed by the N-terminus and GAG binding by the C-terminal helix. For example, the importance of the C-terminal helix in GAG binding had been shown for CXCL4 [97]. However, it appears that this generalization is not true for many chemokines, now that the GAG binding sites have been delineated for more chemokines. In fact, the 20s loop contains residues important both for receptor binding and GAG binding in several chemokines such as CXCL8, CXCL10, CXCL12 and CCL2 [32, 98–100].

The GAG binding sites have now been mapped for several chemokines. The residues on chemokines that interact with GAGs identified to date are represented in Figure 1.6. Heparin binding regions are often defined by a cluster of basic residues, forming either a BBXB or a BBXXB motif (where B is a basic amino acid). These residues have been mutated to Ala in the CCR5 ligands CCL3 [92, 101], CCL4 [91] and CCL5 [93], showing that they constitute the principal heparin binding site(s). While no effect on receptor binding was observed for the R46A mutant of MIP-1β

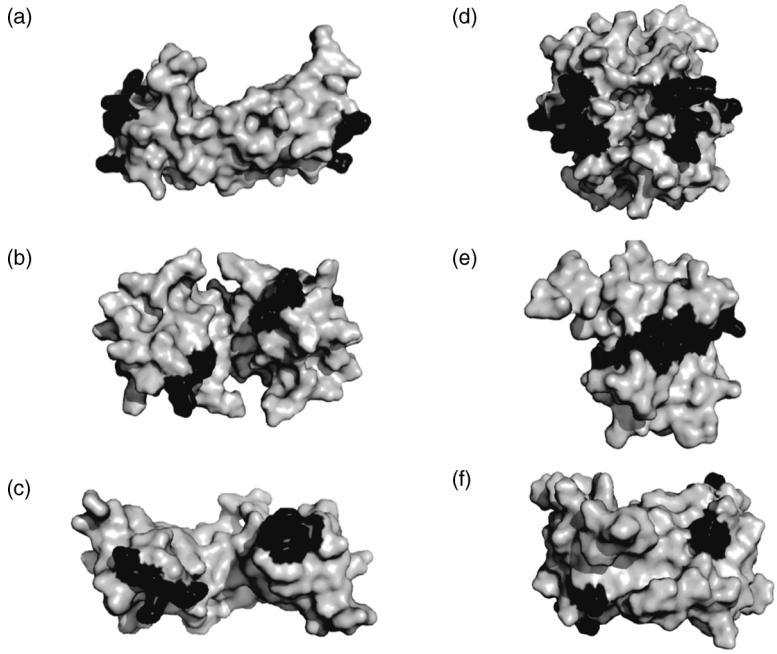

Figure 1.6 Representative dimer structures of CC chemokines (a–c), CXC chemokines **(**d, e**)**, and the C chemokine **(**f**)** with GAG binding epitopes identified by mutagenesis, highlighted in dark gray. (a) CCL2 with GAG epitopes R18, K19, R24, K48, K58, H66. (b) CCL3 with GAG epitopes R18, K45, R46, K48. (c) CCL5 with GAG epitopes R44, K45, R47. (d) CXCL8 with GAG epitopes K20, R60, K64, K67, R68. (e) CXCL12 with GAG epitopes K24, H25, K27, R41, K43. (f) XCL1, low salt high temperature form, with GAG epitopes K23, R43. The figures were generated in PyMol (DeLano Scientific).

to its receptor CCR5, the mutation R46A in MIP-1α abolished binding to CCR1. Our results with CCL5 have shown a similar phenomenon. The mutations of the residues R44, K45 and R47 individually to Ala had no significant effect on all the parameters tested – binding to heparin, CCR1, CCR5 or on *in vitro* chemotaxis. However, the concomitant mutation of the basic residues to Ala in the ^{44}RKNR^{47} motif had a profound effect on heparin binding and a significant effect on binding to CCR1, while wild-type activity was retained on CCR5. However, while GAG binding was not completely abrogated, the mutations were sufficient to eliminate *in vivo* cell migration [102]. CCL5 has a second cluster of basic residues immediately preceding the C-terminal helix, ^{55}KKWVR^{59} but mutation of these residues either individually or as a triple mutation had no effect in all of the above assays. However, using the elegant assay discussed above to involving covalent linking of the protein to a heparin bead, followed by enzymic digestion of the complex and finally N-terminal sequencing of the peptides bound to the heparin, the sequence ^{55}KKWVR^{59} was found when using an increased ratio of chemokine to heparin [90]. Interestingly this region was subsequently found to play a role in binding to tissue in the kidney [103].

The C-terminal helix of CXCL8 presents four basic residues which interact with GAGs: K64 and R68 play the major role, but R60 and K67 also contribute [98]. K20, in the 20s loop which has been shown to be involved in receptor binding, is also involved in GAG interactions. However, in the CXCL8 protein the main receptor interface is spatially separated from the C-terminal helix which is the principal GAG binding region. CXCL12 has introduced an entirely new region that is implicated in GAG binding [100]. In this chemokine, GAG binding is mediated by a BBXB motif, but which is located in the 20s loop, since it consists of the residues ^{24}KHLK^{27}. However these basic amino acids play no role in CXCR4 activation. Interestingly, while no GAG biding is observed by surface Plasmon resonance at concentrations $\leq$200 nM, micromolar concentrations show GAG binding equivalent to the wild type [100]. However the loss in GAG binding is sufficient to abrogate the ability to recruit leukocytes into the peritoneal cavity. The GAG binding residues on MCP-1 were initially described as being located towards the C-terminus, formed by two residues: K58, which is located immediately before the C-terminal helix, and H66, which is found at the end of the helix. The other basic residues in this region, K56, K69 and K75 are not involved in GAG binding [94]. An extensive alanine scanning mutagenesis study of this chemokine delineated residues on the 20s loop, R18 and K19, as being the predominant amino acids for GAG binding, with a lesser contribution provided by R24 [32]. The 20s region is also involved in GAG binding for the chemokine CXCL10. Residues Arg20, Arg22, Ile24, and Lys26, as well as Lys46 and Lys47 were found to constitute the main GAG binding domain, with the mutation of Arg22 resulting in the largest reduction of heparin binding affinity [99].

The C-terminal truncation influences the GAG binding properties of several chemokines. C-terminal cleavage of CXCL11(1–73) to CXCL11(1–58) by MMP-8 or MMP-9 significantly diminishes heparin binding affinity [104]. For the case of CXCL12a, C-terminal cleavage also decreases its heparin binding capacity, as well as its ability to attract B-lymphocytes and to stimulate pre-B cell proliferation [105].

1.6
Chemokine Analogs– Research Tools and Potential Therapeutics?

As discussed above, alterations in the N-termini of certain chemokines have resulted in profound changes in their activity, and there are many examples of modifications of the N-terminus to produce antagonists. N-terminally truncated CCL5, MCP-1 and MCP-3 proteins have antagonized the effect of their parent ligands *in vitro* [106]. Interestingly, removal of the first eight residues from the N-terminus of CCL5 changes its specificity, since the truncated protein is able to bind to CCR2, to which the full-length protein does not bind. The removal of seven residues from the N-terminus of MCP-1 (7ND-MCP-1) forms an antagonist protein that was initially proposed to act by preventing the formation of the active MCP-1 dimer, analogous to dominant negative mutations [107]. Interestingly these proteins have not been extensively studied *in vivo*, and there is only one report of the activity of a truncated chemokine *in vivo*, where the administration of the (9–68)-MCP-1 protein into mice

that spontaneously develop arthritic symptoms was very effective in reducing the inflammatory symptoms, when administered in a therapeutic protocol [108]. However, 7ND-MCP-1 has been extensively used in the form of gene therapy where the cDNA has been administered and has shown to be effective in reducing symptoms in several disease models, for example experimental autoimmune myocarditis (EAM), an animal model of human myocarditis [109] and cancer [110].

The extension of the N-terminus of certain chemokines can also have a profound effect on the biological properties. In the case of CCL5, when the recombinant human protein is produced in *Escherichia coli*, despite being correctly folded as assessed by NMR spectroscopy [36], the chemokine had no activity [111]. It was established that the retention of the initiating methionine was responsible for this effect, and that the protein was a potent antagonist – an entirely serendipitous finding. This analog, Met-CCL5, retains high affinity binding for human CCR1 [111] and CCR5 [112] but only moderate affinity for CCR3 [22, 102, 113, 114], and we have recently shown that it only binds to CCR1 and CCR5 in the mouse system [115]. Met-CCL5 has been tested in numerous rodent inflammatory models which have served to highlight the efficacy of chemokine receptor antagonism in preventing inflammation. Thus Met-CCL5 has been reported to reduce inflammation in models of arthritis [116], nephritis [117], organ transplant [118], colitis [119] and asthma [120]. Since this analog has high affinity for mCCR1 and mCCR5, chemokine receptors expressed on leukocytes implicated in many inflammatory disorders such as T lymphocytes and monocytes, these results indicate that these two receptors are good therapeutic targets. It is however interesting to note that Met-CCL5 is also effective in reducing airways inflammation in the mouse ovalbumin sensitization model, despite the fact that it has no affinity for mCCR3. Since CCR3 is the predominant CC chemokine receptor expressed on eosinophils and this cell type is widely thought to be the main pathogenic culprit in asthma, this receptor was widely believed to be a prime target for this disease. However the use of this analog demonstrates that antagonism of receptors other than CCR3 is effective in reducing airway inflammation.

Our initial studies characterizing Met-CCL5 were carried out on the promonocytic cell line, THP-1, where we found it to be devoid of activity. However, on eosinophils from certain patients, weak partial agonist activity was observed [102]. Through a semisynthetic approach aimed at producing a derivative that was devoid of this partial agonist activity, we identified an analog, AOP-CCL5 (aminooxy pentane CCL5) that had higher affinity for CCL5 receptors than Met-CCL5 [102, 112]. Again the N-terminally extended CCL5 protein was inactive on monocytes, and antagonized the effects of CCL5, CCL3 and CCL4 on this cell type. This analog has since taught us new mechanistic pathways that play a role in disease. AOP-CCL5 was found to be a particularly potent inhibitor of HIV-1 infection, being significantly more potent than CCL5 itself [112]. The inhibition of HIV-1 infection by chemokines could be mediated by two mechanisms – either simply by steric hindrance, or by inducing downregulation of the receptor from the cell surface. Although AOP-CCL5 was initially thought to be an antagonist, subsequent studies demonstrated that it also retained partial agonist activity on CCR1 and CCR3, but was a full agonist of CCR5 [102, 121]. In fact in certain activities, it was even more potent than CCL5

itself [23, 121]. Thus its potent HIV-1 inhibitory properties were attributed to its greater effectiveness in downregulating CCR5 and furthermore in preventing the recycling of the receptor to the cell surface [23, 121]. However AOP-CCL5 did not target the receptor to the lysosomal compartment where it is degraded, as has been shown to occur for other chemokine receptors such as CCR3 [24, 121]. It has subsequently been demonstrated that CCR5 does in fact recycle to the cell surface after AOP-CCL5 treatment, but through a mechanism that remains to be elucidated [122]. Thus modification of the receptor endocytotic pathways is an effective mechanism of receptor antagonism.

Two programs capitalized on this unusual property of AOP-CCL5 and developed variants that were considerably more potent. An optimization program led to a variant, PSC-CCL5 that was 50-fold more potent [123] and showed efficacy as a microbicide by topical application in preventing HIV infection in macaques [124]. However this variant had the disadvantage of being costly to produce since it was made by total chemical synthesis. Therefore a second approach was adopted which was to search for a variant of CCL5 that had the properties of PSC-CCL5, but which consisted entirely of natural amino acids using phage display. Three analogs were identified that exhibited *in vitro* potency against HIV-1 comparable to that of PSC-CCL5 [125]. The first induced prolonged intracellular sequestration of CCR5, the second had no detectable G protein-linked signaling activity nor receptor sequestration and the third induced significant levels of CCR5 internalization without detectable G protein-linked signaling activity. These analogs therefore represent promising candidates for further development as topical HIV prevention strategies. Using phage display to identify chemokine receptor antagonists appears to be a powerful tool – recently an antagonist of CX3CR1 that appears to be totally devoid of agonist activity has been identified by this approach [126].

The essential roles of GAG binding for the biological activity of chemokines *in vivo*, and oligomerisation for certain chemokines, are discussed above. Therefore it is not surprising that interference with these properties could result in anti-inflammatory activities. Modulation of GAG binding has been used with two very different approaches. The first approach used chemokine analogs that had mutations removing basic residues to abrogate GAG binding, while the second introduced basic residues to enhance GAG binding. Not only was the variant ^{44}AANA^{47} CCL5 unable to induce cellular recruitment into the peritoneal cavity, but its administration prior to CCL5 or thioglycollate inhibited the cellular recruitment [47]. This anti-inflammatory property translated into potent inhibition of disease symptoms in the mouse model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) [127] and in atherosclerosis in mice [128]. The inhibitory mechanism appears to be its ability to disaggregate CCL5 oligomers and form heterodimers with the wild-type protein which are devoid of activity *in vivo*. Similar anti-inflammatory properties were described for a mutant of CCL7 which was designed to eliminate heparin binding [129]. The opposite approach has been taken for other inflammatory chemokines where the chemokine has been mutated to abrogate receptor activation, and additional basic residues have been introduced to augment heparin binding with the rationale of displacing the active

chemokine from the endothelial surface [130]. Lastly, interference with oligomerization provides another anti-inflammatory strategy – as discussed above the mutation P8A in CCL2 creates an obligate monomer that is also able to inhibit peritoneal recruitment and to reduce disease symptoms in EAE in mice [131, 132] and antigen induced arthritis (AIA) in rats [133].

In conclusion we have described how understanding the structural relationships of chemokines and their receptors to their biological activity has led to the design of antagonists that have been pivotal in validating the chemokine system as targets for disease. This knowledge, particularly the structure–function relationships of chemokine ligands, will be crucial to the design of novel therapeutic strategies. We now look forward to the next leap in chemokine structural biology, that of solving and understanding the structure and interactions of the receptors.

Acknowledgments

This work was funded by the Lymphoma Research Foundation, the Department of Defense (USAMRAA W81XWH0710446) and NIH (RO1-AI37113 and R21 AI076961) awards to T.M.H., an NRSA postdoctoral fellowship F32GM083463 awarded to D.J.H. and a European Union FP6 INNOCHEM award (LSHB-CT-2005-518167) to A.E.I.P.

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