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Chemokines and their receptors: insights from molecular modeling and crystallography

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Abstract

Chemokines are small secreted proteins that direct cell migration in development, immunity, inflammation, and cancer. They do so by binding and activating specific G protein coupled receptors on the surface of migrating cells. Despite the importance of receptor:chemokine interactions, their structural basis remained unclear for a long time. In 2015, the first atomic resolution insights were obtained with the publication of X-ray structures for two distantly related receptors bound to chemokines. In conjunction with experiment-guided molecular modeling, the structures suggest a conserved receptor:chemokine complex architecture, while highlighting the diverse details and functional roles of individual interaction epitopes. Novel findings promote the development and detailed structural interpretation of the canonical two-site hypothesis of receptor:chemokine recognition, and suggest new avenues for pharmacological modulation of chemokine receptors.

Graphical abstract

Introduction

Interaction of chemokines (chemotactic cytokines) with cell surface receptors is the driving force behind cell migration and plays a major role in development, immunity, inflammation, and cancer [1, 2]. Chemokine receptors belong to the superfamily of G protein-coupled

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Conflict of interest

The author declares no conflict of interest.

receptors (GPCRs) and translate chemokine binding into activation of intracellular G protein- and β -arrestin-mediated pathways. Following activation, receptors are internalized and targeted for degradation or recycling in a receptor- and ligand-dependent manner [3, 4]. The 22 receptors and approximately 45 chemokines expressed in humans form a pharmacologically complex system [5]. Quite frequently, recognition of a single chemokine by different receptors, as well as binding of different chemokines to the same receptor, leads to different signaling and trafficking responses (so-called *functional selectivity*) [4, 6].

A prominent example of polypharmacology and functional selectivity is given by the chemokine receptors CXCR4 and ACKR3 with their shared chemokine ligand CXCL12, the trio extensively studied for its role in shaping the cancer microenvironment [7]. CXCL12 activates both G protein and β -arrestin signaling when acting through CXCR4 but only β -arrestin signaling when binding to ACKR3 [8]. Another intricate subsystem involves chemokine receptors CCR2 and CCR5, both promising targets in inflammatory diseases [9] and cancer immunotherapy [10, 11]. CCR2 and CCR5 are coexpressed on a variety of hematopoietic cells and share several chemokine ligands [5] which vary in their pharmacological action towards the two receptors [6].

Elements of the system are also extensively exploited by pathogens. For example, *P. vivax* utilizes the atypical chemokine receptor ACKR1 and HIV utilizes CCR5 and CXCR4 for host cell entry [5]. Ticks (*Ixodidae*) and parasitic nematodes (*S. mansoni*) express chemokine-binding proteins that interfere with host immune and inflammatory responses [5]. Viruses (*Herpesviridae*) encode in their genomes both chemokines (e.g. vMIP-II) and chemokine receptors (e.g. US28) that integrate into the host signaling cascades and act to viral replicative advantage [12].

In view of the major role of chemokines and chemokine receptors in disease pathology, numerous attempts have been undertaken to target them with small molecule drug candidates [5, 13]. However, as of 2016, only two approved small molecules target chemokine receptors: a CCR5 antagonist Maraviroc (marketed as an HIV entry inhibitor [14]), and a CXCR4 antagonist Plerixafor (used for stem cell mobilization in hematopoietic stem cell transplant patients [15]). Many pre-clinical candidates failed in the clinics due to poor pharmacokinetic properties, lack of efficacy, toxicity, etc. [5, 13]. Like most protein:protein interactions, receptor:chemokine interfaces are *poorly druggable*, i.e. conceptually difficult to inhibit with small molecules; therefore, much effort is being devoted to the development of biologics and biomimetics, including engineered chemokines [4, 16, 17], nanobodies and antibodies against chemokines [18, 19] and receptors [20–23], and therapeutic nucleotides [24–26]. The first anti-CCR4 monoclonal antibody Mogamulizumab was recently approved in Japan for cutaneous T-cell lymphoma [27].

Many questions related to the development of both small molecule and biologic modulators of receptor:chemokine interactions are best answered with structures at hand. Yet until recently, structural understanding of receptor:chemokine interactions was lacking. In 2010 and 2013, X-ray structures were determined for two chemokine receptors, CXCR4 [28] and CCR5 [29], in complexes with small molecule and peptide antagonists; however, these structures did not readily answer the question of how receptors interact with chemokines.

This knowledge gap is now closing: in 2015, the first structures of two receptor:chemokine complexes were solved [30, 31], and experiment-guided molecular modeling is rapidly supplying information about targets not yet amenable to crystallization. The present review summarizes recent advances in the structural understanding of receptor interactions with chemokines, and their pharmacological implications.

Versatility of chemokines

Chemokines are secreted 7–12 kDa proteins. They share a common topology that consists of a flexible N-terminus, a conserved double-cysteine motif, an 8–9 residue unstructured loop called the *N-loop* followed by a single turn of a 3_{10} helix, a three-strand β -sheet, and a C-terminal helix (Figure 1A–D). The pattern of cysteine residues in the N-terminus serves as a basis for chemokine classification: in CC, CXC, and CX₃C chemokines, the cysteines are separated by no, one, and three residues, respectively; while in the two human chemokines of the XC family, one of the cysteines is absent. The conserved cysteines connect the N-terminus to the third β -strand, and (with the exception of the XC family) to the β_1 – β_2 loop (also called 30s loop) (Figure 1A–D).

Many chemokines form dimers in solution. CC chemokines preferentially dimerize through their proximal N-termini involving the CC motifs, and the interaction further extends into the groove bounded by the N-loop on one side and the β_2 – β_3 (or 40s loop) on the other [32, 33] (Figure 1E). By contrast, CXC chemokines dimerize by forming an anti-parallel β -sheet between their β_1 -strands (Figure 1F). The CX₃C chemokine CX₃CL1 (fractalkine) forms a tetramer in the crystal structure [34], representative of its behavior on membranes and extracellular matrices; the primary oligomerization interface is homologous to that of CC chemokines although the relative orientation of the monomers is different (Figure 1G). Finally, the metamorphic XC chemokine lymphotactin is thought to dimerize by adopting an all- β -strand topology [35] that is dramatically different from its canonical chemokine topology in the monomeric form (Figure 1H).

The chemokine dimerization interfaces, as well as their counterparts in other chemokine subfamilies, are actively exploited by proteins that bind chemokines. An increasing number of structural studies demonstrate that despite the lack of universal homology, pathogenic chemokine binding proteins engage human CC and CX₃C chemokines through their proximal N-termini and/or the N-loop/40s loop grooves [36–38]. The homologous interface in CXC chemokines frequently forms secondary packing contacts within the chemokine crystal lattices; additionally, in CXCL12, the N-loop/40s loop groove can accommodate an anti-CXCL12 antibody [19], as well as a small molecule inhibitor [39]. On the other hand, the β_1 strand not only mediates CXC homodimer interactions but also serves as a recognition epitope for antibodies targeting CXCL13 [40] as well as a landing site for the flexible N-terminus in the self-inhibited conformation of this chemokine [41].

These observations demonstrate that with the total solvent accessible surface area of only ~4500 to ~6100 Å², chemokines possess one or two distinct preferred interaction surfaces recognizing diverse binding partners in a structurally similar fashion (Figure 1I–L). Unfortunately, these surfaces lack non-polar, enclosed pockets that are generally required for

high-affinity binding of small molecules. The scarcity and low affinity of known small molecule binders to chemokines [39, 42, 43] reflects this conceptual limitation.

Topology and structure of chemokine receptors

Like all members of the GPCR superfamily, chemokine receptors possess seven transmembrane helices (TM1–TM7), three extracellular (EC) and three intracellular (IC) loops, and (like most GPCRs) a disulfide bond connecting TM3 and ECL2. With the exception of CXCR6, human chemokine receptors also have a disulfide bond connecting their flexible N-termini to ECL3. The X-ray structures of complexes with small molecule antagonists were solved for CXCR4 [28] and CCR5 [29] in 2010 and 2013, respectively. The structure of CXCR4 bound to a 16-residue cyclized peptide antagonist is also published [28]. In 2012, an apo CXCR1 structure was obtained using rotationally aligned solid-state NMR in phospholipid bilayers in conjunction with molecular modeling [44].

The receptor structures featured a conserved β -hairpin topology of ECL2, a large degree of conformational plasticity, and unstructured/disordered N-termini. They demonstrated that despite possessing well-defined binding pockets, chemokine receptors are far from being easily druggable targets. Consistent with the nature of their endogenous ligands (chemokines), their pockets are wide open and very polar. The co-crystallized small molecules demonstrate unusually low degree of enclosure (i.e. a large fraction of their surface is exposed to solvent), and sparse hydrophobic anchoring to the pocket surface [28, 29]. The structures generated much insights for small molecule antagonist development [45]; however, they did not provide an immediate answer regarding structural principles of receptor interactions with chemokines.

Architecture of receptor:chemokine complexes: the two-site hypothesis

As of only two years ago (2014), knowledge of the structural basis for receptor:chemokine recognition was limited to the so-called *two-site hypothesis* [1] that originated from biochemical, biophysical, and functional studies of different receptor:chemokine pairs. The hypothesis states that the interaction involves two distinct epitopes: chemokine recognition site 1 (CRS1) where the extended N-terminus of the receptor binds to the globular core of the chemokine, and CRS2, where the N-terminus of the chemokine reaches into the TM domain pocket of the receptor (Figure 2A). CRS1 is frequently important for binding and relies on receptor tyrosine sulfation, while CRS2 is widely recognized as a critical signaling domain [46].

Higher resolution understanding of the interaction was not available until 2015 because of difficulties associated with structure determination for GPCRs (such as chemokine receptors), and especially for protein:protein complexes involving GPCRs [47, 48]. Attempts to “divide-and-conquer” the system and to structurally characterize CRS1 interaction by NMR outside of the context of the full-length receptor, using N-terminal receptor peptides [43, 49–51], provided ambiguous results, even in determining the orientation of the bound CRS1 with respect to the chemokine (Figure 2B–D). When analyzed retrospectively, this is because the TM domain of the receptor provides some critical anchoring contacts: without

these contacts, the receptor N-terminus is free to bind chemokine in multiple energetically equivalent conformations. Quite informatively though, the preferred chemokine interaction surface involving its proximal N-terminus and/or the N-loop/40s loop groove (darker red in Figure 1I–J) is invariably utilized in all chemokine structures with receptor N-termini (Figure 2B–D) and even with a small molecule [39, 42] (Figure 2E).

The first internally-consistent atomic resolution insights into the geometry of full length receptor:chemokine complexes were obtained by molecular modeling. Given the extent and the flexibility of the interface between the two molecules, and also the expected substantial (but at that time yet unknown) changes in their conformations in the complex as compared to their unbound states, this problem represented an extremely challenging case of flexible protein-protein docking that was, and still is, unfeasible using existing computational technology. Therefore, it was critical that the modeling process was guided by experimental restraints [52]. To this end, our group adopted and refined the technique of *disulfide trapping* where irreversible covalent complexes are generated from transiently interacting proteins by introducing two cysteine residues (one per protein) at strategically selected positions within their interaction interface [53]. The abundance and purity of covalent complexes that are formed provides an estimate for the spatial proximity of the mutated residues. In 2014, we presented a model of an intact CXCR4: CXCL12 complex informed by mutagenesis, functional experiments and disulfide trapping [54]. In 2015, a structurally similar model was obtained by another group using constrained docking of CXCL8 to an NMR structure of CXCR1 [55]. Unlike models obtained purely by energy-based optimization (which is rather inaccurate in view of the expected flexibility of the components), these models reconciled the abundant experimental data while also supporting the two-site hypothesis.

Lessons from the first X-ray structures of receptor:chemokine complexes

Next, under the guidance of complex models, we designed an exceptionally stable irreversible complex between CXCR4 and a herpesvirus-encoded antagonist chemokine vMIP-II. The favorable properties of this complex made it a prime candidate for crystallization, and its structure was determined to 3.1 Å resolution [30] (Figure 2F). Shortly after that, the first structure of a non-covalent receptor:chemokine complex was published – this time of a herpesvirus-encoded receptor US28 with human CX₃CL1 [31] (Figure 2G).

The structures confirmed the existence of the two recognition epitopes, CRS1 and CRS2, but showed that in reality, they are parts of a single contiguous interface and are connected by an intermediate region (“CRS1.5” [30]) that brings the conserved cysteine motifs of the chemokines in proximity to those in the N-termini of the receptors (Figure 2F–G). In CRS1 and CRS1.5, the receptors appear to largely utilize the preferred interaction surfaces of the chemokines (the proximal N-termini and the N-loop/40s loop grooves, Figure 1I,K). Because these surfaces are also involved in CC and CX₃C chemokine dimer formation, their dimerization and receptor binding are mutually exclusive [30], consistent with long known signaling incompetence of CC chemokine dimers [56, 57]. In CRS2, the distal N-termini of the chemokines reach the residues at the base of the receptor orthosteric binding pockets, in agreement with the well-established signaling role of this epitope [46]. Other than this general trend, CRS2 interactions share no structural similarity between the two crystallized

complexes and thus appear to be receptor-specific. CRS1.5 appears to act as a flexible hinge between CRS1 and CRS2, allowing some freedom in positioning of the chemokine body with respect to the receptor. As a result, the chemokine RMSD between the two complex structures [30, 31] after the receptors are superimposed is as large as $\sim 8.3\text{\AA}$.

The structures corroborated the overall correctness of the complex topology and geometry predicted by modeling only a year earlier. The RMSD between the chemokines in the CXCR4: CXCL12 model [54] and in the CXCR4: vMIP-II structure [30], when the receptors are superimposed, is $\sim 9.5\text{\AA}$ (compare to $\sim 8.3\text{\AA}$ between the two experimental structures). However, the expectation of large conformational changes in the receptor that are needed to accommodate a chemokine was fully met, and pre-structure modeling could not predict these changes with acceptable accuracy. The changes involve repositioning of TM helices, re-orienting the receptor N-terminus from almost parallel to the membrane plane to almost perpendicular, and formation of an extra helical turn in TM1 – all to form a “comfortable” CRS1 for the chemokine to bind (Figure 2H).

Along with providing these insights, the structures highlighted major technological challenges: both were made possible by extremely slow (US28: CX₃CL1) or non-existent (CXCR4: vMIP-II) dissociation of the complexes; both feature only a part of CRS1 and completely lack receptor tyrosine sulfation. Furthermore, comparison of the well-resolved CRS2 interactions demonstrates that different receptor: chemokine pairs utilize distinct structural determinants and conformational mechanisms to achieve specificity of binding and signaling: this diversity hinders transfer of structural knowledge between different complexes by homology and emphasizes the importance of detailed studies of individual pairs.

Anatomy of receptor: chemokine interfaces: the two-site hypothesis revisited

By serving as homology modeling templates in relevant conformations, the structures gave a substantial boost to molecular modeling and docking efforts [30, 58]. This helped further expand on structural and functional understanding of the binding interface and the various constituent epitopes in the context of different receptor: chemokine pairs.

The fundamental recognition differences between CC and CXC families are now becoming clear [30]. Modeling and bioinformatics suggest that the affinity of CC complexes is driven by receptor sulfotyrosine recognition at CRS1, as the abundant sulfotyrosines in the proximal N-termini of CC receptors correlate with numerous basic residues in the N-loops and 40s loops of their cognate CC chemokines (Figure 3A–B). Additionally, a conserved base at the N-terminus-TM1 junction of the receptor helps maintain a favorable β -sheet interaction with the CC chemokine homodimerization interface in CRS1.5. By contrast with the CC family, the lack of sulfotyrosines in the proximal N-termini of CXC receptors and the predominantly neutral nature of the N-loop/40s loop regions of CXC chemokines suggest a secondary role for CRS1 in the CXC family recognition. Instead the affinity is driven by a highly conserved acid in CRS2 (D^{6.58} using Ballesteros-Weinstein notation [59]) that provides a complementary charge for a highly conserved base preceding the CXC motif of

the chemokine (Figure 3C–D). The predominant roles of CRS1 and CRS2 residues in binding affinity of CC and CXC complexes, respectively, have been characterized experimentally for several receptors [51, 60–65], supporting this hypothesis.

Modeling also proposed a structural explanation for the role of CRS2 in CXCR4: CXCL12 agonism [30, 58]. It has been long known that a specific sequence in the distal N-terminus of CXCL12 (K1 followed by P2) serves as a critical signaling determinant: even minor modifications to it convert CXCL12 into an equally potent antagonist [60]. In contrast to its signaling capacity, the binding affinity of CXCL12 to CXCR4 is much less sequence-dependent: numerous high affinity antagonists can be obtained by a 1-residue N-terminal extension of CXCL12 and by varying the appended residue simultaneously with residues 1–3 of the native sequence [16]. However, the binding affinity is strongly dependent on the length of the chemokine N-terminus: the affinity loss caused by 3-residue N-terminal truncation cannot be restored by varying residues 4–7 [16]. Complex models suggest that formation of a salt bridge between the N-terminal amine of the chemokine and the side chain of a unique CXCR4 residue, D97^{2.63}, contributes a large fraction of the binding energy, while specific interactions between the side-chain amine of K1 with E288^{7.39} and the side chain of P2 with Y116^{3.32} and W94^{2.60} form a spatial arrangement that initiates signaling [30, 58] (Figure 4A). Residues E^{7.39} and Y^{3.32} are conserved across chemokine receptors. Although their amino acid conservation in other GPCRs is low, the corresponding residue positions are well-characterized ligand binding and signaling determinants in aminergic, opioid, and adenosine receptors [66, 67] (so-called *functional conservation*).

Understanding of structural details of receptor:chemokine interactions is important as it may assist rational design of chemokine- and receptor-based therapeutics and tools. In the past, the desired pharmacological profiles were achieved using selection-based strategies, as in the design of CCL5-based HIV inhibitors with distinct profiles for CCR5 activation, sequestration, and post-endocytic trafficking [4, 68], or as in generation of anti-inflammatory CXCL12 variants [16]. Structures and models enable an alternative, rational approach to these tasks, as in manipulating the selectivity of CX₃CL1 away from its human receptor and towards the virally encoded US28, to create a toxin carrier selectively targeting US28-expressing cells [17], or as in the design of a potent CXCR1-based CXCL8 capture agent for diagnostic applications [55].

Pharmacological roles for novel receptor:chemokine recognition epitopes

While supporting the overall correctness of the canonical two-site hypothesis, the structures and models suggest that it is an incomplete, oversimplified description of the receptor:chemokine complex anatomy. Additional, yet unnamed interaction epitopes become obvious. For example, the CRS1.5 interaction brings the residues in the chemokine N-loop into direct proximity with ECL3 of the receptor (Figure 4B, cyan). On the opposite side of the binding pocket, an unexpected interaction between ECL2 of the receptor and the 30s loop of the chemokine is apparent (Figure 4B, magenta). These epitopes provide a rational basis for previously observed phenomena, such as the critical role of the first N-loop residue in receptor binding and activation by CCL2 [69] and CCL4 [70], or the intriguing role of a basic residue in the 30s loop of CCL2 [61] and especially CCL5. In N-terminally modified

CCL5 variants, this residue (K33) appears to control their ability to internalize CCR5, and the chemokine anti-HIV potency with it – without effects on CCR5 binding or signaling [71]. Thus close examination of the new epitopes may in the future enable rational design of therapeutics with novel, non-canonical pharmacology.

Interestingly, a few studies are now converging on the hypothesis about the involvement of CXC chemokine dimerization interface (i.e. its β_1 -strand) in receptor interactions through formation of an anti-parallel β -sheet with the distal N-terminus of the receptor (Figure 4B, red). Methyl transferred cross-saturation NMR experiments suggested the involvement of CXCL12 β_1 -strand in its interactions with CXCR4 in 2009 [72]. More recently, the interaction was explicitly observed in the NMR structure of monomeric CXCL12 with an N-terminal peptide of CXCR4 [43], although in this case, the observed β -sheet is parallel (Figure 2D). This discrepancy is likely due to the absence of the context normally provided by the receptor TM domain. In the CXCR1: CXCL8 case, N-terminal peptides from the receptor promote chemokine dimer dissociation [73] and mutations at the dimer interface reduce receptor affinity [74]. Finally, for the complex of CXCL12 with its atypical receptor ACKR3, the hypothesis is supported by radiolytic protection of both receptor and chemokine regions in the complex vs. the uncomplexed state [75]. Therefore, it appears that the CXC chemokine dimerization interface is repurposed for receptor interactions in a manner that is analogous and complementary to the repurposing of the CC/CX₃C chemokine dimerization interface observed in crystallized receptor:chemokine complexes [30, 31].

The biological role of this repurposing remains to be elucidated. Unlike the dimerization of CC chemokines that makes them signaling incompetent, CXC chemokine dimerization is not mutually exclusive with receptor binding [30, 50, 76]. Nevertheless, in the CXCR1: CXCL8 complex, dimerization of the chemokine lowers its affinity for the receptor [73]. Even more intriguingly, in the context of the CXCR4: CXCL12 interaction, chemokine dimerization alters its pharmacology by converting it from balanced into a functionally selective (G protein biased) agonist [77]. Thus the pharmacological potential of this interaction epitope may be complex-dependent and deserves further study in different receptors and contexts.

Conclusions

Despite the far-reaching consequences of chemokine receptor signaling in health and disease, structural information on how receptors interact with chemokines has been long lacking. Year 2015 brought the first high-resolution insights into the structural basis of receptor:chemokine recognition. The structures of two crystallized distantly related receptor:chemokine complexes established a conserved complex architecture but suggested that the interaction details vary between different receptors and chemokines. From structure analysis and molecular modeling, a number of new interaction epitopes emerged that, on one hand, provide structural explanation for earlier experimental observations, and on the other, suggest novel approaches to pharmacological modulation of receptor:chemokine interactions.

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Special interest (•)

Outstanding interest (••)

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Highlights

- Chemokine interactions with receptors trigger cell migration in immunity and cancer
- 2015 brought high-resolution insights into the structural basis of these interactions
- Receptor:chemokine complexes share a conserved architecture, but details vary
- Interaction is mediated by multiple functionally distinct epitopes
- Chemokine dimerization interfaces are repurposed for receptor binding
- Novel epitopes hold promise for fine-tuned pharmacological modulation

Figure 1. Chemokines and their preferred interaction interfaces

(A–D) Ribbon diagrams of four families of chemokines found in mammals: CC, CXC, CX₃C, and XC. The different spacing of the conserved N-terminal cysteines causes variations in the conformations of the flexible N-termini, marked by arrows. Ribbon coloring is the same as in (I–L). (E–H) Preferred dimerization geometry of chemokines from different families. (I–L) Representative chemokines from each family are shown as molecular surfaces and colored according to the frequency and strength of inter-molecular

contacts that they make with diverse binding partners in the available crystal structures: darker red color indicates a preferred interaction interface.

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Figure 2. Structural insights into receptor:chemokine recognition

(A) The canonical two-site hypothesis of receptor:chemokine interaction: the flexible sulfotyrosinated N-terminus of the receptor (chemokine recognition site 1, CRS1, important for binding affinity) binds to the globular core of the chemokine, while the transmembrane (TM) domain pocket of the receptor (chemokine recognition site 2, CRS2, critical for signaling) accommodates the distal N-terminus of the chemokine. (B–D) NMR structures of chemokines with isolated N-termini of their receptors represent CRS1 interactions outside the full length receptor context. The structure of CXCL8 with a chemically modified N-

terminus of CXCR1 [49] (**B**) is the only structure where the receptor peptide orientation is consistent with the full-length receptor complex. (**E**) An X-ray structure of CXCL12 in complex with a small molecule inhibitor and a sulfate ion: the inhibitor utilizes the preferred interaction surface of the chemokine. (**F–G**) The first two X-ray structures of full-length receptor:chemokine complexes: the presence of receptor TM domains imposes constraints onto the geometry of CRS1 interactions. (**H**) CXCR4 undergoes a large conformational change in order to accommodate a chemokine.

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Figure 3. Determinants of CC vs CXC receptor:chemokine specificity elucidated by crystallography in conjunction with molecular modeling and bioinformatics
Potential specificity-determining positions are highlighted in the partial sequence alignments and mapped onto structures and models for human CC chemokines (**A**), CC receptors (**B**), CXC chemokines (**C**), and CXC receptors (**D**). The highlighted chemokine and receptor features are complementary and are predicted by modeling to directly interact in complexes that belong to the respective classes.

Figure 4. Receptor:chemokine interaction epitopes

(A) Modeling explains the critical role of chemokine recognition site 2 (CRS2, green) in CXCR4: CXCL12 signaling. (B) The emerging architecture of receptor:chemokine complexes features at least five distinct interaction epitopes, possibly with diverse roles in binding affinity, signaling, and pharmacology. Some of the epitopes are yet unnamed (labeled “CRS?”).