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New paradigms in chemokine receptor signal transduction: moving beyond the two-site model

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Abstract

Chemokine receptor (CKR) signaling forms the basis of essential immune cellular functions, and dysregulated CKR signaling underpins numerous disease processes of the immune system and beyond. CKRs, which belong to the seven transmembrane domain receptor (7TMR) superfamily, initiate signaling upon binding of endogenous, secreted chemokine ligands. Chemokine-CKR interactions are traditionally described by a two-step/two-site mechanism, in which the CKR N-terminus recognizes the chemokine globular core (*i.e.* site 1 interaction), followed by activation when the unstructured chemokine N-terminus is inserted into the receptor TM bundle (*i.e.* site 2 interaction). Several recent studies challenge the structural independence of sites 1 and 2 by demonstrating physical and allosteric links between these supposedly separate sites. Others contest the functional independence of these sites, identifying nuanced roles for site 1 and other interactions in CKR activation. These developments emerge within a rapidly changing landscape in which CKR signaling is influenced by receptor PTMs, chemokine and CKR dimerization, and endogenous non-chemokine ligands. Simultaneous advances in the structural and functional characterization of 7TMR biased signaling have altered how we understand promiscuous chemokine-CKR interactions. In this review, we explore new paradigms in CKR signal transduction by considering studies that depict a more intricate architecture governing the consequences of chemokine-CKR interactions.

Graphical abstract

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Keywords

Seven transmembrane-spanning receptor (7TMR); two-site model; chemokine stoichiometry; 7TMR activation; biased agonism

1. Introduction

Chemokine receptors (CKRs) are cell-surface seven transmembrane domain receptors (7TMRs) that mediate a diverse repertoire of functions, such as immune surveillance and embryonic development, by directly regulating cellular migration, adhesion, growth, and survival. They are also implicated in many pathological processes such as atherosclerosis, HIV infection, tumor metastasis, and autoimmune disorders [1]. Due to their prominent roles in so many disease processes, CKRs have been the target of considerable drug development efforts since the discovery of the chemokine-CKR system in the late 1980s [2, 3].

Chemokines and CKRs demonstrate widespread promiscuity, wherein chemokines may bind multiple receptors and *vice versa*. Of the nearly 50 chemokines and 20 CKRs identified in humans, most bind multiple counterparts, with a minority involved in monogamous interactions. Promiscuous interactions among chemokines and their receptors are increasingly recognized as a mechanism to generate diverse signaling or other functional outcomes using a discrete set of chemokine and CKR components [4, 5]. This characteristic promiscuity may be explained, in part, by their conserved tertiary structure, composed of an unstructured N-terminus, conserved mono- or di-cysteine motif (*e.g.* C, CC, CXC, CX₃C, where X represents a non-cysteine residue), extended loop, three anti-parallel β -strands, and C-terminal α -helix (Fig. 1.A) [1]. One or two conserved disulfides constrain the chemokine fold by linking the cysteine motif with the β 1– β 2 turn (*a.k.a.* the 30s loop) and the β 3-strand.

CKR binding and activation is described as proceeding via a two-step/two-site mechanism, a model which dates back to the mid-1990s. This model is alternatively framed by segregating chemokine-CKR interactions *functionally* (*i.e.* two-step) and *spatially* (*i.e.* two-site). In the functional formulation, site 1 provides affinity and specificity, followed by site 2 which elicits receptor activation. In the spatial formulation, site 1 refers to interactions between the CKR N-terminus (*a.k.a.*, chemokine recognition site 1, CRS1) and the chemokine globular core, and site 2 refers to contacts between residues in the receptor transmembrane (TM) domain (*a.k.a.*, CRS2) and the unstructured chemokine N-terminus [3]. Notably, interactions between chemokines and CKR extracellular loops (ECLs) are variously ascribed to site 1, site 2, or not included in these models at all [1, 6–9].

Isolation of the receptor N-terminal domain has enabled structure determination of several site 1 complexes but numerous difficulties hindered the characterization of full-length receptors. Since 2007, technical innovations have made possible the purification and crystallization of over 100 family A 7TMRs including CKRs. Until recently, only apo structures or those bound to small molecule antagonists were available [10–12]. In 2015, the first structures of chemokine-CKR complexes were solved, detailing chemokine interactions in the TM domain (*i.e.* site 2) [8, 9]. Combination of these site 1 and site 2 structures recently enabled construction of the most detailed chemokine-CKR model to date [13]; and together, these data highlight numerous contacts that fall outside of the conventional spatial and functional definitions of sites 1 and 2. This coupled with an increased awareness of biased agonism (*i.e.* preferential activation of G protein or β -arrestin pathways), non-chemokine ligands (*e.g.* ubiquitin, β -defensins), and the expanding roles of post-translational modifications (PTMs; *e.g.* sulfation, polysialylation) underscores how the two-site model may overlook the complexity and diversity of CKR signaling that we now appreciate two decades after it was proposed [4, 5, 14–17].

The goal of this review is to highlight instances in which the two-site model inadequately addresses more complex features of chemokine-CKR interactions. In doing so, we hope to broaden the reader's appreciation of the mechanistic details involved in CKR signal transduction. We emphasize that the two-site model has served as a useful framework to understand CKR activation, and in some cases may sufficiently describe binding and activation. Nevertheless, we believe it is advantageous to look beyond the functional and structural roles segregated into site 1 or site 2, to delineate new capacities for interactions that have not been well described by either site, and to include new features that have been discovered since the original conception of the two-site model.

2. Beyond Site 1

2.1. Origins of the two-site model and early studies of the site 1 interface

The two-step/two-site model of chemokine-CKR interactions was realized almost 20 years ago through the work of three contemporaneous studies [18–20]. First, Monteclaro and colleagues used a chimera of the CCR2 N-terminal domain and the CCR1 TM region to show that the receptor N-terminus was sufficient to recognize CCL2 with high affinity and recapitulate the native interaction [18]. Notably, the complementary CCR1–CCR2 chimera exhibited a 30-fold decrease in G protein signaling, demonstrating that the CCR2 N-terminus is essential for chemokine recognition but not signaling. In a follow-up study they showed that high affinity CCL2 binding was completely dependent upon the presence of the CCR2 N-terminus and could be fully recapitulated using only a membrane tethered N-terminal peptide [19]. Crump and colleagues also hypothesized a two-site mechanism through studies of the chemokine rather than the receptor. They showed that mutation of the CXCL12 N-terminus attenuated signaling activity without significant loss of affinity (*e.g.* 3–13-fold increase in binding K_d) [20]. Taken together, these studies suggested that the site 1 and site 2 interactions were spatially and functionally independent, with site 1 conferring receptor specificity and affinity, and site 2 mediating receptor activation. Over time, other functional studies led to the consensus that this model was broadly applicable to the

chemokine-CKR system [21]. Interestingly, the two site model of chemokine-CKR interactions was predated by an analogous model described for interactions between the inflammatory protein C5a and its receptor, suggesting broad applicability of this model among other GPCRs with protein ligands [22].

At the same time other studies began to probe the site 1 interface in greater detail. Alanine scanning of CXCL8 identified residues in an extended loop between the conserved N-terminal cysteine(s) and the 3₁₀-helix (a.k.a. the N-loop) [23]. Unlike CXCL8, CXCL1 is a high affinity CXCR2 ligand with weak affinity for CXCR1. Exchange of seven CXCL1 N-loop residues with those of CXCL8, a high affinity CXCR1 ligand, resulted in a molecule capable of recognizing both receptors [24, 25]. Using a similar chimera approach, Crump and colleagues showed that insertion of the CXCL12 N-loop into unrelated CXC-family chemokines (CXCL1 and CXCL10) rendered them capable of binding and activating CXCR4 [20]. Subsequent studies expanded the importance of this region for site 1 interactions to other CC and CXC chemokines, establishing the N-loop as a critical motif for CKR recognition [19, 20, 26, 27].

While these and related functional studies have helped define functional roles for the N-loop and other chemokine domains in signal transduction, NMR titration experiments have historically been used to define structural interactions contributing to site 1 recognition. One of the most common NMR-based approaches has been to titrate unlabeled, CKR N-terminal peptides into purified, [*U*-¹⁵N]-labeled chemokines to identify chemokine residues that participate in direct site 1 binding interactions. This and related approaches have been used to map the chemokine site 1 binding interactions for CCL11:CCR3 [28], CCL21:CCR7 [29], CCL24:CCR3 [30], CXCL8:CXCR1 [31, 32], CXCL10:CXCR3 [26], CXCL12:CXCR4 [13, 33–37], and CX3CL1:CX3CR1 [38]. Collectively, these studies demonstrate the essential role of the chemokine N-loop in directly binding CKR N-termini, and are validated by soluble chemokine-CKR structures (discussed in section 2.4).

2.2. Site 1 interactions: chemokine allostery and conformational dynamics

While early studies of chemokine-CKR interactions suggested the functional and spatial independence of site 1 interactions, more recent studies suggest that site 1 interactions can alter functional outcomes. In a study of CXCL8 activation of CXCR2 and CXCR1, Rajarathnam and colleagues identified an important Gly-Pro (GP) sequence in the 30s-loop that had large conformational effects on CXCL8 when mutated, causing CXCL8 to activate CXCR1 and CXCR2 in unique ways [39]. While some GP mutants activated both receptors with similar potencies, two mutants (G31A and P32G) displayed a modest reduction in affinity at CXCR2 but completely lost the ability to elicit CXCR2-mediated calcium release. These same mutants lost all binding and calcium signaling at CXCR1. The study demonstrated that the change in signaling was due to intramolecular interactions between the GP motif of the 30s-loop and the conserved “ELR” motif of the CXCL8 N-terminus, which is known to be important for CXCR2 activation. The authors employed molecular dynamics (MD) simulations to show conformational switching for some CXCL8 mutants interconverted the 30s-loop between a type-I and type-II β-turn, thereby altering the conformation and orientation of the chemokine N-loop and N-terminus allosterically. They

suggested that chemokines exist in conformational ensembles, and receptor binding and activation involves conformational selection on the part of the ligand and receptor. In this way, different CKR N-termini may selectively bind specific orientations of the chemokine ensemble, thereby eliciting unique functional outcomes at two separate receptors (see Section 5.1 on biased signaling).

In another study, the same group examined the role of the residue sandwiched between the conserved cysteines of CXCL8. Conversion of the CXC motif to a CC motif greatly reduced the binding affinity for both CXCR1 and CXCR2 and rendered it incapable of activating CXCR2 [40]. The mutation did not affect the chemokine fold, dimerization, or glycosaminoglycan (GAG) binding, suggesting that the attenuated binding and signaling properties were a consequence of altered intramolecular dynamics. Supported again by MD simulations, the authors suggested that allosteric site 1 interactions may in effect ‘steer’ the orientation of the chemokine N-terminus within the receptor TM bundle. Similar studies of vMIP-II and CX3CL1 cysteine motifs suggest that “conformational switching” may be a more general phenomenon among the chemokine family [41]. These studies demonstrate that subtle structural changes in one chemokine domain can significantly alter receptor activation by eliciting conformational changes in another domain. In effect, these studies challenge the structural and functional independence of site 1 and site 2 interactions.

2.3. Complex roles for receptor post-translational modifications

Farzan and colleagues expanded the scope of interactions underlying site 1 recognition by showing that sulfation of tyrosines in the CCR5 N-terminus enhanced affinity for CCL3 and CCL4 [42]. This and later studies broadened the repertoire of CKR post-translational modifications (PTMs) to include glycosylation, demonstrating that in addition to enhancing chemokine affinity, PTMs can regulate functional outcomes of site 1 interactions [17, 21].

CKRs undergo enzymatic, O-sulfate modification by tyrosyl protein sulfotransferase (TPST) during processing in the *trans*-Golgi network. The presence of these sulfotyrosine (sTyr or sY) PTMs at receptor N-termini enhances binding of chemokine ligands and affects receptor activation in many chemokine-CKR systems [16, 42–49]. The structural contributions of sTyr modifications to site 1 interactions were defined by the NMR solution structure of a covalently-linked CXCL12 dimer bound to the first 38 amino acids of CXCR4 that was enzymatically sulfated at three tyrosine positions. The CXCL12 locked dimer was engineered out of necessity, as CXCR4 peptide binding altered the CXCL12 monomer-dimer equilibrium, thereby hindering NMR experiments. The observation that tyrosine sulfation enhanced CXCR4 peptide binding, which in turn promotes CXCL12 dimerization, defined an allosteric model in which binding of sTyr peptides at the conserved sTyr pocket causes CXCL12 self-association. For instance, Ziarek and Getschman, *et al.*, found that a sulfated heptapeptide corresponding to the Tyr21 region of CXCR4 specifically and preferentially binds to the CXCL12 dimer while promoting dimerization of WT-CXCL12. These studies represent the first evidence that binding at a pocket, disconnected from the CXC dimer interface, could allosterically regulate chemokine self-association [50]. Specifically, dynamic NMR studies of CXCL12 revealed that to accommodate dimer formation, the α -helix of CXCL12 rearranges to an almost 90° angle, perpendicular to the β -

strands [51]. This large motion is triggered by two residues adjacent to the sulfotyrosine binding pocket that serve as a link or “microswitch” back to the C-terminal α -helix of CXCL12 [52]. Importantly, self-association of CXCL12 has significant functional effects, as the locked, dimeric version of CXCL12 elicits a unique signaling profile compared to WT-CXCL12 [36].

Glycosylation of CKR extracellular domains occurs during processing in the endoplasmic reticulum (*i.e.* N-linkage of asparagine residues) or Golgi (*i.e.* O-linkage of serine/threonine residues). Recent studies described a novel functional role for chemokine receptor glycosylation in which polysialic acid (polySia) addition to receptor glycans allows CKRs to discriminate between chemokine binding partners [17]. CCR7 is polysialylated by the enzymes ST8Sia II and ST8Sia IV on the surface of patrolling dendritic cells. This rare PTM is utilized by CCL21, which has an unusual extended C-terminal tail not shared by the other CCR7 ligand, CCL19. When CCR7 is polysialylated, CCL21 binds CCR7 with high affinity due to an interaction between the polySia of CCR7 and the C-terminus of CCL21. This interaction is thought to release CCL21's tail from an autoinhibitory interaction with its chemokine core, freeing its N-terminus to bind and activate CCR7. In the examples described for both tyrosine sulfation and polysialylation, interactions between these receptor PTMs and chemokine site 1 domains dictate unique functional outcomes. Despite some shared features of PTMs in chemokine-CKR signal transduction (*e.g.* sTyr enhancement of chemokine affinity/potency [16]), many features appear to be context-specific (*e.g.* polySia alteration of CCL21 activity [17]) or even contradictory (*e.g.* sTyr promotes and inhibits chemokine dimerization for different chemokine-CKR pairs [16]), preventing the prediction of PTMs on biological activity in the absence of experimental data.

2.4. Complex interactions between receptor N-termini and the chemokine core

An early model for the interaction of CXCL8 with the CXCR1 N-terminus set the structural precedent for site 1 formation, corroborating the direct involvement of the N-loop and expanding the interface to include the *chemokine cleft*, formed by the N-loop and β 2/ β 3 turn [32]. Nevertheless, this NMR structure required a biased NMR structure refinement procedure due to weak binding of their modified CXCR1 peptide, suggesting the need for more site 1 complexes to validate the site 1 interface [32]. To date, six site 1 complexes (four NMR [13, 32, 35, 53] and two crystallographic [8, 9]) have been determined in which the receptor peptide adopts three different orientations. Indeed, in all structures except the CCL11:CCR3 complex, the receptor lies nearly perpendicular to the β -sheet axis primarily contacting the N-loop, chemokine cleft and β -strands, validating the site 1 interface defined by the CXCL8:CXCR1 structure. Despite this common interface, the site 1 complexes demonstrate considerable architectural diversity. For instance, the orientation of the N- and C-termini of the CXCR4 peptide is inverted when bound to CXCL12 compared to other site 1 complex structures. While a recent review has suggested that this orientation is incompatible with chemokine N-terminal insertion into the TM domain [54], flexible docking of the monomeric CXCL12:CXCR4₁₋₃₈ structure into CXCR4 demonstrates that this distinct directionality may facilitate rotation of CXCL12 relative the CXCR4 TM bundle so that it is positioned to form extensive site 2 and intermediate-site interactions [13]. Specifically, our model predicts that CXCR4 assumes a bent conformation adjacent to the

conserved N-terminal Pro-Cys (PC) motif relative to other chemokine-CKR complexes (Fig. 1B, discussed in Section 4.1) [13]. Despite their differences, all six receptors form apolar and electrostatic contacts, often through a highly conserved tyrosine with the chemokine cleft.

Dimeric CXCL12 was recently identified as a biased agonist that induces G protein signaling but is incapable of promoting β -arrestin recruitment or cellular migration [13, 36, 55]. Regardless of quaternary structure CXCR4 makes specific contacts with the N-loop and chemokine cleft, contributing 50% of the total site 1 binding energy [50], but residues of the CXCR4 N-terminal domain adopt two distinct conformations when bound to CXCL12 monomer and dimer (Fig. 1C–E). For example, CXCR4 residues 7–9 contribute an intermolecular β -strand to monomeric CXCL12 and residues 4–6 tuck into a hydrophobic pocket abutting the C-terminal helix (Fig. 1C, D) [13]. These contacts are supported by mutagenic and NMR experiments with the full-length receptor [13, 56, 57]. Self-association with a second CXCL12 molecule competes for the monomer-specific β 1 and helix contacts and displaces those residues of the receptor, which instead form less stable contacts with the second CXCL12 protomer (Fig. 1E) [35]. Inspection of the CXCL12: CXCR4 hybrid model suggests dimeric CXCL12's distinct signaling profile may result from unique contacts with the ECL and TM regions (discussed in Section 4).

It is reasonable to assert that the degree to which the receptor N-terminus wraps around the globular core modulates the chemokine's orientation and interactions with the receptor ECL and TM regions. In the context of biased agonism, the receptor N-terminus may mask or expose epitopes to the receptor ECLs. Taken together, the site 1 interactions may generate functional complexity via unique interactions rather than simply tethering the chemokine and contributing binding energy. Classic definitions of site 1 fail to recognize the diversity of binding modes and unique domains that can interface with receptor N-termini, suggesting a greater spatial and functional complexity than the traditional model would suggest.

3. Beyond Site 2

3.1. A more complicated site 2: the major and minor binding pockets

The recent crystal and NMR structures of chemokine-CKR complexes provide clues that far from following a two-site convention, interactions are diverse and highly specific for each individual chemokine-CKR pair at the extracellular surface [8, 9, 13]. Similarly, a surfeit of 7TMR crystal structures over the past decade is defining how the conserved TM architecture recognizes diverse ligand types and triggers unique signaling outcomes [58, 59]. In particular, the early 7TMR crystal structures divided the orthosteric-binding site (*i.e.* the 'main' ligand binding pocket) into two subpockets [58, 60–62]: the *major subpocket* consists of the cavity defined by TMs 4, 5, and 6, and the *minor subpocket* by TMs 1 and 2. TMs 3 and 7 occupy the interface between the two subpockets and stabilize ligand-CKR interactions in either subpocket [3, 58, 63, 64].

A 2013 analysis of over 40 7TMR structures revealed the majority of co-crystallized family A 7TMR ligands contacted the major subpocket (especially TMs 3, 5, and 6) with few ligands forming even one contact in the minor subpocket [65]. It should be noted this

analysis was enriched for antagonist contact points since inactive 7TMR structures are more abundant. Likewise, peptide-binding receptors (including CKRs) represented a small minority of the crystallized receptors in this study. Nevertheless, reviews of CKR binding determinants show a more equitable distribution of contact points among major and minor subpockets, with many CKR agonists and antagonists alike preferentially binding the minor subpocket alone [3, 7–10, 12, 64]. This trend is borne out among the five CKR-ligand co-complexes, with three of the five co-crystallized ligands primarily occupying the minor subpocket (IT1t: CXCR4, vMIP-II: CXCR4, and CX3CL1: US28), one ligand primarily occupying the major subpocket (CVX15: CXCR4), and one ligand straddling the two subpockets (maraviroc: CCR5) [8–10, 12].

CKRs possess a number of unique features that may explain why ligands more readily sample the minor binding pocket relative to other family A 7TMR subtypes. Firstly, the extracellular portion of TM1 in all CKR structures is inwardly oriented toward the center of the TM bundle, with CXCR4-IT1t displaced 9 Å relative to a prototypical family A member, β_2 -adrenergic receptor (β_2 AR) [10]. TM1 is positioned closer to the adjacent TM7 and creates a more contiguous helix-helix interface [8–10, 12]. Secondly, compared to the β_2 AR, TM1 is 1–2 turns longer extracellularly when the receptor (CXCR4 or US28) is bound to a chemokine ligand (vMIP-II or CX3CL1) and TM7 is 1–2 turns longer regardless of the associated ligand (discussed in Section 4.1). The overall effect of the elongated TM1 and TM7 helices, and the inward orientation of TM1, is to create a larger minor pocket. Another feature that may enrich minor subpocket contacts is that chemokines almost universally bind receptor N-termini [7]. By forming extensive interactions with CKR N-termini, which themselves are linked to TM7 via a disulfide bond, chemokines are positioned directly above the minor subpocket (Fig. 2A, discussed in Section 5.1). Still, small molecule ligands also demonstrate enriched utilization of the minor binding pocket despite making no contacts with CKR N-termini, suggesting ectodomain interactions are not solely responsible [3].

3.2. Role of subpocket specificity in receptor activation

The diversity of CKR ligand binding sites emphasizes the additional level of regulation built into receptor activation compared to major subpocket-biased 7TMRs. The major and minor subpockets contain unique sets of residues that comprise *molecular switches* [4, 64, 66]. *Molecular switches* are conserved receptor ‘hotspots’ that undergo conformational rearrangements following agonist binding, helping to drive global conformational rearrangements required for 7TMR activation [66]. To complicate matters, CKR agonists and antagonists frequently share a subset of receptor contacts, a general feature of 7TMR ligands [67]. For instance, Glu^{7.39} and Trp^{2.60} (Ballesteros-Weinstein nomenclature [68]) frequently serve as contact points for small molecule CKR ligands [3, 64, 69]. Notably, our recent hybrid model of CXCL12 bound to CXCR4 (based on the IT1t: CXCR4 X-ray and LM: CXCR4_{1–38} NMR structures) suggests that unique positional and rotameric states of multiple CXCR4 residues, relative to antagonist-bound structures, contribute to receptor activation [13]. Consequently, site 2 binding might itself be broken down into a series of “choices” dictated by the ligand: 1) selection of the CKR binding-pocket (*i.e.* major subpocket, minor subpocket or a combination of both), and 2) stabilization of subpocket residues in active (or inactive conformations), both of which will have the effect of engaging

(or preventing engagement of) a particular subset of molecular switch residues required to elicit the ligand-associated functional response (Fig. 2B).

The diverse binding modes in the major and minor subpockets place each ligand in the proximity of a unique subset of molecular switches. Considering the major binding pocket, the “conserved core” interaction between Pro^{5.50}, Ile^{3.40}, and Phe^{6.44}, directly below the ligand binding sites of the β_2 AR and μ -opioid receptor (MOR), propagates conformational changes to the intracellular receptor surface for activation [70–72]. Similarly, below the minor pocket is the TxPxW motif (*i.e.* Thr^{2.56}-x-Pro^{2.58}-x-Trp^{2.60}) conserved among most chemokine receptors, although its specific role in receptor activation is not well understood [64, 73]. Pro^{2.58} is important for receptor activation in multiple receptors, including CCR5 [64, 74]. Trp^{2.60} has been consistently identified as a principal binding contact for CKR small molecule antagonists, and when mutated, disrupts their inhibitory effects [3, 75]. Finally, our CXCL12: CXCR4 hybrid model suggests that interactions between the TxPxW motif and residues in TMs 3 and 7 may initiate a concerted rearrangement of a group of hydrophobic residues in the TM region, resulting in receptor activation [13]. Given the unique distribution of both ligand contacts and residues involved in conserved motifs among different TM domains, it is becoming clear that ligand-specific receptor outcomes are a consequence of the stabilization of specific rotameric states in the receptor-binding pocket followed by engagement of unique subsets of molecular switches.

A chemokine’s subpocket “preference” may also depend upon its N-terminal cysteine motif. Qin and colleagues aligned multiple chemokine structures belonging to the CC and CXC subgroups, and noted that CXC chemokines display a characteristic bend immediately preceding the CXC motif, causing their N-terminus to run parallel to the N-loop [8]. In models of CXCL12 bound to CXCR4, they predicted that the bend directs the N-terminus toward the major pocket, whereas vMIP-II (a viral CC chemokine) directs its N-terminus towards the minor subpocket. Since most chemokines are thought to form interactions with receptor N-termini, CC chemokines might be predicted to preferentially utilize the minor pocket, whereas CXC chemokines would be able to take advantage of the major binding pocket by redirecting their N-termini via the CXC bend. Despite possessing a distinct “bulge” at its CX3C motif, The N-terminus of CX3CL1 inserts into US28’s minor subpocket [9, 38]. Nevertheless, the drastic deviations in chemokine orientations from those seen in recent structures and models suggests that subpocket preference may be more complicated than can be predicted by the CC/CXC/CX3C motif [8, 13]. More structures of chemokine-CKR complexes will be needed to see if the cysteine-motif elicits subpocket binding preferences.

3.3. Role of binding depth and chemokine N-terminal length in receptor activation

In addition to the ligand’s “choices” to 1) specify a binding pocket, and 2) stabilize subpocket residues, a ligand may also “choose” to bind at a particular depth within that pocket. While four of five CKR-ligand complexes bind high in the orthosteric-binding pocket relative to other 7TMRs, maraviroc binds CCR5 at a depth resembling that of many aminergic ligands [8–10, 12]. A review of mutagenic and functional studies suggests diversity in the depths at which different chemokines contact their respective receptors, with

some N-termini potentially achieving depths comparable to those of deep-binding aminergic ligands [7]. Additional complex structures will be needed to validate that chemokines may contact receptors at different depths within the TM domain, however current data suggest that depth variation presents yet another level of complexity within site 2 manipulated by chemokines to achieve specific signaling outcomes.

Chemokine N-terminal length does not necessarily correlate with the chemokine's binding depth, or its functional properties. Early chemokine structure-function studies showed that truncation of the chemokine N-terminus transforms chemokine agonists into antagonists [20, 76, 77]. Recent studies of the CCL5 N-terminus demonstrate that extension of the chemokine N-terminus produces variant-specific functional outcomes, such as receptor internalization, degradation, recycling, or biased signaling [54, 77]. Similar approaches have since been applied to other chemokines [77]. A recent study by Hanes and colleagues utilized phage display and modeling to suggest how N-terminal length influences receptor function [78]. The authors screened two phage display libraries of CXCL12 for CXCR4 antagonists: a "N-addition" library with a single amino acid addition to CXCL12 and the first four residues of the lengthened chemokine randomized, and a "N-truncation library," where the first four residues were deleted and residues 5–8 randomized. Two results were conclusively found: 1) the N-addition library produced more antagonists, whereas the N-truncation library produced none, and 2) of the N-addition antagonists found, many bound with greater affinity than WT-CXCL12. Interestingly, the screen selected for a subset of variants possessing neutral polar and aliphatic residues, independent of amino acid sequence. The authors propose that despite the "scrambled sequence," similar intermolecular contacts form due to the conformational dynamics of the chemokine N-terminus and receptor pocket. These results are consistent with recent NMR studies of the MOR peptide agonist dynorphin, which was highly dynamic even in a receptor-bound state [78, 79]. In sum, these studies suggest that it may be difficult to make generalizations with respect to chemokine N-terminal length as it relates to CKR activation, as examples of elongated and shortened chemokine variants demonstrate diverse outcomes. Moreover, the dynamic nature of the chemokine N-terminus suggests that elongated peptides may adopt a more folded structure in the orthosteric pocket, as opposed to "diving" more deeply into the TM bundle [78]. Indeed, comparison of the vMIP-II: CXCR4 structure and our CXCL12: CXCR4 model suggests that despite vMIP-II's two additional N-terminal residues, both chemokines reach the same depth by virtue of vMIP-II forming a short N-terminal helix [13].

4. Beyond Site 1.5

4.1. Defining unique, non-site 1, non-site 2 interactions at the receptor surface

The first chemokine-CKR crystal structure showed that in contrast to recognizing spatially distinct receptor domains, the chemokine formed interactions spanning from the receptor N-terminus (*i.e.* site 1) to the receptor TM domain (*i.e.* site 2) [8]. Noting a region that lacked precedence as either site 1 or site 2, Qin and colleagues named an interaction between the chemokine's CC motif and the receptor N-terminal base chemokine recognition site 1.5 (CRS1.5) (Fig. 2.A) [8]. Similar interactions were observed in the CX3CL1:US28 structure, confirming previous predictions that the N-terminal stalk region (in the context of its

disulfide interaction with TM7) serves a direct and essential role in chemokine recognition [9, 80]. More recently we identified analogous sites (*i.e.* CRS1.5-*like*) in our hybrid CXCL12:CXCR4 model and experimentally validated their role in binding and activation [13]. The existence of multiple structurally-validated intermediate interfaces calls into question the assumed *spatial* and *functional* separation between sites 1 and 2, suggesting that other interactions may be overlooked by the two-site model. This section will highlight these and other intermediate chemokine-CKR interactions that do not fall into traditional spatial designations of sites 1 or 2, and will speculate on the functional implications of these interactions.

- *Diverse chemokine orientations:* The most striking difference between the vMIP-II:CXCR4 and CX3CL1:US28 structures is the substantial deviation in chemokine orientation relative to the orthosteric pocket of the two receptors [8, 9]. Specifically, vMIP-II and CX3CL1 are rotated $\sim 35^\circ$ about the C-terminal ends of their C-terminal α -helices (Fig. 3A, B). The CXCL12:CXCR4 hybrid model diverges even more drastically, with CXCL12 rotated $\sim 80^\circ$ relative to vMIP-II [13]. The CXCL12:CXCR4 model was produced through a combination of rigid body docking and computational relaxation such that the backbone, side chain and rigid body positions were optimized. Extensive hydrophobic, polar and charged interactions spanning nearly half of CXCL12's total surface area supports the plausibility of a substantial deviation in orientation relative to vMIP-II. Importantly, variation in chemokine orientation allows these ligands to form unique but overlapping subsets of interactions with receptor ECLs and TM domains.
- *ECL2 links a 'disulfide network':* The orientations of CX3CL1 and vMIP-II relative to their receptor binding pockets demonstrates that CX3CL1, but not vMIP-II, is ideally positioned to interact with ECL2 (Fig. 3A, B). CX3CL1 forms multiple interactions between its 30s-loop and ECL2 of US28, which, intriguingly, completes a *disulfide network* spanning from TM3 to TMs 1 and 7. In addition to a family A-conserved disulfide bond between ECL2 and TM3, most CKRs possess a disulfide that connects the N-terminal stalk with TM7 to form an additional ECL, termed "ECL4" (reviewed in 52) [80]. Owing to these two CKR disulfides, rigid body motions of receptor TM domains elicited by a chemokine at one site (*e.g.* N-loop interactions with TM7) could be efficiently communicated to a distant receptor domain (*e.g.* TM3) via a third interface (*i.e.* ECL2-30s-loop interactions), as suggested by Rajagopalan and colleagues [6]. In short, this structure may provide a glimpse of how multiple-site coupling at extracellular domains might influence chemokine receptor conformation via *lateral (through-chemokine) allostery*.
- *Chemokine loop engagement:* The two chemokine-CKR co-crystal structures exhibit an extended TM interface (relative to other non-CKR family A 7TMRs), formed by 1–2 additional α -helical loops at the extracellular portions of TMs 1 and 7, and the disulfide bond linking TM7

to the receptor N-terminus (Fig. 2.A) [8, 9]. This extended TM1–TM7 interface may also be important for chemokine binding and orientation, analogous to the 30s-loop-ECL2 interactions of CX3CL1 and US28. For instance, Leu13 in the vMIP-II N-loop forms contacts, albeit weakly, with the extended TM1–TM7 interface (*i.e.* residues Cys274^{7,25} and Glu277^{7,28}), as well as the closely positioned Gly273^{ECL3}. These interactions in turn may contribute to the rotation of vMIP-II relative to CX3CL1, causing the vMIP-II 30s-loop to be spatially sequestered, preventing the formation of multiple interactions with CXCR4 (*i.e.* vMIP-II is *30s-loop deficient*). In effect, these chemokine-CKR co-crystal structures suggest that chemokines utilize different loops to stabilize intermediate (*i.e.* non site 1/site 2) interactions with CKRs, thereby guiding chemokine orientation and likely influencing the signaling behavior of unique chemokine-CKR complexes.

- *Diverse uses of ECL2:* Some descriptions of the two site model categorize chemokine interactions with ECLs as site 1 interactions due to their contribution toward specificity and affinity, as well as their interactions with the chemokine body [3, 9]. Nevertheless, ECLs also interact with chemokine N-termini and strongly influence CKR activation, as was recently shown by Chevigné and colleagues at CXCR4 [6, 7, 81, 82]. Chemokine-CKR structures also support the resistance of ECL interactions to site 1 or site 2 classification: CXCR4 preferentially uses ECL2 to stabilize the vMIP-II N-terminus, whereas US28 preferentially uses ECL2 to stabilize the CX3CL1 30s loop, making few N-terminal contacts (Fig. 3A, B) [8, 9]. Evidently, each CKR utilizes ECL2 for different purposes, likely contributing to the unique binding modes of the respective chemokines.

These structural examples illustrate that in some instances, *spatial* delineation of site 1 and site 2 may be artificial. Moreover, each chemokine-CKR complex utilizes distinct combinations of structural domains to specify unique functional outcomes. Having now seen the first comprehensive structural evidence of multiple intermediate, non-site 1/2 interactions, we will consider how receptor ECLs take on *functional* characteristics of sites 1 and 2 alike.

4.2. A “multi-site model” accounts for diverse, interdependent chemokine-CKR interactions

While we are only now beginning to appreciate the extent and diversity of chemokine-CKR interactions following high-resolution structural data, pharmacological evidence predating the two-site model supported a more complex “multi-site model” of CKR activation [83, 84]. Studies of CXCR1 and CXCR2 in the 1990s established a number of important principles concerning CKR recognition and activation as they relate to the receptor extracellular surface, including: 1) different chemokines utilize unique combinations of extracellular domains for the binding and activation of a single CKR [85, 86], 2) a single chemokine may utilize unique combinations of extracellular domains when binding different

CKRs [85], and more generally 3) CKR binding and activation is a consequence of multiple, interdependent variables, particularly the identity of the chemokine and the simultaneous interactions it makes with all adjacent extracellular domains (i.e. N-terminus, ECLs 1–3) (Fig. 2A, B) [83–86]. Similar pharmacological and structural studies expanded these principles to other chemokine-CKR pairs, including CCR1 [81, 87, 88], CCR2 [18, 89, 90], CCR3 [91, 92], CCR5 [18, 88, 93–96], CXCR1 [97–99], CXCR2 [97, 98], CXCR3 [100], CXCR4 [82, 101], CX3CR1 [102].

CXCR3 provides an illustrative example of the interdependence of chemokine “multi-site” binding, chemokine preference for unique CKR conformations, and associated functional outcomes. Xanthou and colleagues disputed the universality of the two-site model for chemokine ligands, proposing instead a “multi-site model in which several distinct extracellular domains are required for efficient ligand binding and receptor activation” [103]. The authors created “gain-of-function” chimeras by individually swapping the N-terminus, ECL1, ECL2, and ECL3 of CXCR3 into CXCR1 (which does not share ligands with CXCR3), and *vice versa* to create “loss-of-function” chimeras. They found that while absence of ECL2 did not abolish chemokine binding, it completely attenuated CXCR3-mediated signaling, supporting a role beyond chemokine recognition. In addition, they showed that each chemokine required a unique subset of interactions to activate CXCR3: CXCL9 required ECL2 and ECL3; CXCL10 required all EC domains; and CXCL11 required the N-terminus, ECL1, and ECL2.

From these experiments it is clear that in addition to possessing *spatial* characteristics of sites 1 and 2, ECL2 may in some instances possess *functional* characteristics of sites 1 and 2. Moreover, this study suggests a “multi-site” mechanism by which different chemokines could, in principle, elicit functionally distinct downstream outcomes. Two lines of evidence support such a mechanism. A recent study demonstrated that the three CXCR3 ligands elicit unique patterns of Gα_i activation, β-arrestin recruitment, and internalization following CXCR3 stimulation [5]. Secondly, CXCL10 preferentially binds inactive conformations of CXCR3, whereas CXCL11 binds both inactive and active conformations [104]. Intriguingly, the distinct functional outcomes elicited by CXCL10 and CXCL11 downstream of CXCR3 can drive opposing cellular and physiologic consequences. For instance, CXCL11 signaling promotes Th1 cell polarization into an immunotolerant T cell subset, resulting in an anti-inflammatory phenotype in a mouse model of multiple sclerosis, whereas CXCL10 promotes pro-inflammatory effects [105].

In all, these data show that chemokines can preferentially bind unique subsets of EC domains and/or available receptor conformations to elicit specific functional outcomes. By illustrating the interdependence of multiple extracellular domains on CKR signal transduction, these studies undermine the functional separation of sites 1 and 2 into recognition and activation, and imply that cooperative interactions influence chemokine recognition at extracellular CKR domains and subsequent functional outcomes. Moreover, unique interactions between each chemokine-CKR pair suggest therapeutic approaches that might target non-overlapping chemokine binding sites to selectively disrupt one chemokine-CKR interaction while maintaining another. In fact, several such *selective allosteric*

modulators have been described for CCR1 and CCR5, demonstrating the feasibility of this approach for the development of highly targeted CKR therapies [3, 106–108].

4.3. Molecular switches at the extracellular surface: allosteric coupling to the TM region

As suggested in the previous section, the role of ECLs is more nuanced than static stabilization of chemokine ligands. Dynamic interactions between ECL and TM residues may act as molecular switches regulating receptor activation, especially in the case of ECL2. While ECL2 displays high variability in sequence, length, and structure even among related receptor subtypes, an impressive number of family A receptors seem to utilize ECL2 in this capacity, including rhodopsin [109–112], the serotonin 5-HT₄ receptor [113], the V(1a) vasopressin receptor (V1aR) [114], the cannabinoid receptor 1 (CB1) [115–117], the β_2 AR [118], the angiotensin II type 1a receptor (AT1aR) [119, 120], the D2 dopamine receptor (D2R) [121], the C5a complement receptor (C5aR) [122], and protease activated receptor 1 (PAR-1) [123], among others [124, 125]. Considering CXCR4, we recently suggested that manipulation of ECL2 by CXCL12 draws ECL2 toward the orthosteric pocket, thereby moving TMs 2 and 3 closer to one another [13]. These TM movements may then help initiate receptor activation, in an analogous mechanism to that predicted for CCR5 [126].

In addition to ECL2, ECLs 3 and 4 have been suggested to act as a tandem molecular switch required for CXCR4 activation [80, 101]. Using mutagenesis and a yeast-based G α_i protein activation screen, Rana and colleagues showed that interaction between TM7 and the CXCR4 N-terminus is essential for receptor activation, and that replacement of the disulfide-bonded cysteines with an electrostatic pair (Arg-Glu) conserves CXCR4 signaling. The authors suggest a model in which the N-terminal-TM7 disulfide undergoes a conformational change during receptor activation that is transmitted to ECL3 and TM6. Comparison of the active state CX3CL1:US28 and inactive state vMIP-II: CXCR4 structures supports this model (Fig. 3.B) [8, 9]. Compared to CXCR4, US28 demonstrates an inward (*i.e.* toward the TM domain) motion of ECL4, seemingly driving an inward motion of ECL3 and the extracellular portion of TM6. In a well characterized mechanism, inward motion of the top of TM6 causes it to rotate about a conserved proline “kink,” resulting in substantial outward movement at the intracellular face to accommodate G protein binding [70].

Similar ECL4 motions may contribute to CXCR4 activation, as suggested by our recent CXCL12: CXCR4 model. Hydrogen bonding between the backbone carbonyl of CXCR4 Phe29^{N-term}, which is adjacent to the N-term-TM7 disulfide, and CXCL12 Ser6, “pulls” the extracellular domain of TM7 toward the orthosteric pocket, which in turn may facilitate rearrangement of residues in CXCR4’s TM domain during activation [13]. These examples suggest that chemokine binding to extracellular domains “primes” the receptor for activation by stabilizing an intermediate conformation, followed by chemokine N-terminal insertion into the receptor TM core and intracellular coupling of signaling effectors (*i.e.* G protein or β -arrestin). More structural studies will be needed to validate the role of ECL4 in the activation of CXCR4, US28, and expand its role to other CKRs. Nevertheless, it is becoming clear that chemokine-ECL interactions serve complex functional roles in CKR recognition and signaling.

5. Beyond canonical chemokine receptor signaling

5.1. Beyond 1:1 interactions: stoichiometry of chemokine-receptor interactions

Chemokine dimerization—Initially thought to be a crystallization artifact, chemokine dimerization has been reexamined over the past years in numerous structural and biochemical studies. It appears now that the vast majority of chemokines are able to form dimeric species, with the monomer-dimer equilibrium being regulated by factors such as pH, anions and interactions with glycosaminoglycans [127, 128].

Depending on the family, chemokines adopt two main oligomeric states with unique structural arrangements and interaction interfaces. CC chemokines form flexible and extended dimers mainly through residues surrounding the cysteine motif [129, 130], whereas CXC chemokines self-associate in more compact dimers via interactions involving the first β -strand [128, 131, 132]. In both types of interactions the N-termini of the two monomers are pointing in opposite directions. Chemokines of the C family, XCL1 and XCL2, have recently been shown to exist in a monomer-dimer equilibrium, unusually requiring complete protein unfolding. XCL dimers adopt a novel dimer conformation that also creates a six-stranded β -sheet [133, 134]. CX3CL1, the only member of the CX3C family, dimerizes in a similar manner to that of CC chemokines [135]. Additionally, some chemokines have been observed to form tetramers (*e.g.* CCL2, CCL5, CCL27, CXCL4, and CX3CL1) or higher-order oligomers [130, 135–139]. Heterodimers of two different CC or CXC chemokines as well as cross-family CC/CXC heterodimers have also been reported [140–142]. Furthermore, HMGB1 (High mobility group protein B1) protein was reported to form complexes with CXCL12 promoting different conformational rearrangements of CXCR4 from that of CXCL12 alone [143]. These findings further challenge the two-step binding model for chemokine-CKR interactions and complicate the question of which stoichiometries are capable of generating functional responses.

Immobilization of chemokines on glycosaminoglycans (GAGs) is an important step for chemokine function as it creates a gradient to direct cell migration and regulates the local chemokine concentration and availability for their receptors. Likewise, GAG binding can favor dimer formation as demonstrated for CCR2-binding chemokines [141], CXCL8 [144, 145], CXCL12 [146–149], XCL1 and XCL2 [133, 134]. Oligomerization has also been shown to increase GAG affinity by creating a more extensive surface for interactions [128].

The biological relevance of chemokine dimerization is still a matter of debate and its impact on receptor binding, stoichiometry and biased signaling remains to be unraveled [128, 132, 150, 151]. As an illustration it has been demonstrated that monomeric and dimeric CXCL12 induce different intracellular signaling responses and opposite effects on cell migration, but other recent studies suggested that this receptor interacts with CXCL12 in a 1:1 stoichiometry [36, 57].

Receptor dimerization—Throughout the past two decades, it has been assumed that CKRs exist as monomers, which behave as fully competent signaling units. This assumption, in part, forms the basis of the classical two-site binding model. However, a number of studies demonstrated that CKRs can form homodimers and/or heterodimers (Fig. 4A) [152].

CKR dimerization has been investigated by various biochemical approaches such as co-immunoprecipitation (co-IP) [153–156], protein fragment complementation (PFC) [157, 158], Förster/Bioluminescence Resonance Energy Transfer (FRET/BRET) [159–161] and GPCR Heteromer Identification Technique (GPCR HIT) [162, 163]. The first structural evidence of CKR dimerization however was provided by the first inactive-state crystal structures of CXCR4 in which the receptor was present as a dimer with the interface between the subunits located at the top of TM5 and TM6 and stabilized by hydrogen bonds [10]. CKRs from all four subfamilies (C, CC, CXC, CX3C) have now been described to form homo- or heterodimers *in vitro* [154, 164–166] and some of them, including CXCR4 and CCR5, were shown to interact with other families of GPCRs such as the α 1A/B-adrenergic receptors [167], opioid receptors [168] or non-GPCR membrane proteins that modulate the activity of the receptor or act as coreceptor for certain non-conventional ligands (Fig. 4A) [169]. Receptor dimerization has been shown to modify ligand binding properties [155, 170] and receptor signaling [153, 167, 171, 172] as well as intracellular trafficking [158]. However, so far there is no *in vivo* data reporting the existence of CKR dimers and therefore their biological relevance remains controversial [152, 173].

Stoichiometry of chemokine-receptor complex—Another poorly understood and highly debated facet of chemokine-CKR interactions is their stoichiometry in functional signaling complexes. As both chemokines and receptors can homo- and heterodimerize, novel hypotheses around the stoichiometry of their interactions have emerged, leading to more complex models than the initially proposed two-step/two-site model. Among them, the 1:2 stoichiometry model where one chemokine binds two receptors simultaneously (Fig. 4B), the 2:1 stoichiometry model in which a chemokine dimer binds one receptor (Fig. 4C), and finally, the 2:2 stoichiometry model in which both the chemokine and the receptor interact as dimers (Fig. 4D) [8, 21, 57, 174]. Complementation studies carried out with CXCR4 mutants partially deficient in site 1 (*i.e.* CRS1) or site 2 (*i.e.* CRS2) were inconsistent with a 1:2 stoichiometry model and supported CXCR4 monomers as fully competent signaling units [57]. These results were later supported by the crystal structure resolution of the viral chemokine vMIP-II in complex with CXCR4 and CX3CL1 in complex with US28, both revealing a 1:1 stoichiometry interaction and an extensive contact surface between the two partners [8, 9]. However, more recent investigation of the preferential binding of monomeric CXCL12 to either monomeric (1:1) or dimeric (1:2) CXCR4 by molecular dynamics simulations proposed that in the 1:2 stoichiometry model, the N-terminus of the chemokine could make more tight contacts with the CRS2 of the second monomer to more efficiently favor signaling than in the 1:1 stoichiometry [174]. Finally, studies of CXCR4:ACKR3 heterodimers suggest that upon CXCL11 binding to CXCR7, conformational changes propagate through the dimer interface activating CXCR4 without the need of its own ligand (1:2*stoichiometry) (Fig. 4E) [172]. Gathering structural and mechanistic information on receptor dimerization, chemokine/receptor stoichiometry and relating it to functional observations remains challenging and necessitates state-of-the-art techniques to strengthen or to invalidate the currently accepted but oversimplified two-step/two-site model [9, 21, 57].

5.2. Beyond G protein signaling: biased signaling at chemokine receptors

Once proposed to serve redundant signaling and functional roles, promiscuous chemokine-CKR interactions are widely believed to confer signaling and functional complexity to the CKR axis [1, 4–6]. Individual chemokines regulate multiple essential functions via independent CKR interactions. In turn, the consequences of each unique interaction depends upon concurrent spatial and temporal expression of both partners [175]. To complicate matters, CKRs were until recently accepted to signal exclusively through canonical G protein pathways and to couple exclusively to the $G_{\alpha_{i/o}}$ G protein subtype. However, mounting evidence shows that some CKRs may also signal through other G protein subtypes (G_{α_s} , $G_{\alpha_{q/11}}$ or $G_{\alpha_{12/13}}$) and activate G protein-independent signaling cascades (e.g. via β -arrestin) in ligand- and cell-specific contexts [176–178]. Analogous findings have been described for countless non-CKR 7TMRs for over a decade, signifying the new paradigm known as biased signaling or functional selectivity [179, 180]. Biased signaling appears to be ubiquitous among CKRs, and it provides a framework for understanding how a finite cast of ligands generates myriad functionally diverse outcomes. Biased signaling has been subdivided into three categories: ligand bias, receptor bias and cellular or tissue bias [4, 5, 181, 182].

Chemokine ligand bias occurs when different chemokines bind the same receptor to elicit distinct cellular responses. Ligand bias has been well documented for both CC and CXC chemokines, including CCL19 and CCL21 at CCR7 [177, 183, 184], CCL27 and CCL28 at CCR10 [5], three chemokine ligands at CXCR3 [5, 184], CXCL7 and CXCL8 at CXCR2 [185], as well as for chemokine ligands at CCR1 [5, 186], CCR2 [187] and CCR4 [188, 189]. Intriguingly, biased responses may be elicited by chemokines bearing unique PTMs, including truncation, citrullination or dimerization as reported for CCL14 [190] and CXCL12 [36]. The characteristic promiscuity of the chemokine-CKR network and poor sequence identity among chemokines, may partly explain the pervasiveness of ligand bias among chemokine ligands [5]. Indeed, variation of the structural interactions discussed in the article (summarized in Fig. 2B) such as chemokine orientation, ECL contacts, and major/minor subpocket selection likely stabilizes distinct active forms of the receptor, eliciting preferential coupling to different intracellular effectors [191]. While we are far from defining the precise structural mechanisms underpinning biased signaling, studies of other family A 7TMRs suggest a role for helical movements of and direct physical interactions with TMs 5 and 6 for G protein and TM7 for β -arrestin coupling, respectively [4, 70, 191–194].

In a peculiar twist, a chemokine agonist at one receptor can antagonize another receptor. Chemokines activating CXCR3 can also bind CCR3, blocking CCL11-induced cell migration and G protein signaling [195]. Similarly, CXCL11 and CCL7, well-characterized agonists at CXCR3 and CCR1/CCR2, respectively, were found to be antagonists at CCR5 [196–198]. In another example of *dual activity* chemokines, vMIP-II binds human and viral CKRs across all four families, acting as an antagonist or an agonist at different receptors [199–201]. Dual activity is often observed in cross-family interactions and could relate to differences in chemokine N-terminal orientation among CC and CXC chemokines

(discussed in Section 3.2) [8]. Nevertheless, antagonism within the same family has also been reported, suggesting that other determinants may also play a role [198, 202].

Receptor bias occurs when a particular receptor preferentially or exclusively couples to a particular effector even in the context of multiple different ligands. Receptor bias has been well- characterized at atypical and viral CKRs [203]. For instance, CXCL12 binding to CXCR4 elicits both G protein and β -arrestin signaling [176, 204], whereas binding to ACKR3 (a.k.a. CXCR7) elicits G protein-independent β -arrestin signaling exclusively [205].

Cellular or tissue bias occurs when the same chemokine-CKR triggers distinct signaling pathways or cellular responses in different cellular contexts. For instance, CCL19 binding to CCR7 induces chemotaxis only in certain cell types [206, 207]. Such cellular bias is unsurprising considering the large variety of cells expressing CKRs, each of which carry unique expression profiles of signaling effectors (*e.g.* G protein subtypes and β -arrestin isoforms), receptor modifying enzymes (*e.g.* GRKs, TPSTs), as well as of other CKRs or receptor modulating partners involved in dimeric receptor interactions.

5.3. Beyond chemokines: binding and signaling by non-chemokine ligands

The chemokine receptors CCR2, CCR3, CCR5, CXCR2, CXCR4 and ACKR3 bind endogenous or virus-encoded ligands other than chemokines. These unconventional ligands vary widely in size, ranging from large proteins (*e.g.* > 100 kDa) to peptides, and often have no sequence or structural similarities with chemokines [15, 169, 208, 209]. Despite their structural dissimilarities, non-chemokine CKR ligands can trigger signaling pathways similar to those induced by endogenous chemokines, although in some cases they initiate unconventional signaling responses [15, 169, 208–212]. For some, binding and signaling relies on the CKR alone [15], while for others, the CKR operates in tandem with another membrane protein that usually serves as primary receptor [210, 213].

One of the best-known examples of non-chemokine CKR ligands is the HIV envelope protein gp120 (120 kDa), which uses CCR5 and CXCR4 as co-receptors for cell type-specific recognition and entry into host cells. To initiate viral membrane fusion with host cell membranes, gp120 first binds to CD4, a primary single TM segment receptor, initiating conformational changes (Fig. 4F). These changes then expose gp120's third variable loop (*i.e.* V3 loop), which in turn interacts with CCR5 or CXCR4 [213–215]. The second interaction was suggested to occur in a two-site binding mechanism similar to that initially proposed for chemokines, with common interacting determinants [211]. Importantly, not only is the interaction of gp120 with CXCR4 or CCR5 required for cell-specific HIV entry but it also leads to the activation of signaling pathways such as JNK and MAPKs, facilitating the early steps of viral replication [216, 217]. Tat, the HIV-trans-activating protein (14 kDa) released extracellularly by infected cells, triggers G protein-mediated signaling and chemotaxis through CCR2 and CCR3 [218, 219] and acts as an antagonist of CXCR4 [220]. Similarly, the HIV-1 matrix protein p17 binds CXCR1 and CXCR2, inducing chemokine-like activity on monocytes through Rho/ROCK activation [221, 222].

More recently, the pseudo-chemokine MIF (macrophage migration inhibitory factor), a pleiotropic and proinflammatory chemotactic cytokine of 12.3 kDa highly expressed by

tumor cells, has been identified as a ligand for CXCR2 [209], CXCR4 [169] and ACKR3 [210], inducing ERK1/2 and ZAP-70 signaling and chemotaxis. As with gp120, the binding of MIF to CKRs requires a primary receptor, CD74, a single segment membrane-spanning protein also known as HLA class II histocompatibility antigen gamma chain (Fig 4G). Although MIF possesses some chemokine-like features, including a pseudo-ELR motif (D45-X-R129) and an N-loop-like region (amino acids 48–57), it lacks the canonical cysteine motif and is therefore classified among the chemokine-like function (CLF) chemokines.

In addition to CXCL12, CXCR4 also binds extracellular ubiquitin (eUb, 8.6 kDa). The eUb-CXCR4 interaction was proposed to follow a two-site binding mode, leading to G protein signaling similar to that induced by CXCL12 [15]. Other endogenous non-chemokine ligands such as human β 3-defensin (HDB-3) (5.1 kDa) [223] and EPI-X4 (1.8 kDa) a 16-amino acid peptide derived from human albumin [224] also interact with CXCR4 but fail to induce intracellular signaling. Finally, human cytosolic proteins such as histidyl- and asparaginyl-tRNA synthetases, released in some inflammatory pathologies, were shown to induce leukocyte migration through CCR3 and CCR5 [225]. Similar results were reported for parasitic asparaginyl-tRNA synthetases which act as agonists for CXCR1 and CXCR2 [226].

The identification of non-cognate ligands for CKRs, some exclusive to a single receptor, others interacting with several receptors across several subfamilies, further emphasizes the complexity of the chemokine receptor network, which seems now more promiscuous and predisposed to bias than initially thought. These new ligands will certainly help to uncover other important physiological and pathological functions for this family of receptors, explain past observations and provide new therapeutic opportunities to modulate chemokine/CKR activity.

6. Conclusion

The two-site model has served as a valuable conceptual framework in which to understand chemokine-CKR signal transduction over the past twenty years. Nevertheless, a growing body of evidence supports a more complex model regulating how chemokines interact with their receptors to mediate an increasingly diverse complement of outcomes. The first structures depicting the complete extracellular and TM chemokine-CKR interfaces identified new interactions resisting site 1 and site 2 categorization. In parallel, advances in our understanding of 7TMR structure, dynamics, and activation are helping to define mechanisms by which chemokine binding is translated into receptor activation. Finally, paradigm shifts from within and outside of the chemokine realm are altering how we understand the complexity of the chemokine system. A more complete, “multi-site model” [103] of how chemokine-CKR interactions elicit functional outcomes will require active-state CKR structures and complementary studies probing the dynamics and functional specificity of unique ligand-receptor pairings.

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References

- Allen SJ, Crown SE, Handel TM. Chemokine: receptor structure, interactions, and antagonism. *Annual review of immunology*. 2007; 25:787–820.
- Horuk R. Chemokine receptor antagonists: overcoming developmental hurdles. *Nature reviews Drug discovery*. 2009; 8:23–33. [PubMed: 19079127]
- Scholten DJ, Canals M, Maussang D, Roumen L, Smit MJ, Wijtmans M, et al. Pharmacological modulation of chemokine receptor function. *British journal of pharmacology*. 2012; 165:1617–43. [PubMed: 21699506]
- Steen A, Larsen O, Thiele S, Rosenkilde MM. Biased and G protein-independent signaling of chemokine receptors. *Frontiers in immunology*. 2014; 5:277. [PubMed: 25002861]
- Rajagopal S, Bassoni DL, Campbell JJ, Gerard NP, Gerard C, Wehrman TS. Biased agonism as a mechanism for differential signaling by chemokine receptors. *The Journal of biological chemistry*. 2013; 288:35039–48. [PubMed: 24145037]
- Rajagopalan L, Rajarathnam K. Structural basis of chemokine receptor function—a model for binding affinity and ligand selectivity. *Biosci Rep*. 2006; 26:325–39. [PubMed: 17024562]
- Thiele S, Rosenkilde MM. Interaction of chemokines with their receptors—from initial chemokine binding to receptor activating steps. *Current medicinal chemistry*. 2014; 21:3594–614. [PubMed: 25039782]
- Qin L, Kufareva I, Holden LG, Wang C, Zheng Y, Zhao C, et al. Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. *Science*. 2015; 347:1117–22. [PubMed: 25612609]
- Burg JS, Ingram JR, Venkatakrishnan AJ, Jude KM, Dukkipati A, Feinberg EN, et al. Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. *Science*. 2015; 347:1113–7. [PubMed: 25745166]
- Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, et al. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science*. 2010; 330:1066–71. [PubMed: 20929726]
- Park SH, Das BB, Casagrande F, Tian Y, Nothnagel HJ, Chu M, et al. Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature*. 2012; 491:779–83. [PubMed: 23086146]
- Tan Q, Zhu Y, Li J, Chen Z, Han GW, Kufareva I, et al. Structure of the CCR5 chemokine receptor-HIV entry inhibitor maraviroc complex. *Science*. 2013; 341:1387–90. [PubMed: 24030490]
- Ziarek JJ, Kleist AB, London N, Raveh B, Malik R, Montpas N, et al. Structural basis for balanced signaling of the chemokine receptor CXCR4 by constitutively monomeric CXCL12. Submitted.
- Lee AY, Phan TK, Hulett MD, Korner H. The relationship between CCR6 and its binding partners: does the CCR6-CCL20 axis have to be extended? *Cytokine*. 2015; 72:97–101. [PubMed: 25585877]
- Saini V, Marchese A, Majetschak M. CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin. *The Journal of biological chemistry*. 2010; 285:15566–76. [PubMed: 20228059]
- Ludeman JP, Stone MJ. The structural role of receptor tyrosine sulfation in chemokine recognition. *British journal of pharmacology*. 2014; 171:1167–79. [PubMed: 24116930]
- Kiermaier E, Moussion C, Veldkamp CT, Gerardy-Schahn R, de Vries I, Williams LG, et al. Polysialylation controls dendritic cell trafficking by regulating chemokine recognition. *Science*. 2016; 351:186–90. [PubMed: 26657283]

18. Monteclaro FS, Charo IF. The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1 α receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *The Journal of biological chemistry*. 1996; 271:19084–92. [PubMed: 8702581]
19. Monteclaro FS, Charo IF. The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1. Receptor activation by a pseudo-tethered ligand. *The Journal of biological chemistry*. 1997; 272:23186–90. [PubMed: 9287323]
20. Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, et al. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *The EMBO journal*. 1997; 16:6996–7007. [PubMed: 9384579]
21. Szpakowska M, Fievez V, Arumugan K, van Nuland N, Schmit JC, Chevigne A. Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. *Biochemical pharmacology*. 2012; 84:1366–80. [PubMed: 22935450]
22. Siciliano SJ, Rollins TE, DeMartino J, Konteatis Z, Malkowitz L, Van Riper G, et al. Two-site binding of C5a by its receptor: an alternative binding paradigm for G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91:1214–8. [PubMed: 8108389]
23. Hebert CA, Vitangcol RV, Baker JB. Scanning mutagenesis of interleukin-8 identifies a cluster of residues required for receptor binding. *The Journal of biological chemistry*. 1991; 266:18989–94. [PubMed: 1918013]
24. Lowman HB, Slagle PH, DeForge LE, Wirth CM, Gillece-Castro BL, Bourell JH, et al. Exchanging interleukin-8 and melanoma growth-stimulating activity receptor binding specificities. *The Journal of biological chemistry*. 1996; 271:14344–52. [PubMed: 8662882]
25. Lee J, Horuk R, Rice GC, Bennett GL, Camerato T, Wood WI. Characterization of two high affinity human interleukin-8 receptors. *The Journal of biological chemistry*. 1992; 267:16283–7. [PubMed: 1379593]
26. Booth V, Keizer DW, Kamphuis MB, Clark-Lewis I, Sykes BD. The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions. *Biochemistry*. 2002; 41:10418–25. [PubMed: 12173928]
27. Fernando H, Nagle GT, Rajarathnam K. Thermodynamic characterization of interleukin-8 monomer binding to CXCR1 receptor N-terminal domain. *The FEBS journal*. 2007; 274:241–51. [PubMed: 17222184]
28. Ye J, Kohli LL, Stone MJ. Characterization of binding between the chemokine eotaxin and peptides derived from the chemokine receptor CCR3. *The Journal of biological chemistry*. 2000; 275:27250–7. [PubMed: 10859315]
29. Love M, Sandberg JL, Ziarek JJ, Gerarden KP, Rode RR, Jensen DR, et al. Solution structure of CCL21 and identification of a putative CCR7 binding site. *Biochemistry*. 2012; 51:733–5. [PubMed: 22221265]
30. Mayer KL, Stone MJ. NMR solution structure and receptor peptide binding of the CC chemokine eotaxin-2. *Biochemistry*. 2000; 39:8382–95. [PubMed: 10913244]
31. Clubb RT, Omichinski JG, Clore GM, Gronenborn AM. Mapping the binding surface of interleukin-8 complexed with an N-terminal fragment of the type 1 human interleukin-8 receptor. *FEBS letters*. 1994; 338:93–7. [PubMed: 8307164]
32. Skelton NJ, Quan C, Reilly D, Lowman H. Structure of a CXC chemokine-receptor fragment in complex with interleukin-8. *Structure*. 1999; 7:157–68. [PubMed: 10368283]
33. Veldkamp CT, Seibert C, Peterson FC, Sakmar TP, Volkman BF. Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-derived factor-1 α (SDF-1 α /CXCL12). *Journal of molecular biology*. 2006; 359:1400–9. [PubMed: 16725153]
34. Seibert C, Veldkamp CT, Peterson FC, Chait BT, Volkman BF, Sakmar TP. Sequential tyrosine sulfation of CXCR4 by tyrosylprotein sulfotransferases. *Biochemistry*. 2008; 47:11251–62. [PubMed: 18834145]

35. Veldkamp CT, Seibert C, Peterson FC, De la Cruz NB, Haugner JC 3rd, Basnet H, et al. Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. *Science signaling*. 2008; 1:ra4. [PubMed: 18799424]
36. Drury LJ, Ziarek JJ, Gravel S, Veldkamp CT, Takekoshi T, Hwang ST, et al. Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:17655–60. [PubMed: 21990345]
37. Gozansky EK, Louis JM, Caffrey M, Clore GM. Mapping the binding of the N-terminal extracellular tail of the CXCR4 receptor to stromal cell-derived factor-1alpha. *Journal of molecular biology*. 2005; 345:651–8. [PubMed: 15588815]
38. Mizoue LS, Bazan JF, Johnson EC, Handel TM. Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1. *Biochemistry*. 1999; 38:1402–14. [PubMed: 9931005]
39. Joseph PR, Sawant KV, Isley A, Pedroza M, Garofalo RP, Richardson RM, et al. Dynamic conformational switching in the chemokine ligand is essential for G-protein-coupled receptor activation. *The Biochemical journal*. 2013; 456:241–51. [PubMed: 24032673]
40. Joseph PR, Sarmiento JM, Mishra AK, Das ST, Garofalo RP, Navarro J, et al. Probing the role of CXC motif in chemokine CXCL8 for high affinity binding and activation of CXCR1 and CXCR2 receptors. *The Journal of biological chemistry*. 2010; 285:29262–9. [PubMed: 20630874]
41. Davis CN, Zujovic V, Harrison JK. Viral macrophage inflammatory protein-II and fractalkine (CX3CL1) chimeras identify molecular determinants of affinity, efficacy, and selectivity at CX3CR1. *Molecular pharmacology*. 2004; 66:1431–9. [PubMed: 15361546]
42. Farzan M, Mirzabekov T, Kolchinsky P, Wyatt R, Cayabyab M, Gerard NP, et al. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell*. 1999; 96:667–76. [PubMed: 10089882]
43. Stone MJ, Chuang S, Hou X, Shoham M, Zhu JZ. Tyrosine sulfation: an increasingly recognised post-translational modification of secreted proteins. *New biotechnology*. 2009; 25:299–317. [PubMed: 19658209]
44. Stone MJ, Payne RJ. Homogeneous sulfopeptides and sulfoproteins: synthetic approaches and applications to characterize the effects of tyrosine sulfation on biochemical function. *Accounts of chemical research*. 2015; 48:2251–61. [PubMed: 26196117]
45. Ouyang Y, Lane WS, Moore KL. Tyrosylprotein sulfotransferase: purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:2896–901. [PubMed: 9501187]
46. Kehoe JW, Bertozzi CR. Tyrosine sulfation: a modulator of extracellular protein-protein interactions. *Chemistry & biology*. 2000; 7:R57–61. [PubMed: 10712936]
47. Fong AM, Alam SM, Imai T, Haribabu B, Patel DD. CX3CR1 tyrosine sulfation enhances fractalkine-induced cell adhesion. *The Journal of biological chemistry*. 2002; 277:19418–23. [PubMed: 11909868]
48. Farzan M, Chung S, Li W, Vasilieva N, Wright PL, Schnitzler CE, et al. Tyrosine-sulfated peptides functionally reconstitute a CCR5 variant lacking a critical amino-terminal region. *The Journal of biological chemistry*. 2002; 277:40397–402. [PubMed: 12183462]
49. Seibert C, Cadene M, Sanfiz A, Chait BT, Sakmar TP. Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:11031–6. [PubMed: 12169668]
50. Ziarek JJ, Getschman AE, Butler SJ, Taleski D, Stephens B, Kufareva I, et al. Sulfopeptide probes of the CXCR4/CXCL12 interface reveal oligomer-specific contacts and chemokine allostery. *ACS chemical biology*. 2013; 8:1955–63. [PubMed: 23802178]
51. Veldkamp CT, Ziarek JJ, Su J, Basnet H, Lennertz R, Weiner JJ, et al. Monomeric structure of the cardioprotective chemokine SDF-1/CXCL12. *Protein science : a publication of the Protein Society*. 2009; 18:1359–69. [PubMed: 19551879]

52. Baryshnikova OK, Sykes BD. Backbone dynamics of SDF-1 α determined by NMR: interpretation in the presence of monomer-dimer equilibrium. *Protein science : a publication of the Protein Society*. 2006; 15:2568–78. [PubMed: 17075134]
53. Millard CJ, Ludeman JP, Canals M, Bridgford JL, Hinds MG, Clayton DJ, et al. Structural basis of receptor sulfotyrosine recognition by a CC chemokine: the N-terminal region of CCR3 bound to CCL11/eotaxin-1. *Structure*. 2014; 22:1571–81. [PubMed: 25450766]
54. Kufareva I, Salanga CL, Handel TM. Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. *Immunology and cell biology*. 2015; 93:372–83. [PubMed: 25708536]
55. Roy I, McAllister DM, Gorse E, Dixon K, Piper CT, Zimmerman NP, et al. Pancreatic Cancer Cell Migration and Metastasis Is Regulated by Chemokine-Biased Agonism and Bioenergetic Signaling. *Cancer research*. 2015; 75:3529–42. [PubMed: 26330165]
56. Kofuku Y, Yoshiura C, Ueda T, Terasawa H, Hirai T, Tominaga S, et al. Structural basis of the interaction between chemokine stromal cell-derived factor-1/CXCL12 and its G-protein-coupled receptor CXCR4. *The Journal of biological chemistry*. 2009; 284:35240–50. [PubMed: 19837984]
57. Kufareva I, Stephens BS, Holden LG, Qin L, Zhao C, Kawamura T, et al. Stoichiometry and geometry of the CXCR4 chemokine receptor 4 complex with CXCL12 ligand: molecular modeling and experimental validation. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111:E5363–72. [PubMed: 25468967]
58. Surguchov JS, Rodrigo J, Kellenberger E, Rognan D. A chemogenomic analysis of the transmembrane binding cavity of human G-protein-coupled receptors. *Proteins*. 2006; 62:509–38. [PubMed: 16294340]
59. Katritch V, Cherezov V, Stevens RC. Diversity and modularity of G protein-coupled receptor structures. *Trends in pharmacological sciences*. 2012; 33:17–27. [PubMed: 22032986]
60. Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human β_2 adrenergic G-protein-coupled receptor. *Nature*. 2007; 450:383–7. [PubMed: 17952055]
61. Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, et al. GPCR engineering yields high-resolution structural insights into β_2 -adrenergic receptor function. *Science*. 2007; 318:1266–73. [PubMed: 17962519]
62. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, et al. High-resolution crystal structure of an engineered human β_2 -adrenergic G protein-coupled receptor. *Science*. 2007; 318:1258–65. [PubMed: 17962520]
63. Schwartz TW, Frimurer TM, Holst B, Rosenkilde MM, Elling CE. Molecular mechanism of 7TM receptor activation--a global toggle switch model. *Annual review of pharmacology and toxicology*. 2006; 46:481–519.
64. Rosenkilde MM, Benned-Jensen T, Frimurer TM, Schwartz TW. The minor binding pocket: a major player in 7TM receptor activation. *Trends in pharmacological sciences*. 2010; 31:567–74. [PubMed: 20870300]
65. Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM. Molecular signatures of G-protein-coupled receptors. *Nature*. 2013; 494:185–94. [PubMed: 23407534]
66. Nygaard R, Frimurer TM, Holst B, Rosenkilde MM, Schwartz TW. Ligand binding and micro-switches in 7TM receptor structures. *Trends in pharmacological sciences*. 2009; 30:249–59. [PubMed: 19375807]
67. Katritch V, Cherezov V, Stevens RC. Structure-function of the G protein-coupled receptor superfamily. *Annual review of pharmacology and toxicology*. 2013; 53:531–56.
68. Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods in Neurosciences*. 1995; 25:366–428.
69. Rosenkilde MM, Schwartz TW. GluVII:06--a highly conserved and selective anchor point for non-peptide ligands in chemokine receptors. *Current topics in medicinal chemistry*. 2006; 6:1319–33. [PubMed: 16918451]

70. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature*. 2011; 477:549–55. [PubMed: 21772288]
71. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, et al. Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature*. 2011; 469:175–80. [PubMed: 21228869]
72. Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, Sanborn AL, et al. Structural insights into micro-opioid receptor activation. *Nature*. 2015; 524:315–21. [PubMed: 26245379]
73. Nomiya H, Yoshie O. Functional roles of evolutionary conserved motifs and residues in vertebrate chemokine receptors. *Journal of leukocyte biology*. 2015; 97:39–47. [PubMed: 25416815]
74. Govaerts C, Blanpain C, Deupi X, Ballet S, Ballesteros JA, Wodak SJ, et al. The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation. *The Journal of biological chemistry*. 2001; 276:13217–25. [PubMed: 11278962]
75. Hall SE, Mao A, Nicolaidou V, Finelli M, Wise EL, Nedjai B, et al. Elucidation of binding sites of dual antagonists in the human chemokine receptors CCR2 and CCR5. *Molecular pharmacology*. 2009; 75:1325–36. [PubMed: 19297521]
76. Clark-Lewis I, Schumacher C, Baggiolini M, Moser B. Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *The Journal of biological chemistry*. 1991; 266:23128–34. [PubMed: 1744111]
77. Chevigne A, Fievez V, Schmit JC, Deroo S. Engineering and screening the N-terminus of chemokines for drug discovery. *Biochemical pharmacology*. 2011; 82:1438–56. [PubMed: 21824467]
78. Hanes MS, Salanga CL, Chowdry AB, Comerford I, McColl SR, Kufareva I, et al. Dual Targeting of the Chemokine Receptors CXCR4 and ACKR3 with Novel Engineered Chemokines. *The Journal of biological chemistry*. 2015; 290:22385–97. [PubMed: 26216880]
79. O'Connor C, White KL, Doncescu N, Didenko T, Roth BL, Czaplicki G, et al. NMR structure and dynamics of the agonist dynorphin peptide bound to the human kappa opioid receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; 112:11852–7. [PubMed: 26372966]
80. Szpakowska M, Perez Bercoff D, Chevigne A. Closing the ring: a fourth extracellular loop in chemokine receptors. *Science signaling*. 2014; 7:pe21. [PubMed: 25185155]
81. Zoffmann S, Chollet A, Galzi JL. Identification of the extracellular loop 2 as the point of interaction between the N terminus of the chemokine MIP-1alpha and its CCR1 receptor. *Molecular pharmacology*. 2002; 62:729–36. [PubMed: 12181451]
82. Chevigne A, Fievez V, Szpakowska M, Fischer A, Counson M, Plessier JM, et al. Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1 infection. *Biochimica et biophysica acta*. 2014; 1843:1031–41. [PubMed: 24480462]
83. Hebert CA, Chuntharapai A, Smith M, Colby T, Kim J, Horuk R. Partial functional mapping of the human interleukin-8 type A receptor. Identification of a major ligand binding domain. *The Journal of biological chemistry*. 1993; 268:18549–53. [PubMed: 8103045]
84. Leong SR, Kabakoff RC, Hebert CA. Complete mutagenesis of the extracellular domain of interleukin-8 (IL-8) type A receptor identifies charged residues mediating IL-8 binding and signal transduction. *The Journal of biological chemistry*. 1994; 269:19343–8. [PubMed: 8034699]
85. Ahuja SK, Lee JC, Murphy PM. CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct. *The Journal of biological chemistry*. 1996; 271:225–32. [PubMed: 8550564]
86. Katancik JA, Sharma A, Radcliff SJ, De Nardin E. Mapping of the extracellular binding regions of the human interleukin-8 type B receptor. *Biochemical and biophysical research communications*. 1997; 232:663–8. [PubMed: 9126332]

87. Pease JE, Wang J, Ponath PD, Murphy PM. The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1 α and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. *The Journal of biological chemistry*. 1998; 273:19972–6. [PubMed: 9685332]
88. Yoshiura C, Ueda T, Kofuku Y, Matsumoto M, Okude J, Kondo K, et al. Elucidation of the CCR1- and CCR5-binding modes of MIP-1 α by application of an NMR spectra reconstruction method to the transferred cross-saturation experiments. *Journal of biomolecular NMR*. 2015; 63:333–40. [PubMed: 26472202]
89. Han KH, Green SR, Tangirala RK, Tanaka S, Quehenberger O. Role of the first extracellular loop in the functional activation of CCR2. The first extracellular loop contains distinct domains necessary for both agonist binding and transmembrane signaling. *The Journal of biological chemistry*. 1999; 274:32055–62. [PubMed: 10542238]
90. Datta-Mannan A, Stone MJ. Chemokine-binding specificity of soluble chemokine-receptor analogues: identification of interacting elements by chimera complementation. *Biochemistry*. 2004; 43:14602–11. [PubMed: 15544331]
91. Duchesnes CE, Murphy PM, Williams TJ, Pease JE. Alanine scanning mutagenesis of the chemokine receptor CCR3 reveals distinct extracellular residues involved in recognition of the eotaxin family of chemokines. *Molecular immunology*. 2006; 43:1221–31. [PubMed: 16102831]
92. Datta A, Stone MJ. Soluble mimics of a chemokine receptor: chemokine binding by receptor elements juxtaposed on a soluble scaffold. *Protein science : a publication of the Protein Society*. 2003; 12:2482–91. [PubMed: 14573861]
93. Blanpain C, Doranz BJ, Bondue A, Govaerts C, De Leener A, Vassart G, et al. The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle. *J Biol Chem*. 2003; 278:5179–87. [PubMed: 12466283]
94. Samson M, LaRosa G, Libert F, Paindavoine P, Detheux M, Vassart G, et al. The second extracellular loop of CCR5 is the major determinant of ligand specificity. *The Journal of biological chemistry*. 1997; 272:24934–41. [PubMed: 9312096]
95. Yoshiura C, Kofuku Y, Ueda T, Mase Y, Yokogawa M, Osawa M, et al. NMR analyses of the interaction between CCR5 and its ligand using functional reconstitution of CCR5 in lipid bilayers. *Journal of the American Chemical Society*. 2010; 132:6768–77. [PubMed: 20423099]
96. Schnur E, Kessler N, Zherdev Y, Noah E, Scherf T, Ding FX, et al. NMR mapping of RANTES surfaces interacting with CCR5 using linked extracellular domains. *The FEBS journal*. 2013; 280:2068–84. [PubMed: 23480650]
97. Nasser MW, Raghuwanshi SK, Malloy KM, Gangavarapu P, Shim JY, Rajarathnam K, et al. CXCR1 and CXCR2 activation and regulation. Role of aspartate 199 of the second extracellular loop of CXCR2 in CXCL8-mediated rapid receptor internalization. *The Journal of biological chemistry*. 2007; 282:6906–15. [PubMed: 17204468]
98. Barter EF, Stone MJ. Synergistic interactions between chemokine receptor elements in recognition of interleukin-8 by soluble receptor mimics. *Biochemistry*. 2012; 51:1322–31. [PubMed: 22242662]
99. Park SH, Casagrande F, Cho L, Albrecht L, Opella SJ. Interactions of interleukin-8 with the human chemokine receptor CXCR1 in phospholipid bilayers by NMR spectroscopy. *Journal of molecular biology*. 2011; 414:194–203. [PubMed: 22019593]
100. Colvin RA, Campanella GS, Manice LA, Luster AD. CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Molecular and cellular biology*. 2006; 26:5838–49. [PubMed: 16847335]
101. Rana S, Baranski TJ. Third extracellular loop (EC3)-N terminus interaction is important for seven-transmembrane domain receptor function: implications for an activation microswitch region. *The Journal of biological chemistry*. 2010; 285:31472–83. [PubMed: 20663868]
102. Chen Y, Green SR, Almazan F, Quehenberger O. The amino terminus and the third extracellular loop of CX3CR1 contain determinants critical for distinct receptor functions. *Molecular pharmacology*. 2006; 69:857–65. [PubMed: 16317113]
103. Xanthou G, Williams TJ, Pease JE. Molecular characterization of the chemokine receptor CXCR3: evidence for the involvement of distinct extracellular domains in a multi-step model of

- ligand binding and receptor activation. *European journal of immunology*. 2003; 33:2927–36. [PubMed: 14515277]
104. Cox MA, Jenh CH, Gonsiorek W, Fine J, Narula SK, Zavodny PJ, et al. Human interferon-inducible 10-kDa protein and human interferon-inducible T cell alpha chemoattractant are allotropic ligands for human CXCR3: differential binding to receptor states. *Molecular pharmacology*. 2001; 59:707–15. [PubMed: 11259614]
105. Zohar Y, Wildbaum G, Novak R, Salzman AL, Thelen M, Alon R, et al. CXCL11-dependent induction of FOXP3-negative regulatory T cells suppresses autoimmune encephalomyelitis. *The Journal of clinical investigation*. 2014; 124:2009–22. [PubMed: 24713654]
106. Jensen PC, Thiele S, Ulven T, Schwartz TW, Rosenkilde MM. Positive versus negative modulation of different endogenous chemokines for CC-chemokine receptor 1 by small molecule agonists through allosteric versus orthosteric binding. *The Journal of biological chemistry*. 2008; 283:23121–8. [PubMed: 18559339]
107. Vaidehi N, Schlyer S, Trabanino RJ, Floriano WB, Abrol R, Sharma S, et al. Predictions of CCR1 chemokine receptor structure and BX 471 antagonist binding followed by experimental validation. *The Journal of biological chemistry*. 2006; 281:27613–20. [PubMed: 16837468]
108. Watson C, Jenkinson S, Kazmierski W, Kenakin T. The CCR5 receptor-based mechanism of action of 873140, a potent allosteric noncompetitive HIV entry inhibitor. *Molecular pharmacology*. 2005; 67:1268–82. [PubMed: 15644495]
109. Ahuja S, Hornak V, Yan EC, Syrett N, Goncalves JA, Hirshfeld A, et al. Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. *Nature structural & molecular biology*. 2009; 16:168–75.
110. Yan EC, Kazmi MA, De S, Chang BS, Seibert C, Marin EP, et al. Function of extracellular loop 2 in rhodopsin: glutamic acid 181 modulates stability and absorption wavelength of metarhodopsin II. *Biochemistry*. 2002; 41:3620–7. [PubMed: 11888278]
111. Yan EC, Kazmi MA, Ganim Z, Hou JM, Pan D, Chang BS, et al. Retinal counterion switch in the photoactivation of the G protein-coupled receptor rhodopsin. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:9262–7. [PubMed: 12835420]
112. Ludeke S, Beck M, Yan EC, Sakmar TP, Siebert F, Vogel R. The role of Glu181 in the photoactivation of rhodopsin. *Journal of molecular biology*. 2005; 353:345–56. [PubMed: 16169009]
113. Baneres JL, Mesnier D, Martin A, Joubert L, Dumuis A, Bockaert J. Molecular characterization of a purified 5-HT4 receptor: a structural basis for drug efficacy. *The Journal of biological chemistry*. 2005; 280:20253–60. [PubMed: 15774473]
114. Conner M, Hawtin SR, Simms J, Wootten D, Lawson Z, Conner AC, et al. Systematic analysis of the entire second extracellular loop of the V(1a) vasopressin receptor: key residues, conserved throughout a G-protein-coupled receptor family, identified. *The Journal of biological chemistry*. 2007; 282:17405–12. [PubMed: 17403667]
115. Ahn KH, Bertalovitz AC, Mierke DF, Kendall DA. Dual role of the second extracellular loop of the cannabinoid receptor 1: ligand binding and receptor localization. *Molecular pharmacology*. 2009; 76:833–42. [PubMed: 19643997]
116. Bertalovitz AC, Ahn KH, Kendall DA. Ligand Binding Sensitivity of the Extracellular Loop Two of the Cannabinoid Receptor 1. *Drug Dev Res*. 2010; 71:404–11. [PubMed: 21170298]
117. Shore DM, Baillie GL, Hurst DH, Navas F 3rd, Seltzman HH, Marcu JP, et al. Allosteric modulation of a cannabinoid G protein-coupled receptor: binding site elucidation and relationship to G protein signaling. *The Journal of biological chemistry*. 2014; 289:5828–45. [PubMed: 24366865]
118. Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, et al. Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature*. 2010; 463:108–12. [PubMed: 20054398]
119. Unal H, Jagannathan R, Bhat MB, Karnik SS. Ligand-specific conformation of extracellular loop-2 in the angiotensin II type 1 receptor. *The Journal of biological chemistry*. 2010; 285:16341–50. [PubMed: 20299456]

120. Unal H, Jagannathan R, Bhatnagar A, Tirupula K, Desnoyer R, Karnik SS. Long range effect of mutations on specific conformational changes in the extracellular loop 2 of angiotensin II type 1 receptor. *The Journal of biological chemistry*. 2013; 288:540–51. [PubMed: 23139413]
121. Shi L, Javitch JA. The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:440–5. [PubMed: 14704269]
122. Klco JM, Wiegand CB, Narzinski K, Baranski TJ. Essential role for the second extracellular loop in C5a receptor activation. *Nature structural & molecular biology*. 2005; 12:320–6.
123. Soto AG, Smith TH, Chen B, Bhattacharya S, Cordova IC, Kenakin T, et al. N-linked glycosylation of protease-activated receptor-1 at extracellular loop 2 regulates G-protein signaling bias. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; 112:E3600–8. [PubMed: 26100877]
124. Unal H, Karnik SS. Domain coupling in GPCRs: the engine for induced conformational changes. *Trends in pharmacological sciences*. 2012; 33:79–88. [PubMed: 22037017]
125. Wheatley M, Wootten D, Conner MT, Simms J, Kendrick R, Logan RT, et al. Lifting the lid on GPCRs: the role of extracellular loops. *British journal of pharmacology*. 2012; 165:1688–703. [PubMed: 21864311]
126. Govaerts C, Bondue A, Springael JY, Olivella M, Deupi X, Le Poul E, et al. Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3. *The Journal of biological chemistry*. 2003; 278:1892–903. [PubMed: 12411445]
127. Veldkamp CT, Peterson FC, Pelzek AJ, Volkman BF. The monomer-dimer equilibrium of stromal cell-derived factor-1 (CXCL 12) is altered by pH, phosphate, sulfate, and heparin. *Protein science : a publication of the Protein Society*. 2005; 14:1071–81. [PubMed: 15741341]
128. Dyer DP, Salanga CL, Volkman BF, Kawamura T, Handel TM. The dependence of chemokine-glycosaminoglycan interactions on chemokine oligomerization. *Glycobiology*. 2015
129. Paavola CD, Hemmerich S, Grunberger D, Polsky I, Bloom A, Freedman R, et al. Monomeric monocyte chemoattractant protein-1 (MCP-1) binds and activates the MCP-1 receptor CCR2B. *The Journal of biological chemistry*. 1998; 273:33157–65. [PubMed: 9837883]
130. Wang X, Watson C, Sharp JS, Handel TM, Prestegard JH. Oligomeric structure of the chemokine CCL5/RANTES from NMR, MS, and SAXS data. *Structure*. 2011; 19:1138–48. [PubMed: 21827949]
131. Rajarathnam K, Clark-Lewis I, Sykes BD. ¹H NMR studies of interleukin 8 analogs: characterization of the domains essential for function. *Biochemistry*. 1994; 33:6623–30. [PubMed: 8204599]
132. Rajarathnam K, Sykes BD, Kay CM, Dewald B, Geiser T, Baggiolini M, et al. Neutrophil activation by monomeric interleukin-8. *Science*. 1994; 264:90–2. [PubMed: 8140420]
133. Fox JC, Nakayama T, Tyler RC, Sander TL, Yoshie O, Volkman BF. Structural and agonist properties of XCL2, the other member of the C-chemokine subfamily. *Cytokine*. 2015; 71:302–11. [PubMed: 25497737]
134. Fox JC, Tyler RC, Guzzo C, Tuinstra RL, Peterson FC, Lusso P, et al. Engineering Metamorphic Chemokine Lymphotactin/XCL1 into the GAG-Binding, HIV-Inhibitory Dimer Conformation. *ACS chemical biology*. 2015; 10:2580–8. [PubMed: 26302421]
135. Hoover DM, Mizoue LS, Handel TM, Lubkowski J. The crystal structure of the chemokine domain of fractalkine shows a novel quaternary arrangement. *The Journal of biological chemistry*. 2000; 275:23187–93. [PubMed: 10770945]
136. Jansma AL, Kirkpatrick JP, Hsu AR, Handel TM, Nietlispach D. NMR analysis of the structure, dynamics, and unique oligomerization properties of the chemokine CCL27. *The Journal of biological chemistry*. 2010; 285:14424–37. [PubMed: 20200157]
137. Lau EK, Paavola CD, Johnson Z, Gaudry JP, Geretti E, Borlat F, et al. Identification of the glycosaminoglycan binding site of the CC chemokine, MCP-1: implications for structure and function in vivo. *The Journal of biological chemistry*. 2004; 279:22294–305. [PubMed: 15033992]
138. Zhang X, Chen L, Bancroft DP, Lai CK, Maione TE. Crystal structure of recombinant human platelet factor 4. *Biochemistry*. 1994; 33:8361–6. [PubMed: 8031770]

139. Ren M, Guo Q, Guo L, Lenz M, Qian F, Koenen RR, et al. Polymerization of MIP-1 chemokine (CCL3 and CCL4) and clearance of MIP-1 by insulin-degrading enzyme. *The EMBO journal*. 2010; 29:3952–66. [PubMed: 20959807]
140. Iida Y, Xu B, Xuan H, Glover KJ, Tanaka H, Hu X, et al. Peptide inhibitor of CXCL4-CCL5 heterodimer formation, MKEY, inhibits experimental aortic aneurysm initiation and progression. *Arteriosclerosis, thrombosis, and vascular biology*. 2013; 33:718–26.
141. Crown SE, Yu Y, Sweeney MD, Leary JA, Handel TM. Heterodimerization of CCR2 chemokines and regulation by glycosaminoglycan binding. *The Journal of biological chemistry*. 2006; 281:25438–46. [PubMed: 16803905]
142. Nesmelova IV, Sham Y, Gao J, Mayo KH. CXC and CC chemokines form mixed heterodimers: association free energies from molecular dynamics simulations and experimental correlations. *The Journal of biological chemistry*. 2008; 283:24155–66. [PubMed: 18550532]
143. Schiraldi M, Raucci A, Munoz LM, Livoti E, Celona B, Venereau E, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *The Journal of experimental medicine*. 2012; 209:551–63. [PubMed: 22370717]
144. Gangavarapu P, Rajagopalan L, Kolli D, Guerrero-Plata A, Garofalo RP, Rajarathnam K. The monomer-dimer equilibrium and glycosaminoglycan interactions of chemokine CXCL8 regulate tissue-specific neutrophil recruitment. *Journal of leukocyte biology*. 2012; 91:259–65. [PubMed: 22140266]
145. Joseph PR, Mosier PD, Desai UR, Rajarathnam K. Solution NMR characterization of chemokine CXCL8/IL-8 monomer and dimer binding to glycosaminoglycans: structural plasticity mediates differential binding interactions. *The Biochemical journal*. 2015; 472:121–33. [PubMed: 26371375]
146. Amara A, Lorthioir O, Valenzuela A, Magerus A, Thelen M, Montes M, et al. Stromal cell-derived factor-1alpha associates with heparan sulfates through the first beta-strand of the chemokine. *The Journal of biological chemistry*. 1999; 274:23916–25. [PubMed: 10446158]
147. Mbemba E, Gluckman JC, Gattegno L. Glycan and glycosaminoglycan binding properties of stromal cell-derived factor (SDF)-1alpha. *Glycobiology*. 2000; 10:21–9. [PubMed: 10570220]
148. Sadir R, Baleux F, Grosdidier A, Imberty A, Lortat-Jacob H. Characterization of the stromal cell-derived factor-1alpha-heparin complex. *The Journal of biological chemistry*. 2001; 276:8288–96. [PubMed: 11087743]
149. Ziarek JJ, Veldkamp CT, Zhang F, Murray NJ, Kartz GA, Liang X, et al. Heparin oligosaccharides inhibit chemokine (CXC motif) ligand 12 (CXCL12) cardioprotection by binding orthogonal to the dimerization interface, promoting oligomerization, and competing with the chemokine (CXC motif) receptor 4 (CXCR4) N terminus. *The Journal of biological chemistry*. 2013; 288:737–46. [PubMed: 23148226]
150. Nasser MW, Raghuwanshi SK, Grant DJ, Jala VR, Rajarathnam K, Richardson RM. Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. *Journal of immunology*. 2009; 183:3425–32.
151. Das ST, Rajagopalan L, Guerrero-Plata A, Sai J, Richmond A, Garofalo RP, et al. Monomeric and dimeric CXCL8 are both essential for in vivo neutrophil recruitment. *PloS one*. 2010; 5:e11754. [PubMed: 20668677]
152. Stephens B, Handel TM. Chemokine receptor oligomerization and allostery. *Progress in molecular biology and translational science*. 2013; 115:375–420. [PubMed: 23415099]
153. Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, Fernandez S, Martin de Ana A, Jones DR, et al. Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *The EMBO journal*. 2001; 20:2497–507. [PubMed: 11350939]
154. Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Albar JP, Martinez AC, Mellado M. The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:3628–33. [PubMed: 10097088]

155. El-Asmar L, Springael JY, Ballet S, Andrieu EU, Vassart G, Parmentier M. Evidence for negative binding cooperativity within CCR5-CCR2b heterodimers. *Molecular pharmacology*. 2005; 67:460–9. [PubMed: 15509716]
156. Vila-Coro AJ, Rodriguez-Frade JM, Martin De Ana A, Moreno-Ortiz MC, Martinez AC, Mellado M. The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1999; 13:1699–710. [PubMed: 10506573]
157. Hammad MM, Kuang YQ, Yan R, Allen H, Dupre DJ. Na⁺/H⁺ exchanger regulatory factor-1 is involved in chemokine receptor homodimer CCR5 internalization and signal transduction but does not affect CXCR4 homodimer or CXCR4-CCR5 heterodimer. *The Journal of biological chemistry*. 2010; 285:34653–64. [PubMed: 20801883]
158. Charette N, Holland P, Frazer J, Allen H, Dupre DJ. Dependence on different Rab GTPases for the trafficking of CXCR4 and CCR5 homo or heterodimers between the endoplasmic reticulum and plasma membrane in Jurkat cells. *Cellular signalling*. 2011; 23:1738–49. [PubMed: 21723943]
159. Percherancier Y, Berchiche YA, Slight I, Volkmer-Engert R, Tamamura H, Fujii N, et al. Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *The Journal of biological chemistry*. 2005; 280:9895–903. [PubMed: 15632118]
160. Blanpain C, Vanderwinden JM, Cihak J, Wittamer V, Le Poul E, Issafras H, et al. Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Molecular biology of the cell*. 2002; 13:723–37. [PubMed: 11854425]
161. Watts AO, van Lipzig MM, Jaeger WC, Seeber RM, van Zwam M, Vinet J, et al. Identification and profiling of CXCR3–CXCR4 chemokine receptor heteromer complexes. *British journal of pharmacology*. 2013; 168:1662–74. [PubMed: 23170857]
162. Mustafa S, Pflieger KD. G protein-coupled receptor heteromer identification technology: identification and profiling of GPCR heteromers. *Journal of laboratory automation*. 2011; 16:285–91. [PubMed: 21764024]
163. See HB, Seeber RM, Kocan M, Eidne KA, Pflieger KD. Application of G protein-coupled receptor-heteromer identification technology to monitor beta-arrestin recruitment to G protein-coupled receptor heteromers. *Assay and drug development technologies*. 2011; 9:21–30. [PubMed: 21133678]
164. Springael JY, Le Minh PN, Urizar E, Costagliola S, Vassart G, Parmentier M. Allosteric modulation of binding properties between units of chemokine receptor homo- and hetero-oligomers. *Molecular pharmacology*. 2006; 69:1652–61. [PubMed: 16467191]
165. Wilson S, Wilkinson G, Milligan G. The CXCR1 and CXCR2 receptors form constitutive homo- and heterodimers selectively and with equal apparent affinities. *The Journal of biological chemistry*. 2005; 280:28663–74. [PubMed: 15946947]
166. Martinez Munoz L, Lucas P, Navarro G, Checa AI, Franco R, Martinez AC, et al. Dynamic regulation of CXCR1 and CXCR2 homo- and heterodimers. *Journal of immunology*. 2009; 183:7337–46.
167. Tripathi A, Vana PG, Chavan TS, Brueggemann LI, Byron KL, Tarasova NI, et al. Heteromerization of chemokine (C-X-C motif) receptor 4 with alpha1A/B-adrenergic receptors controls alpha1-adrenergic receptor function. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; 112:E1659–68. [PubMed: 25775528]
168. Suzuki S, Chuang LF, Yau P, Doi RH, Chuang RY. Interactions of opioid and chemokine receptors: oligomerization of mu, kappa, and delta with CCR5 on immune cells. *Experimental cell research*. 2002; 280:192–200. [PubMed: 12413885]
169. Klasen C, Ohl K, Sternkopf M, Shachar I, Schmitz C, Heussen N, et al. MIF promotes B cell chemotaxis through the receptors CXCR4 and CD74 and ZAP-70 signaling. *Journal of immunology*. 2014; 192:5273–84.
170. Sohdy D, Parmentier M, Springael JY. Allosteric transinhibition by specific antagonists in CCR2/CXCR4 heterodimers. *The Journal of biological chemistry*. 2007; 282:30062–9. [PubMed: 17715128]

171. Contento RL, Molon B, Boullaran C, Pozzan T, Manes S, Marullo S, et al. CXCR4-CCR5: a couple modulating T cell functions. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:10101–6. [PubMed: 18632580]
172. Decaillet FM, Kazmi MA, Lin Y, Ray-Saha S, Sakmar TP, Sachdev P. CXCR7/CXCR4 heterodimer constitutively recruits beta-arrestin to enhance cell migration. *The Journal of biological chemistry*. 2011; 286:32188–97. [PubMed: 21730065]
173. Kraemer S, Alampour-Rajabi S, El Bounkari O, Bernhagen J. Hetero-oligomerization of chemokine receptors: diversity and relevance for function. *Current medicinal chemistry*. 2013; 20:2524–36. [PubMed: 23432589]
174. Cutolo P, Basdevant N, Bernadat G, Bachelier F, Ha-Duong T. Interaction of chemokine receptor CXCR4 in monomeric and dimeric state with its endogenous ligand CXCL12: Coarse-grained simulations identify differences. *Journal of biomolecular structure & dynamics*. 2016:1–56.
175. Mantovani A. The chemokine system: redundancy for robust outputs. *Immunology today*. 1999; 20:254–7. [PubMed: 10354549]
176. Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, et al. The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *The Journal of biological chemistry*. 1998; 273:23169–75. [PubMed: 9722546]
177. Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, Struthers RS. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *The Journal of biological chemistry*. 2004; 279:23214–22. [PubMed: 15054093]
178. Corbisier J, Gales C, Huszagh A, Parmentier M, Springael JY. Biased signaling at chemokine receptors. *The Journal of biological chemistry*. 2015; 290:9542–54. [PubMed: 25614627]
179. Masuho I, Ostrovskaya O, Kramer GM, Jones CD, Xie K, Martemyanov KA. Distinct profiles of functional discrimination among G proteins determine the actions of G protein-coupled receptors. *Science signaling*. 2015; 8:ra123. [PubMed: 26628681]
180. Wisler JW, Xiao K, Thomsen AR, Lefkowitz RJ. Recent developments in biased agonism. *Current opinion in cell biology*. 2014; 27:18–24. [PubMed: 24680426]
181. Luttrell LM, Kenakin TP. Refining efficacy: allosterism and bias in G protein-coupled receptor signaling. *Methods in molecular biology*. 2011; 756:3–35. [PubMed: 21870218]
182. Zweemer AJ, Toraskar J, Heitman LH, APIJ. Bias in chemokine receptor signalling. *Trends in immunology*. 2014; 35:243–52. [PubMed: 24679437]
183. Byers MA, Calloway PA, Shannon L, Cunningham HD, Smith S, Li F, et al. Arrestin 3 mediates endocytosis of CCR7 following ligation of CCL19 but not CCL21. *Journal of immunology*. 2008; 181:4723–32.
184. Sauty A, Colvin RA, Wagner L, Rochat S, Spertini F, Luster AD. CXCR3 internalization following T cell-endothelial cell contact: preferential role of IFN-inducible T cell alpha chemoattractant (CXCL11). *Journal of immunology*. 2001; 167:7084–93.
185. Feniger-Barish R, Ran M, Zaslaver A, Ben-Baruch A. Differential modes of regulation of cxc chemokine-induced internalization and recycling of human CXCR1 and CXCR2. *Cytokine*. 1999; 11:996–1009. [PubMed: 10623425]
186. Chou CC, Fine JS, Pugliese-Sivo C, Gonsiorek W, Davies L, Deno G, et al. Pharmacological characterization of the chemokine receptor, hCCR1 in a stable transfectant and differentiated HL-60 cells: antagonism of hCCR1 activation by MIP-1beta. *British journal of pharmacology*. 2002; 137:663–75. [PubMed: 12381680]
187. Berchiche YA, Gravel S, Pelletier ME, St-Onge G, Heveker N. Different effects of the different natural CC chemokine receptor 2b ligands on beta-arrestin recruitment, Galpha signaling, and receptor internalization. *Molecular pharmacology*. 2011; 79:488–98. [PubMed: 21088225]
188. Mariani M, Lang R, Binda E, Panina-Bordignon P, D'Ambrosio D. Dominance of CCL22 over CCL17 in induction of chemokine receptor CCR4 desensitization and internalization on human Th2 cells. *European journal of immunology*. 2004; 34:231–40. [PubMed: 14971049]

189. Ajram L, Begg M, Slack R, Cryan J, Hall D, Hodgson S, et al. Internalization of the chemokine receptor CCR4 can be evoked by orthosteric and allosteric receptor antagonists. *European journal of pharmacology*. 2014; 729:75–85. [PubMed: 24534492]
190. Savino B, Borroni EM, Torres NM, Proost P, Struyf S, Mortier A, et al. Recognition versus adaptive up-regulation and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence. *The Journal of biological chemistry*. 2009; 284:26207–15. [PubMed: 19632987]
191. Liu JJ, Horst R, Katritch V, Stevens RC, Wuthrich K. Biased signaling pathways in beta2-adrenergic receptor characterized by 19F-NMR. *Science*. 2012; 335:1106–10. [PubMed: 22267580]
192. Warne T, Edwards PC, Leslie AG, Tate CG. Crystal structures of a stabilized beta1-adrenoceptor bound to the biased agonists bucindolol and carvedilol. *Structure*. 2012; 20:841–9. [PubMed: 22579251]
193. Wacker D, Wang C, Katritch V, Han GW, Huang XP, Vardy E, et al. Structural features for functional selectivity at serotonin receptors. *Science*. 2013; 340:615–9. [PubMed: 23519215]
194. Szczepek M, Beyriere F, Hofmann KP, Elgeti M, Kazmin R, Rose A, et al. Crystal structure of a common GPCR-binding interface for G protein and arrestin. *Nature communications*. 2014; 5:4801.
195. Loetscher P, Pellegrino A, Gong JH, Mattioli I, Loetscher M, Bardi G, et al. The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP10, are natural antagonists for CCR3. *The Journal of biological chemistry*. 2001; 276:2986–91. [PubMed: 11110785]
196. Petkovic V, Moghini C, Paoletti S, Uguccioni M, Gerber B. I-TAC/CXCL11 is a natural antagonist for CCR5. *Journal of leukocyte biology*. 2004; 76:701–8. [PubMed: 15178708]
197. Napier C, Sale H, Mosley M, Rickett G, Dorr P, Mansfield R, et al. Molecular cloning and radioligand binding characterization of the chemokine receptor CCR5 from rhesus macaque and human. *Biochemical pharmacology*. 2005; 71:163–72. [PubMed: 16298345]
198. Blanpain C, Migeotte I, Lee B, Vakili J, Doranz BJ, Govaerts C, et al. CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. *Blood*. 1999; 94:1899–905. [PubMed: 10477718]
199. Kledal TN, Rosenkilde MM, Coulin F, Simmons G, Johnsen AH, Alouani S, et al. A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science*. 1997; 277:1656–9. [PubMed: 9287217]
200. Lutichau HR, Johnsen AH, Jurlander J, Rosenkilde MM, Schwartz TW. Kaposi sarcoma-associated herpes virus targets the lymphotactin receptor with both a broad spectrum antagonist vCCL2 and a highly selective and potent agonist vCCL3. *The Journal of biological chemistry*. 2007; 282:17794–805. [PubMed: 17403668]
201. Szpakowska M, Chevigne A. vCCL2/vMIP-II, the viral master KEYmokine. *Journal of leukocyte biology*. 2015
202. Petkovic V, Moghini C, Paoletti S, Uguccioni M, Gerber B. Eotaxin-3/CCL26 is a natural antagonist for CC chemokine receptors 1 and 5. A human chemokine with a regulatory role. *The Journal of biological chemistry*. 2004; 279:23357–63. [PubMed: 15039444]
203. de Munnik SM, Smit MJ, Leurs R, Vischer HF. Modulation of cellular signaling by herpesvirus-encoded G protein-coupled receptors. *Frontiers in pharmacology*. 2015; 6:40. [PubMed: 25805993]
204. Sun Y, Cheng Z, Ma L, Pei G. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *The Journal of biological chemistry*. 2002; 277:49212–9. [PubMed: 12370187]
205. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, et al. Beta-arrestin- but not G protein-mediated signaling by the “decoy” receptor CXCR7. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:628–32. [PubMed: 20018651]
206. Ricart BG, John B, Lee D, Hunter CA, Hammer DA. Dendritic cells distinguish individual chemokine signals through CCR7 and CXCR4. *Journal of immunology*. 2011; 186:53–61.
207. Nandagopal S, Wu D, Lin F. Combinatorial guidance by CCR7 ligands for T lymphocytes migration in co-existing chemokine fields. *PloS one*. 2011; 6:e18183. [PubMed: 21464944]

208. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, et al. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science*. 1996; 272:1955–8. [PubMed: 8658171]
209. Hsieh CY, Chen CL, Lin YS, Yeh TM, Tsai TT, Hong MY, et al. Macrophage migration inhibitory factor triggers chemotaxis of CD74+CXCR2+ NKT cells in chemically induced IFN-gamma-mediated skin inflammation. *Journal of immunology*. 2014; 193:3693–703.
210. Alampour-Rajabi S, El Bounkari O, Rot A, Muller-Newen G, Bachelier F, Gawaz M, et al. MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2015; 29:4497–511. [PubMed: 26139098]
211. Cormier EG, Dragic T. The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. *J Virol*. 2002; 76:8953–7. [PubMed: 12163614]
212. Tarnowski M, Grymula K, Liu R, Tarnowska J, Drukala J, Ratajczak J, et al. Macrophage migration inhibitory factor is secreted by rhabdomyosarcoma cells, modulates tumor metastasis by binding to CXCR4 and CXCR7 receptors and inhibits recruitment of cancer-associated fibroblasts. *Molecular cancer research : MCR*. 2010; 8:1328–43. [PubMed: 20861157]
213. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature*. 1996; 381:667–73. [PubMed: 8649512]
214. Dragic T. An overview of the determinants of CCR5 and CXCR4 co-receptor function. *The Journal of general virology*. 2001; 82:1807–14. [PubMed: 11457985]
215. Chevigne A, Delhalle S, Counson M, Beaupain N, Rybicki A, Verschueren C, et al. Isolation of an HIV-1 neutralizing peptide mimicking the CXCR4 and CCR5 surface from the heavy-chain complementary determining region 3 repertoire of a viremic controller. *Aids*. 2016; 30:377–82. [PubMed: 26760231]
216. Popik W, Pitha PM. Early activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase in response to binding of simian immunodeficiency virus to Jurkat T cells expressing CCR5 receptor. *Virology*. 1998; 252:210–7. [PubMed: 9875330]
217. Lee C, Liu QH, Tomkowicz B, Yi Y, Freedman BD, Collman RG. Macrophage activation through CCR5- and CXCR4-mediated gp120-elicited signaling pathways. *Journal of leukocyte biology*. 2003; 74:676–82. [PubMed: 12960231]
218. de Paulis A, De Palma R, Di Gioia L, Carfora M, Prevete N, Tosi G, et al. Tat protein is an HIV-1-encoded beta-chemokine homolog that promotes migration and up-regulates CCR3 expression on human Fc epsilon RI+ cells. *Journal of immunology*. 2000; 165:7171–9.
219. Albin A, Ferrini S, Benelli R, Sforzini S, Giunciuglio D, Aluigi MG, et al. HIV-1 Tat protein mimicry of chemokines. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:13153–8. [PubMed: 9789057]
220. Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, et al. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:11466–71. [PubMed: 11027346]
221. Giagulli C, Magiera AK, Bugatti A, Caccuri F, Marsico S, Rusnati M, et al. HIV-1 matrix protein p17 binds to the IL-8 receptor CXCR1 and shows IL-8-like chemokine activity on monocytes through Rho/ROCK activation. *Blood*. 2012; 119:2274–83. [PubMed: 22262769]
222. Caccuri F, Marsico S, Fiorentini S, Caruso A, Giagulli C. HIV-1 Matrix Protein p17 and its Receptors. *Current drug targets*. 2016; 17:23–32. [PubMed: 26302809]
223. Feng Z, Dubyak GR, Lederman MM, Weinberg A. Cutting edge: human beta defensin 3--a novel antagonist of the HIV-1 coreceptor CXCR4. *Journal of immunology*. 2006; 177:782–6.
224. Zirafi O, Kim KA, Standker L, Mohr KB, Sauter D, Heigle A, et al. Discovery and characterization of an endogenous CXCR4 antagonist. *Cell reports*. 2015; 11:737–47. [PubMed: 25921529]

225. Howard OM, Dong HF, Yang D, Raben N, Nagaraju K, Rosen A, et al. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. *The Journal of experimental medicine*. 2002; 196:781–91. [PubMed: 12235211]
226. Ramirez BL, Howard OM, Dong HF, Edamatsu T, Gao P, Hartlein M, et al. *Brugia malayi* asparaginyl-transfer RNA synthetase induces chemotaxis of human leukocytes and activates G-protein-coupled receptors CXCR1 and CXCR2. *The Journal of infectious diseases*. 2006; 193:1164–71. [PubMed: 16544258]

Fig. 1. Chemokine tertiary structure and implications of site 1 binding modes in chemokine-CKR interactions. (A) The structure of monomeric CXCL12: CXCR4₁₋₃₈ (PDB ID 2N55) modeled into full-length CXCR4 (PDB ID 3ODU) demonstrates conserved features of chemokine tertiary structure [13]. The two steps/sites of the two-step/two-site model are also depicted. (B) Binding sites of the CKR N-termini are shown on chemokines from the two recent chemokine-CKR co-crystal structures with the chemokine represented in the same view (vMIPII: CXCR4: PDB ID 4RWS; CX3CL1: US28: PDB ID 4TX1) [8, 9]. Only TM1 from each receptor is depicted for clarity. The CXCL12: CXCR4₁₋₃₈ structure-based model from Ziarek, et al., is shown for comparison [13]. The location of the conserved Pro-Cys (PC) motif is indicated for each receptor. The orientation of the receptor N-termini varies among the three complexes, suggesting that site 1 contacts may alter subsequent chemokine-CKR interactions. (C) The CXCL12: CXCR4 model based on the CXCL12-monomer: CXCR4₁₋₃₈ structure is shown (i.e. "CXCR4: CXCL12 monomer") along with the CXCR4 N-terminus from the CXCL12-dimer: CXCR4₁₋₃₈ structure (i.e. "CXCR4: CXCL12 dimer"; PDB ID 2K01) [35]. CXCL12 dimerization alters the binding orientation of the CXCR4 N-terminus, which may cause unique binding modes at CXCR4 and ultimately specify functional outcomes. (D) Close-up view of CXCL12: CXCR4 contacts from (C). (E) The CXCL12 dimer is shown with one subunit in orange (aligned to the CXCL12 monomer from C) and the second subunit in grey. CXCL12 dimerization occludes the binding site of CXCR4 N-terminus residues 1-9.

Fig. 2. (A) Chemokine (CK) -chemokine receptor (CKR) interactions encompass many more interactions than those between the chemokine core and the receptor N-terminus (*i.e.* site 1) and those between the chemokine N-terminus and the receptor TM domains (*i.e.* site 2). (B) A “multi-site”/multiple-variable model of CKR activation [103]. Functionally distinct outcomes following chemokine-CKR interactions may be generated by chemokine “selection” of a unique subset of interactions, binding modes, and conformations. These variables are listed numerically to demonstrate how pairs of chemokine-CKR interactions generate complex outcomes.

Fig. 3.

Unique intermediate interactions specify chemokine orientation in two chemokine-CCR complexes. (A) vMIP2 binds CXCR4 such that its 30s-loop is sequestered from forming extensive interactions with extracellular or TM domains of CXCR4 (PDB ID 4RWS). ECL2 of CXCR4 interacts with the vMIP2 N-terminus, boxed. (B) Unlike the vMIP2, CX3CL1 forms extensive intermediate interactions with US28 (PDB ID 4TX1) using its 30s-loop, such that CX3CL1 is stabilized extensively at two separate receptor sites: the N-terminus (*i.e.*, site 1) and ECL2. These extensive interactions may help stabilize US28 in an active state by simultaneously drawing together extracellular domains of US28, aided by a disulfide network comprised of receptor (*i.e.*, N-terminus-TM7 or “ECL4” and TM3-ECL2) and chemokine (CX3C-30s-loop and CX3C-N-loop) disulfides. The active-state US28 structure is overlaid with an inactive-state CXCR4 structure (PDB ID 4RWS) to demonstrate how this disulfide network could facilitate receptor activation (arrows). Unlike the vMIP-II:CXCR4 structure, ECL2 of US28 primarily stabilizes interactions with the 30s-loop of CX3CL1, boxed.

Fig. 4.

CKR homo- and heterodimerization, models of chemokine-CKR stoichiometry, and non-canonical CKR ligands. (A) Interactions between receptors are represented by dots. CC, CXC and atypical chemokine receptor subfamilies are represented in yellow, blue and green, respectively. Non-CKR GPCRs and non-GPCRs are represented in black and orange, respectively. Homodimers are indicated with one-color dots and heterodimers between receptors from different families by two-color dots (monomer 1/ monomer 2). (B) 1:2 stoichiometry in which a chemokine monomer binds a receptor dimer. The disulfide bridges between N-terminus/ECL3 and ECL2/TM3 are depicted as red lines. (C) 2:1 stoichiometry in which a chemokine dimer binds a receptor monomer. (D) 2:2 stoichiometry in which a receptor dimer binds a chemokines dimer. (E) 1:2* stoichiometry in which a receptor dimer interacts with a monomeric chemokine. Upon the binding of the chemokine to one monomer (receptor 1, blue), the conformational changes induced in receptor 1 are propagated to receptor 2 (green) through the dimer interface, activating receptor 2 without the need of chemokine binding. (F) Binding and activation of chemokine receptors CCR5 or CXCR4 by the HIV gp120 envelope protein require another membrane protein, CD4 (red), which acts as a primary receptor for gp120, inducing conformational rearrangements to expose the third variable loop (V3 loop). (G) Binding and activation of CXCR2, CXCR4 and CXCR7 by the pseudo-chemokine MIF also requires the presence of a primary receptor, CD74 (yellow). Like chemokine-CKR interactions, the stoichiometry of MIF:CD74:CKRs is not well established.