

Review

How do chemokines navigate neutrophils to the target site: Dissecting the structural mechanisms and signaling pathways

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A B S T R A C T

Chemokines play crucial roles in combating microbial infection and initiating tissue repair by recruiting neutrophils in a timely and coordinated manner. In humans, no less than seven chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) and two receptors (CXCR1 and CXCR2) mediate neutrophil functions but in a context dependent manner. Neutrophil-activating chemokines reversibly exist as monomers and dimers, and their receptor binding triggers conformational changes that are coupled to G-protein and β -arrestin signaling pathways. G-protein signaling activates a variety of effectors including Ca^{2+} channels and phospholipase C. β -arrestin serves as a multifunctional adaptor and is coupled to several signaling hubs including MAP kinase and tyrosine kinase pathways. Both G-protein and β -arrestin signaling pathways play important non-overlapping roles in neutrophil trafficking and activation. Functional studies have established many similarities but distinct differences for a given chemokine and between chemokines at the level of monomer vs. dimer, CXCR1 vs. CXCR2 activation, and G-protein vs. β -arrestin pathways. We propose that two forms of the ligand binding two receptors and activating two signaling pathways enables fine-tuned neutrophil function compared to a single form, a single receptor, or a single pathway. We summarize the current knowledge on the molecular mechanisms by which chemokine monomers/dimers activate CXCR1/CXCR2 and how these interactions trigger G-protein/ β -arrestin-coupled signaling pathways. We also discuss current challenges and knowledge gaps, and likely advances in the near future that will lead to a better understanding of the relationship between the chemokine-CXCR1/CXCR2-G-protein/ β -arrestin axis and neutrophil function.

1. Introduction

Neutrophils, which constitute the largest subset of circulating leukocytes, function as first responders against microbial infection and tissue injury [1–5]. Chemokines, released in response to insult, play the dual roles of recruiting neutrophils from the vasculature to the tissue and then activating neutrophils in the tissue for microbial killing and/or initiating tissue repair [6,7] (Fig. 1). Precise spatiotemporal control of these processes is essential to mount an effective innate immune response. Humans express seven neutrophil-activating chemokines (NACs) – CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8. NACs exist as monomers and dimers, and exert their function by activating CXCR1 and CXCR2 receptors [8–19]. CXCR1/CXCR2 activation is coupled to G-protein and β -arrestin-mediated signaling cascades, which in turn, are coupled to ultrastructural changes and molecular processes that define neutrophil phenotype and function [20–28]. During the early stages, in the vasculature and in the extracellular matrix, signaling events must promote ultrastructural changes that lead to trafficking of neutrophils to the target tissue. At the end stage, in the

target tissue, signaling events must promote molecular processes for eliminating the invading pathogens or clearing debris and initiating tissue repair. We propose that two forms (monomer and dimer) of the chemokine, activating two receptors, and two signaling pathways, provides better spatiotemporal control of neutrophil function compared to a single form, single receptor, or a single pathway (Fig. 2). At the same time, under pathological conditions, impairment of these processes could result in too few or too many neutrophils and/or hyper-activated or underactivated neutrophils. A dysregulation in chemokine-neutrophil axis has been implicated in acute and chronic diseases of most organs including acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), tuberculosis (TB), arthritis, cystic fibrosis, myocardial infarction, organ rejection, traumatic brain injury (TBI), sepsis, inflammatory bowel disease (IBD), allergies, and several cancers [29–48]. Describing the *in vivo* function of NACs requires not only knowledge of properties such as potency and efficacy for various activities for each of the two receptors but also an appreciation of how these properties apply to *in vivo* NAC levels and oligomeric state as a function of space and time.

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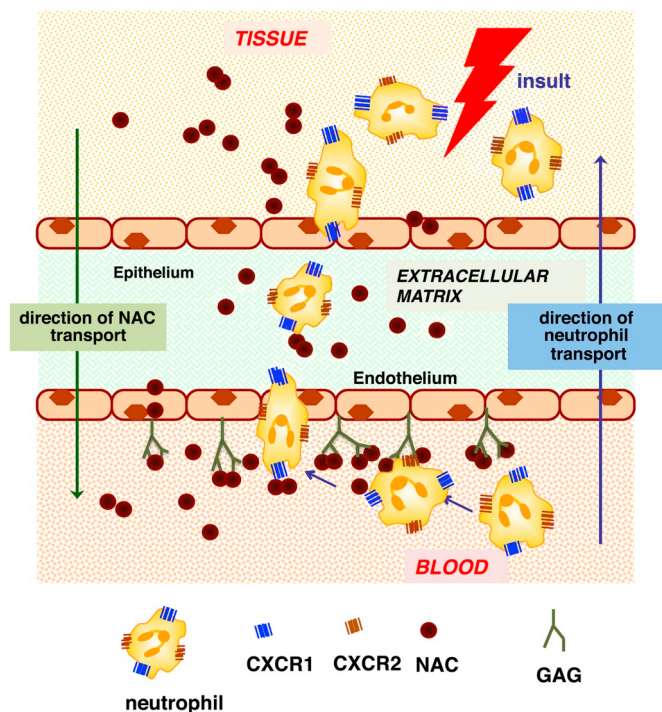


Fig. 1. A schematic showing chemokine-mediated neutrophil recruitment.

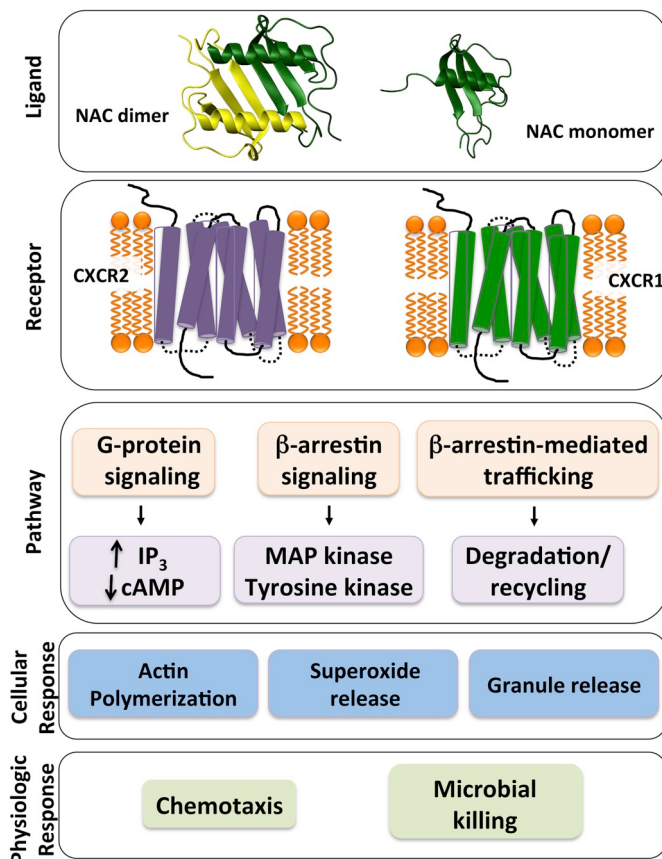


Fig. 2. A schematic linking chemokine monomer and dimer activation of CXCR1 and CXCR2 receptors, G protein and β -arrestin signaling pathways, and neutrophil recruitment and microbial killing functions.

During active neutrophil recruitment, *in vivo* NAC concentrations will vary due to multiple factors that come into play — expression levels at the site of insult, transportation to the blood, presentation on the endothelial apical surface, internalization by neutrophils, and blood flow velocity. Unlike chemokine levels, levels of the receptor and different proteins of the signaling machinery are essentially invariant in circulating neutrophils. Neutrophils are considered as terminally differentiated cells and so any transcriptional activity is non-existent or minimal. However, it does not mean neutrophils are homogeneous, and in fact, its functional phenotype is dynamic and varies as a function of space and time and is defined by its interactions and environment. In particular, the early encounter with chemokines in the blood and tissue defines the neutrophil phenotype for microbial killing. In addition to triggering signaling, chemokine binding also results in receptor internalization that varies between monomer and dimer and between CXCR1 and CXCR2. Therefore, receptor levels at the infected site will be different and the role of the two receptors in terms of granule and superoxide release for microbial killing is also different. Subsequent phenotypic changes that involve interactions with macrophages and monocytes promote phagocytosis, apoptosis, efferocytosis, and clearance.

Activity profiles of a NAC and between NACs can be quite complex. For a given receptor activity, cellular studies have shown that differences among the seven NACs range from subtle to profound. Considering NACs share the same structural fold, chemokine-specific structural features must play a major role in dictating differences in receptor affinity, efficacy, and differences in activation of signaling pathways. Selectivity of chemokines becomes quite evident if we consider their expression profile in disease conditions. CXCL8 is preferentially expressed in several lung pathologies, whereas CXCL7 is highly expressed and released from platelets regulating neutrophil-platelet crosstalk and thrombosis-related diseases [47,48]. The basis for these differences will be discussed in the following sections.

2. Neutrophil-activating Chemokines and CXCR1/CXCR2 Receptors

Chemokines are small molecular weight proteins (~8 to 10 kDa). Most are highly basic and share the following characteristics: existing reversibly as monomers, dimers, and some as high order oligomers; activating receptors that belong to the GPCR class; and binding to sulfated glycosaminoglycans (GAGs) [49–51]. On the basis of conserved cysteines, chemokines are broadly classified into CXC and CC sub-families. NACs are CXC chemokines that are characterized by the conserved 'ELR' motif preceding the N-terminal Cys (Fig. 3). The dimerization constants (K_{M-D}) of NACs are around 0.1 to 10 μ M, and K_{M-D} is also sensitive to solution conditions such as pH and ionic strength [14,18,52–54]. CXCL7 alone dimerizes weakly (K_{M-D} ~50 to 100 μ M) but forms a tetramer at higher concentrations [55].

Chemokine receptors are classified along the same division as the ligands. While there are more than 45 chemokine ligands, the corresponding receptors are fewer (~20). In general, CXC chemokines bind CXC receptors and CC chemokines bind CC receptors. Some ligands are highly selective for a single receptor, whereas others bind multiple receptors. Human neutrophils co-express CXCR1 and CXCR2 at similar levels, but the activity profiles between the receptors can vary for a given chemokine and between chemokines. All NACs bind CXCR2 with high affinity (K_d ~1 nM), but the activity profiles and downstream signaling activities can vary between the members [56–58]. In contrast to CXCR2, NACs show a range of affinities for CXCR1 with CXCL8 monomer alone showing the highest CXCR1 activity [10,11,16,17].

Though CXCL8 binds both CXCR1 and CXCR2 with high affinity, binding CXCR1 alone elicits superoxide release and phospholipase D (PLD) activity [21]. CXCR1 and CXCR2 share 78% amino acid sequence

		Site-II	Site-I (N-loop)	$\beta 1$	30s loop	$\beta 2$	$\beta 3$	C-helix
CXCL1	ASVAT	ELR CQC	LQTLQ-GIHPKN	IQSVNVKS	PGPHCAQTE	VIATL KNGR	KACLN	PASPI VKKIIIEKMLNSDKSN
CXCL2	APLAT	ELR CQC	LQTLQ-GIHLKN	IQSVKVK	PGPHCAQTE	VIATL KNGQ	KACLN	PASPM VKKIIIEKMLKNGKSN
CXCL3	ASVVT	ELR CQC	LQTLQ-GIHLKN	IQSVNVR	PGPHCAQTE	VIATL KNGK	KACLN	PASPM VQKIIIEKILNKGSTN
CXCL5	PAAAVLR	ELR CVC	LQTTQ-GVHPKM	ISNLQVFA	IGPQCSKVE	VVASL KNGK	EICLD	PEAPF LKKVIQKILDGGNKEN
CXCL6	GPVSAVLT	ELR CTC	LRVTLR-VNPKT	IGKLQVFP	AGPQCSKVE	VVASL KNGK	QVCLD	PEAPF LKKVIQKILDGSGNKKN
CXCL7	A	ELR CMC	IKTTS-GIHPKN	IQSLLEVIG	KGTHCNQVE	VIATL KDGR	KICLD	PDAPR IKKIVQKKLAGDESAD
CXCL8	SAK	ELR CQC	IKTYSKPFHPKF	IKELRVIE	SGPHCANTE	IIVKL SDGR	ELCLD	PKENW VQRVVEKFLKRAENS

Fig. 3. Sequences of neutrophil-activating chemokines. The conserved cysteines (blue), ELR (green), the CXC motif, the N-loop residues (red), and N-loop lysines/arginines (purple) are highlighted.

identity, with differences largely clustered in the N-terminal and C-terminal domains and the second extracellular loop. Structure-function data reveal that receptor activation involves interactions between the chemokine N-terminal loop (N-loop) and receptor N-terminal residues (defined as Site-I) and between chemokine N-terminal and receptor extracellular loop residues (defined as Site-II).

In this section, we will discuss the shared structural elements that determine how NACs bind and activate CXCR1 and CXCR2. The functional outcome of a given NAC can be modulated by tuning levels of monomer vs. dimer, and/or tuning Site-I vs. Site-II interactions, and/or tuning CXCR1 vs. CXCR2 interactions, and/or tuning G-protein vs. β -arrestin signaling pathways. Therefore, functional differences between two NACs can arise due to differences in one or more of these interactions or properties. Importantly, a similar readout for a particular receptor activity does not mean that there are no functional differences between the two. A similar or very different functional phenotype can arise due to a small difference at one of the steps that can be amplified due to differential tuning at other steps. Therefore, a comprehensive knowledge of all the individual steps that comprise a functional phenotype for a given chemokine and between chemokines is essential. We will discuss the current knowledge on monomer and dimer function of a given chemokine, differences between chemokines, and between the receptors. CXCL8 is the best-studied member, and this chemokine also stands out as it alone in the monomeric form functions as a potent CXCR1 agonist. The next best-studied NAC CXCL1 essentially functions as a CXCR2 agonist. Studies from these two chemokines capture the complexity of the NAC-CXCR1/CXCR2 system and neutrophil function.

2.1. Chemokine and Receptor Structures: Implications for Function

Structures of the native CXCL1, CXCL2, CXCL5, and CXCL8 dimers and of CXCL7 tetramer have been solved by nuclear magnetic resonance (NMR) and/or X-ray crystallography [54,59–64]. Solution and crystal structures are determined at high concentrations, and so it is not

surprising that the structures correspond to that of the highest oligomer. At the monomer level, all NACs share the same structural fold consisting of an unstructured N-terminal domain (N-domain) and an extended N-loop, followed by three β -strands, and an α -helix (Fig. 4). Conserved cysteines form disulfides, and mutational studies have shown that the disulfides are absolutely essential for stability and function [65–67]. The structures of designed CXCL8 monomers, one containing a methyl group for a backbone amide of a residue at the dimer interface and the other missing the last six residues of the C-terminal helix, are similar to that of the monomer in the dimer [68,69]. Whereas the methyl substitution traps the protein in the monomeric state and prevents dimerization, deletion of the C-terminal residues highly impairs the dimerization process. These monomers are as or more potent than the native chemokine in cellular functional assays [15,17,70–72]. Several other designed CXCL8 monomers and CXCL1 and CXCL7 monomers are also as active as the native chemokines in functional assays [14,18,73].

The structure of CXCR1 was solved by solid-state NMR spectroscopy [74], a major achievement in itself, as all other GPCR structures to date were solved by X-ray crystallography. Structures of the CXCR2 receptor or of ligand-bound receptors are not known. The CXCR1 structure reveals that the N-domain is unstructured and highly dynamic [74]. Sequence analyses also indicate that the N-domain is natively unfolded. On the other hand, the strategy of ‘divide and conquer’ approach of characterizing the binding to CXCR1 N-domain peptides has proven to be useful to describe Site-I interactions. A solution structure of a CXCR1 N-domain peptidomimetic bound to dimeric CXCL8 is known, which has provided some insights into Site-I interactions [75].

NACs dimerize at micromolar (μ M) concentrations and *in vitro* cellular assays have shown that NACs are active at nanomolar (nM) concentrations. At nM concentrations, NACs are essentially monomers. Therefore, activity of a native dimer cannot be determined in cellular assays due to contribution from the native monomer at any given concentration. This bottleneck has been circumvented by designing

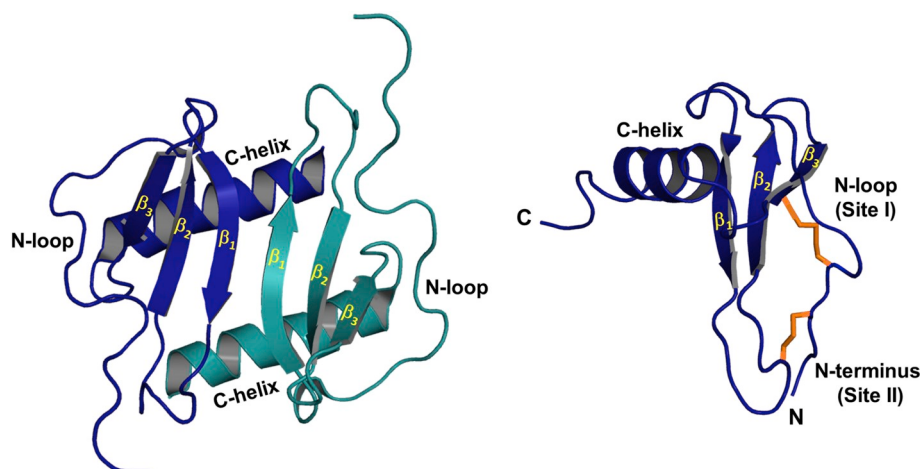


Fig. 4. Structure of a neutrophil-activating chemokine. All NACs have the same structural fold at monomer and dimer level. The monomers in dimer are shown in different colors for clarity. Different structural and functional regions are labeled both in the monomer and dimer.

trapped non-dissociating variants that contain a disulfide at the dimer interface. CXCL8 dimer shows lower affinity and activity for CXCR1 but higher affinity and activity for CXCR2 [16,17]. A trapped CXCL1 dimer is as potent as the monomer for CXCR2 activity in a cell-based Ca^{2+} release assay [13].

Like many GPCRs, chemokine receptors are known to dimerize. CXCR2, but not CXCR1, has been shown to exist as dimer [76], and the NMR structure of CXCR1 reveals it to be a monomer. The observation that trapped CXCL1, CXCL7, and CXCL8 monomers containing a bulky methyl group at the dimer interface are potent agonists indicate that binding-induced chemokine dimerization is not essential for functionality [14–17]. Considering that CXCL1 and CXCL8 dimers are potent CXCR2 agonists and mutating dimer interface residues does not influence function, the dimer interface cannot be involved in receptor interactions [13,17,66,77]. Modeling studies also indicate that only one monomeric unit of the dimer mediates binding with the other monomer pointing away from the receptor. These observations indicate that receptor dimerization most likely regulates function that is unrelated to how the CXCL8 monomer and dimer bind a monomeric receptor.

2.2. Why monomers and dimers? – relevance for *in vivo* function

The ability of NACs to reversibly exist as monomers and dimers must confer advantages compared to existing as one or the other alone. The host immune response during active infection is a dynamic and complex process, and the process of neutrophil recruitment must be highly coordinated, with regulation at the levels of the chemokine, receptor, and signaling pathways. Neutrophils are rapidly recruited after insult and continue to be recruited for many hours during an acute response and for even longer periods during chronic inflammatory conditions. However, too many neutrophils or their undue recruitment can cause collateral tissue damage and disease [78,79]. Not surprisingly, impaired recruitment of too few neutrophils to the insult site results in runaway inflammation. Animal model studies have shown that elevated blood chemokine levels result in receptor desensitization and impaired recruitment, and accumulation of neutrophils on the luminal side of the endothelium or epithelium [80,81]. That chemokine and receptor levels regulate recruitment is also evident from epidemiological and clinical data. Elevated CXCL8 and reduced CXCR1/CXCR2 levels are observed in patients suffering from pulmonary diseases, and polymorphisms that dysregulate CXCL8 and receptor levels lead to higher incidences of infection and disease [44,82–84].

How do chemokines accomplish the delicate mission of recruiting the right number of neutrophils at the right time? We propose that the ability to exist as monomers and dimers and interactions with GAGs regulate the levels of free chemokine available and makeup and nature of the gradients, which direct neutrophils from the blood across the endothelium to the infected tissue. We have characterized the structural basis and molecular mechanisms by which four human (CXCL1, CXCL5, CXCL7, and CXCL8) and two mouse chemokines (KC/mCXCL1 and MIP2/mCXCL2) interact with GAGs [85–90]. These chemokines show a diversity of GAG-binding surfaces, indicating that location and distribution of both conserved and chemokine-specific residues in the context of three-dimensional structure determine binding geometry. For all chemokines, we observe that the dimer compared to the monomer binds GAGs with higher affinity. There is also evidence that some of the chemokines form heterodimers and that GAG interactions determine heterodimer levels [91,92]. For chemokines CXCL7 and CXCL8, we have characterized the binding of both the monomer and dimer to GAG heparin, and observe that the binding surface of a monomer is similar to the monomer within the dimer [55,85].

At any given time, NACs exist in dynamic equilibrium between four forms – monomer in the free and GAG bound forms and dimer in the free and GAG bound forms. The relative levels of the four forms are dictated by equilibrium constants between monomer and dimer, monomer and monomer-bound GAG, and dimer and dimer-bound GAG.

It must be remembered that there is always free dimer due to the equilibrium process. At equilibrium, the rate of dimer binding to GAG and the rate of dimer release from the GAG-bound form are the same, and so the levels of the free dimer remain the same. The levels of the free dimer will change when the total chemokine concentration either increases or decreases. Monomers and dimers differentially activate CXCR1 and CXCR2, which allow additional levels of regulation. During active infection, chemokines that are pre-stored and those that are synthesized by the host machinery are released into the blood stream [93]. Chemokines must cross several barriers including the epithelium, extracellular matrix (ECM), and the endothelium. Once in the vasculature, they bind to the endothelial cell surface GAGs. GAG-bound and soluble gradients direct blood neutrophils across the endothelium, ECM, and the epithelium to the injury site [94,95]. Gradients are dictated by local concentrations, and local concentrations are dictated by local environment, architecture, and interactions.

During the course of neutrophil recruitment from the time of infection to eventual resolution, local chemokine concentration can vary spatiotemporally by orders of magnitude, and so will the monomer/dimer ratio. Concentration by definition is the number of moles of the solute divided by volume. However, the concept of concentration, which is easily grasped in the context of *in vitro* assays due to a defined volume, cannot be easily defined in the context of *in vivo* function. The *in vivo* extracellular environment is complex and heterogeneous [96–98], and so defining a ‘local’ volume is not straightforward due to lack of a strict boundary. Chemokine levels in the immediate proximity of the cell membrane are much higher due to interactions with GAGs than more towards the center of the vessel. ECM can be considered as a mesh of macromolecular complexes comprised of matrix proteins such as collagen and proteoglycans and so defining a local volume is challenging. Further, dimensions of the endothelial venules and capillaries where neutrophils extravasate to the injury site can vary. Nevertheless, we can visualize that the space is restricted, and so chemokines can easily dimerize at different locations.

At this time, there are no technologies or methodologies to unambiguously detect monomeric or dimeric forms of native chemokines (or any protein) *in vivo*. Using a mouse model, we have characterized and compared the recruitment profiles of exogenously administered trapped monomer, trapped dimer, and native forms of CXCL8 and CXCL1 at different doses, time points, and different tissues [99–102]. The activities of the trapped monomer and trapped dimer reflect the activity of the native monomer and dimer. These studies indicate that the recruitment activity of monomers and dimers can be distinct, monomer or dimer can be more active than the native protein (whose activity is a composite of both monomer and dimer), and that the monomer-dimer equilibrium regulates recruitment. At low doses, which could correspond to the very early or late stages of recruitment, the monomer is more active than the dimer. At higher doses, dimer-mediated neutrophil recruitment could be quite robust suggesting dimerization acts as an on-switch. However, persistent high dimer levels are not desirable, as they could elicit massive recruitment and a runaway inflammatory response. On the other hand, monomers at high doses result in reduced recruitment that could be attributed to reduced receptor levels and/or sub-optimal gradients [99].

Chemokine concentrations are low in the blood distant from the site of infection/injury and relatively higher at the site of insult. Therefore, *in vitro* activities measured at low concentrations could reflect signaling events that occur in the blood early after infection and at high concentrations reflect signaling events that occur at the infection site. These observations also suggest that CXCR2 plays a pivotal role in directing neutrophil recruitment. At the site of infection/injury, CXCR2 is no more available due to its rapid endocytosis at high chemokine concentrations, and CXCR1 would play an active role in initiating proinflammatory responses. Therefore, chemokines’ ability to continuously redistribute between monomeric and dimeric forms with differential activation of CXCR1 and CXCR2 could be essential for

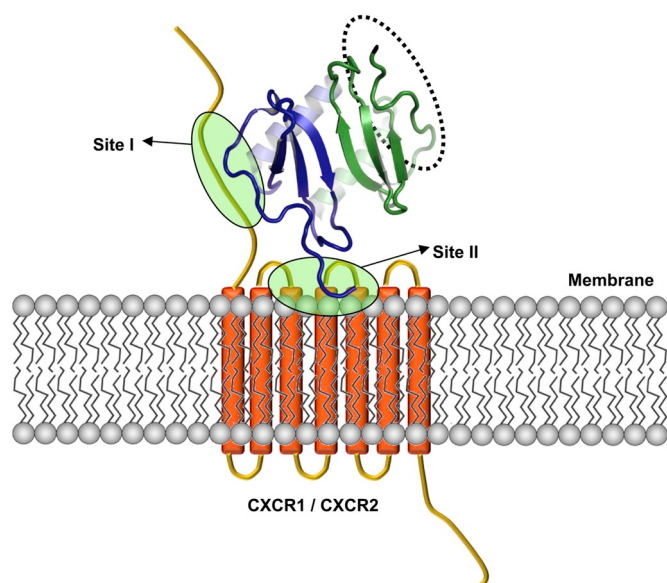


Fig. 5. A schematic of two-site multistep binding of NACs to CXCR1/CXCR2. The initial binding interactions at Site-I results in structural/dynamic changes that are essential for subsequent binding of N-terminal ELR residues to receptor transmembrane/extracellular loop residues and receptor activation (Site-II). The figure is not to scale and is for illustrative purposes only.

regulated neutrophil recruitment in a healthy proinflammatory response. These observations suggest that in the setting of disease, dysregulation of chemokine expression and/or disruption of the monomer/dimer ratio and/or receptor signaling could lead to either low or uncontrolled neutrophil trafficking, resulting in unresolved inflammation and significant collateral tissue damage and disease.

2.3. Structural Basis for Binding and Receptor Activation – A Tale of Two Sites

NACs and CXCR1 and CXCR2 were discovered in the late 80s and early 90s. During the next decade, significant effort went into studying the structural basis of how NACs bind and engage their receptors. These studies involved site-specific mutagenesis or creating chimeric proteins by swapping identical domains between two NACs or between CXCR1 and CXCR2. In CXCL8, essentially every single surface residue has been mutated, and also several CXCL8 chimeric proteins with domains from CXCL1, CXCL4, and CXCL10 were generated [12,67,103–111]. In the case of CXCR1 alone, most extracellular loop residues have been mutated [112,113], and several studies have also characterized CXCR1/CXCR2 chimera in which the N-terminal or one or more extracellular loops were swapped [11,114–116]. These studies collectively indicate that receptor activation involves interactions between the chemokine N-loop and receptor N-terminal residues (Site-I) and between chemokine N-terminal and receptor extracellular loop residues (Site-II) (Fig. 5).

N-loop and N-terminal ELR residues mediate binding to CXCR1 and CXCR2 receptors, but the relative contribution of individual residues can vary. Some are crucial for both receptors, and some play a more prominent role for one receptor and not the other. Further, individual residues play a prominent role for some functions and not for other. The ELR motif is absolutely conserved whereas the N-loop residues are not, suggesting Site-I interactions play an important role in determining receptor-specific activity. CXCL10, a chemokine that binds CXCR3, on grafting the CXCL8 N-terminal, N-loop and 30s turn residues that are in the proximity of N-terminal and N-loop residues, showed CXCL8-like function [66]. CXCL1 and CXCL8 structures show the largest difference for the N-loop residues [59–62]. CXCL1 gained CXCL8 like function and

vice versa on swapping the N-loop residues suggesting that the site-I interaction plays an important role in receptor selectivity [67].

Studies using CXCR1/CXCR2 chimera indicate that the N-domain and extracellular loops residues mediate affinity, selectivity, and function, and that Site-I and site-II interactions are not additive but coupled [11,114–116]. More recently, on the basis of mutations of 30s loop and disulfide residues, it has been proposed that the initial Site-I interactions result in structural and dynamic changes both in the ligand and receptors and that these changes are essential for subsequent Site-II interactions [70,72,117]. The most direct evidence for the two-site model comes from the observation that CXCL8 binds the isolated CXCR1 N-domain with an affinity similar to that for the N-domain in the intact receptor [110,118]. Structures of NAC bound to CXCR1 or CXCR2 are not available. However, structures of other chemokine receptor complexes show evidence for the two-site interactions [119,120]. A structure of the CXCL8 dimer bound to CXCR1 N-domain peptide also shows that only N-loop residues interact with the receptor N-domain [75].

What is the structural basis for Site-I and Site-II interactions? Clearly, this information is encoded both in the ligand and receptor residues. NAC sequences reveal distinct differences in the N-loop region, with some residues highly conserved and some less conserved including gaps in the alignment. In particular, distribution of the basic residues lysine (Lys) and arginine (Arg) stands out (Fig. 3). The CXCL8 N-loop is unique due to insertion of a lysine (Lys15), and also has two additional lysines. The CXCL6 N-loop sequence is the closest to CXCL8, but it is a weak CXCR1 agonist [19], suggesting that the properties of Lys15 are unique in the context of CXCL8 structure and CXCR1 activation. The CXCR1 and CXCR2 N-domain sequences share low sequence homology (~ 40%), reveal gaps in the alignment, and contain several negatively charged Asp/Glu residues, some conserved and some not. Mutational studies and structure of the Site-I complex reveal that a combination of hydrophobic, electrostatic, and polar interactions mediate binding interactions [75]. However, differences in monomer and dimer structures also play a role, which vary between NACs and between receptors. CXCL8 monomer compared to dimer binds CXCR1 with much higher affinity though the same residues in monomer and dimer seem to mediate binding interactions [118,121]. On the other hand, CXCL1 monomer and dimer bind CXCR2 with similar high affinity. Several studies have shown that selective and differential engagement of Site-I N-loop residues dictates monomer vs. dimer affinity and CXCR1 vs. CXCR2 selectivity [13,118]. Solution structures of CXCL8 monomer and dimer and dynamics measurements reveal that the N-loop residues are structured but conformationally flexible, suggesting a role for dynamics in mediating function [59,68,122].

Among the CXC ligands, the N-terminal ELR motif is restricted to those that bind CXCR1 and CXCR2. Unlike Site-I interactions, much less is known regarding which receptor residues interact with ELR residues. CXCR1 and CXCR2 sequences and functional and modeling studies reveal that the charged residues in ECL2 and ECL3 and the proximal TMs as potential candidates for interacting with E4 and R6 of the ELR motif [112,113,123]. The structure of the NAC-bound receptors and a detailed mutational study of both receptors for additional NACs beside CXCL8 are essential to appreciate Site-I interactions and to answer how the two-site interactions mediate receptor functions.

Mutational studies in CXCL8 indicate that the disulfides, the CXC motif, and 30s loop residues that are in the proximity of N-terminal and N-loop residues impact function [65,70,72], suggesting that Site-I and Site-II interactions are not independent but coupled. Structures reveal that the two disulfides are buried and ‘tether’ the functionally important N-terminal and N-loop residues, and in particular, that the disulfide that links the N-terminal and 30s loop residues is conformationally flexible. The Cys7-Cys34 disulfide in CXCL8 has been shown to be essential for function as deleting or modifying the disulfide by introducing non-natural cysteine analogs results in substantial loss of activity [117]. The CXC motif in CXCL8 is also critical as deleting the

intervening Gln8 residue results in impaired activity for both receptors, which was more severe for CXCR2 than for CXCR1 [72]. Considering that the disulfides and the CXC motif are buried and thus cannot be involved in direct receptor interactions, reduced function must be due to indirect coupled interactions. Recently, an additional site defined as Site 1.5, which involves chemokine residues that link Site-I and Site-II, has been also proposed to mediate receptor interactions on the basis of a crystal structure of the complex of a viral chemokine bound to CXCR4 [119]. The structure seems to indicate that Site-I and Site-II interactions are not distinct but a continuum. However, whether this is the case for NACs is not known, and must await determination of chemokine bound CXCR1/CXCR2 structures.

2.4. Chemokine function – A tale of two (CXCR1/CXCR2) receptors and two (G-protein and β -arrestin) signaling pathways

NAC monomers and dimers, CXCR1/CXCR2 activation, and G-protein and β -arrestin-coupled signaling pathways, acting in concert, orchestrate the movement of blood neutrophils to the infected/injured tissue (Figs. 1 and 2). At the same time, these interactions also determine the phenotype of the recruited neutrophils for eliminating the microbes and/or initiating repair at the insult site. Both G-protein and β -arrestin pathways play important non-overlapping roles in neutrophil trafficking and inflammatory responses by triggering neutrophil granule release of cytotoxic enzymes and reactive oxygen species (ROS). Many GPCRs signal via both G protein and β -arrestin pathways, and therefore, it is not surprising that CXCR1 and CXCR2 share many commonalities with other GPCRs. [124] Cellular studies show fundamental differences in potency and efficacy and the time frame of functional response between G-protein and β -arrestin-mediated signaling events. Potency can be defined by EC_{50} , the ligand concentration that corresponds to 50% of the maximum measured activity. Efficacy can be defined as the maximum effect of the agonist at a saturating dose. In general, G-protein-mediated signaling is characterized by a rapid onset followed by waning intensity that is complete over a period of seconds and minutes, whereas β -arrestin-mediated events are characterized by slower onset and sustained duration [125].

CXCR1 and CXCR2 signal predominantly via the G_i class of pertussis toxin-sensitive G proteins [126,127]. G-protein signaling involves dissociation of the receptor-bound heterotrimeric G protein into GTP-bound G_α and $G_\beta\gamma$ subunits, which independently activate a variety of effectors including PLC, GTPases, and Ca^{2+} channels. A well-studied G-protein-coupled molecular event is IP_3 generation that is coupled to intracellular Ca^{2+} release and activation of kinases. There is also compelling evidence for phosphatidylinositol 3-kinase (PI3K) activation in chemokine-mediated signaling [128]. G-protein signaling is modulated and regulated by AGS (activators of G-protein signaling) proteins, RGS (regulators of G protein signaling) proteins, G protein-coupled receptor kinase (GRK) phosphorylation, and β -arrestin binding [125,129,130]. Binding of arrestin plays three distinct non-overlapping roles: (i) termination of G-protein-coupled signaling; (ii) serving as an adaptor for recruiting molecules including those that signal via the MAPK and tyrosine kinase pathways; and (iii) mediating endocytosis and trafficking of the chemokine-chemokine receptor complexes that effectively reduces receptor levels available for further chemokine signaling thus desensitizing the chemokine response [25–28]. β -arrestins have been implicated in spatial and temporal control of activities of several signaling molecules including cytoskeletal regulators, Src kinases, RhoA, MAP kinases ERK1/2, c-Jun N-terminal kinase (JNK) and p38 [131–137]. These signaling events have emerged as key regulators of cellular processes such as cytoskeletal reorganization and chemotaxis (Fig. 6) [138,139].

Early functional studies carried out in freshly isolated neutrophils used antibodies against CXCR1 or CXCR2 to dissect the activity of each of the two receptors. Activation of both receptors triggers increase in intracellular Ca^{2+} levels, release of granule proteins, and chemotaxis.

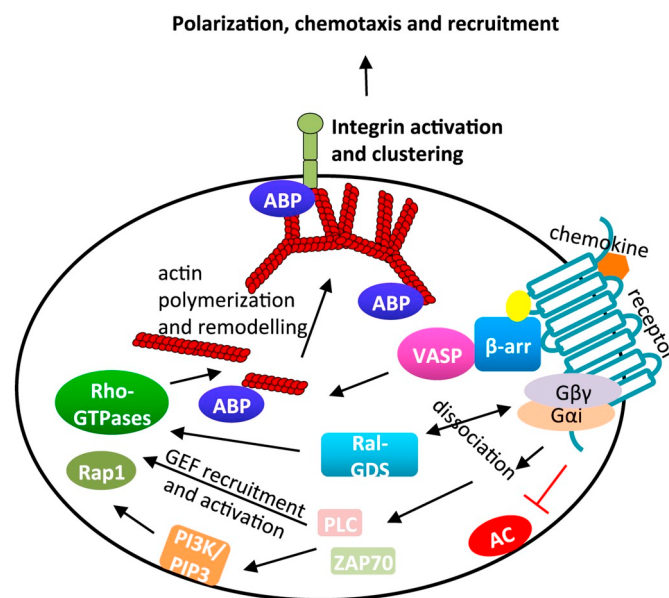


Fig. 6. A schematic showing G protein- and β -arrestin-mediated actin re-modeling, leading to adhesion, chemotaxis and recruitment. ABP: actin-binding proteins such as talin-1, kindlin-3, WASP, Arp2/3, cofilin, LSP1, paxillin or HSP1; PLC: phospholipase C; AC: adenylyl cyclase.

CXCL8/CXCR1 axis alone triggered PLD activation and respiratory burst [21,57]. Subsequently, several studies characterized receptor activity by stably expressing each of the two receptors in Jurkat, rat basophilic leukemia (RBL-2H3), and CHO cells [140–143]. Studies using CXCR1-Jurkat and CXCR2-Jurkat cells confirmed CXCL8 as a high affinity agonist for both receptors in several functional assays, and that both receptors activate ERK1/2 kinases [140,141]. These early studies were critical in establishing that stably transfected cell lines are a viable approach to gaining detailed mechanistic insights. Subsequent and more recent studies using animal models including KO mice have shown that both G protein and β -arrestin signaling events mediate many of the neutrophil trafficking events and neutrophil activation at the insult site. For instance, it is now known that they act in sequence and in distinct compartments to promote integrin-dependent adhesion during leukocyte extravasation.

Considering all NACs bind CXCR2 with nM affinities raises the question whether they trigger similar downstream G protein and β -arrestin signaling events and share functional phenotypes. Cell-based studies have shown that this is not the case and that there are differences. For instance, in neutrophils and CXCR2-HEK293 cells, CXCL1, but not CXCL8, induces influx of extracellular Ca^{2+} [56]. The capacity of neutrophils to produce superoxide is enhanced by prior exposure to several factors including chemokines. Interestingly, CXCL8 on binding CXCR1 (and not CXCR2) and CXCL1 and CXCL5 on binding CXCR2 prime neutrophils for augmented superoxide release upon stimulation with the bacterial peptide fMLP [22]. CXCL8, compared to CXCL7, induces higher level of CXCR2 phosphorylation in CXCR2-HEK 293 cells [144]. These observations indicate affinity is distinct from activity, and that differences in binding-induced receptor conformational states and/or binding to distinct conformers can lead to differential coupling to its downstream effectors and function. There is ample evidence that GPCRs exist as a conformational ensemble and that different conformers are coupled to different effectors resulting in different functional outcomes [145]. We discuss the potential role of signaling bias in the context of NAC monomers and dimers in Section 2.4.3.

2.4.1. Role of accessory proteins and receptor function. Phosphorylation of CXCR1/CXCR2 C-terminal serines and threonines by GRKs plays a critical role in G-protein dissociation and β -arrestin association. In transfected RBL-2H3 cells, CXCR1 and CXCR2 were

shown to couple to distinct GRKs to mediate and regulate cellular functions. Whereas CXCR1 is predominantly phosphorylated by GRK2, CXCR2 is phosphorylated by GRK6 (58). Inhibition of GRK2 or GRK6 using siRNAs prolonged CXCL8-mediated cellular responses to CXCR1 and CXCR2, respectively [58]. Depletion of GRK6 in murine neutrophils increased cellular responses to CXCR2 including chemotaxis, intracellular Ca^{2+} mobilization, superoxide production, and resistance to receptor internalization [146,147]. Whereas GRK2 is recruited from the cytoplasm to the activated receptor in the membrane via $\text{G}_{\beta\gamma}$ interaction, GRK6 is a membrane-bound kinase [148]. Whether differences in monomer vs. dimer interaction impact the ability of CXCR1 and CXCR2 to couple to different GRKs and regulate neutrophil function remains to be explored.

AGS also known as G-protein signaling modulators (GPSM), influence chemokine-mediated neutrophil functions [130,149]. This family of GPCR accessory proteins comprise three subfamilies: GPSM I, GPSM II and GPSM III. CXCL8 activation of CXCR2, but not CXCR1, was shown to complex with the AGS3/GPSM1 and modulate cellular responses such as Ca^{2+} mobilization, MAP kinase activation, and cell migration [150]. AGS3/GPSM1 acts as a guanine nucleotide dissociation inhibitor (GDI), inhibiting dissociation of GDP from $\text{G}\alpha$ subunits as well as competitively preventing $\text{G}\beta\gamma$ subunits from coupling $\text{G}\alpha$ subunits [151,152]. In addition to its regulatory role in GPCRs signaling, AGS3 has been shown to mediate several cellular functions [153]. These observations suggest that differences in monomer and dimer NAC binding to CXCR1/CXCR2 could impact AGS interactions and hence neutrophil function.

2.4.2. Signaling, actin dynamics, and neutrophil migration. Dynamic actin remodeling is fundamental to neutrophil migration. Neutrophils respond to chemokine receptor activation by recruiting and/or releasing signaling molecules of both G-protein and β -arrestin-coupled downstream signaling cascades that contribute to actin remodeling and integrin activation (Fig. 6). Chemokine-mediated integrin activation is required for neutrophil adhesion to the vessel wall and subsequent transendothelial migration [154–156]. Integrin activation depends heavily on recruitment of actin-binding proteins (ABP) such as kindlin-3, talin-1, paxillin, and HS1 [157–160]. Most of our current knowledge regarding chemokine-mediated actin remodeling has been inferred for CXCL1 and CXCL8 from cellular assays and mouse models.

G protein-mediated actin dynamics. Several lines of evidence have shown a role for G protein signaling in actin remodeling. The $\text{G}\alpha_i$ subunit canonically inhibits adenylate cyclase isoforms and thus the generation of cAMP, and subsequent activation of PKA, EPAC, and PLC [161,162], leading to altered actin cytoskeletal remodeling [163]. Moreover, G_i -induced recruitment and activation of Rho GTPases and PIP_2 generation are critical events during chemokine-triggered integrin activation and leukocyte recruitment [164]. Another G_i -triggered key event is the PLC-dependent activation of Rap1 [165], which is a global phenomenon in different leukocyte subsets. Moreover, chemokine-mediated PLC activation leads to increased Ca^{2+} flux and subsequent calpain activation required for dissociation of integrins from actin filaments to improve integrin membrane motility and clustering [166]. PLC isoforms play complex roles during chemotaxis that are reviewed elsewhere [167].

In neutrophils, chemokine-activated PLC regulates cofilin activity via the phosphatase slingshot 2, which dephosphorylates and activates cofilin to induce F-actin depolymerization [168,169]. Moreover, G-protein-mediated Rac1 activation is critical for actin dynamics as it activates nucleation-promoting factors required for Arp2/3 complex activation, and branched actin network formation at the leading edge of neutrophils during migration [170]. ELMO/Dock protein complexes have emerged as critical guanine nucleotide exchange factors (GEFs) for Rac1 activation [171]. In fact, chemokines induce interaction of ELMO1 with the $\text{G}\beta\gamma$ subunit and subsequent translocation to the plasma membrane, where it also interacts with Dock1 to form a functional GEF activating Rac1 leading to efficient actin dynamics and chemotaxis

[172]. Dock2 has also been shown to activate Rac1, efficient actin polymerization at the leading edge and chemotaxis [173]. Dock2 dynamics are dependent on PIP_3 , phosphatidic acid, and PLD [174], but Dock2-ELMO1 interactions have so far only been described in migrating lymphocytes [175]. Moreover, chemokine-induced neutrophil migration modes involving actomyosin dynamics also strongly depend on the substrate they are migrating on or in (2D vs. 3D migration; for example, on the vascular endothelium vs. within interstitial tissues). Given the complexity of this topic, the reader is referred to another excellent comprehensive review [176].

β -arrestin-mediated actin dynamics. Studies linking β -arrestins as critical regulators of actin dynamics were mostly inferred in cell types other than neutrophils or for chemoattractants other than chemokines [177,178]. β -arrestin interacts with a number of proteins that are critical for cytoskeletal organization. For instance, β -arrestin sequestered GEF Ral-GDS was released upon neutrophil activation by fMLP, resulting in Rac1/RhoA-dependent actin polymerization and chemotaxis [131]. Moreover, downregulation of β -arrestin-1 resulted in reduced fMLP-mediated chemotaxis. Mechanistically, β -arrestin-1 was required for activation of Rap2 and actin accumulation at the leading edge. Although these processes occurred downstream of the fMLP receptor FPR, it is likely that similar signaling downstream of CXCR1/CXCR2 mediate actin remodeling and neutrophil recruitment, as CXCL1 also induced activation of small GTPases in a manner dependent on the ABP HS1 leading to integrin activation and effective neutrophil recruitment [157,179].

Vasodilator-stimulated phosphoprotein (VASP), an ABP that is important for actin polymerization, has been shown to interact with CXCR2 [180,181]. Direct VASP interaction with CXCR2 triggered by CXCL8 was observed in HL60 cells, which contributed to neutrophil polarization and chemotaxis [181]. Although a link to β -arrestin was not shown in that study, later it has been shown that VASP formed part of a molecular complex containing CCR2 and β -arrestin-2, and was required for CCR2 internalization [180]. Additionally, VASP is required for Rap1-mediated integrin activation, which is a prerequisite during neutrophil recruitment [182].

These studies highlight the importance of β -arrestins and G-proteins for actin dynamics and migratory responses in neutrophils, but many aspects of the relationship between NAC properties and β -arrestin and G protein-mediated signaling are poorly understood. In particular, how NAC monomers and dimers activating either of the receptors impact actin remodeling is not known. It is also important to remember that chemokine-dependent actin dynamics and migration experiments always have to be interpreted in a context-dependent manner — the chemokine used, the receptor being targeted, the substrate, and the *in vitro* or *in vivo* experimental setting.

2.4.3. NAC monomers and dimers and biased signaling. Functional bias can be achieved at the level of ligand (monomer vs. dimer), receptor (CXCR1 vs. CXCR2) and/or signaling (G-protein vs. β -arrestin). Regulation at multiple levels allow fine-tuning of neutrophil functions but at the same time can also lead to dysregulation and disease. All of the G-protein and arrestin-mediated signaling pathways downstream of a GPCR can be activated or blocked by conventional ‘balanced’ agonists or antagonists, respectively. In addition, these signaling pathways can also be selectively activated by a biased agonist for controlling both efficacy and potency. A biased agonist binding to a GPCR promotes a response that can result in selective signaling through one of its downstream pathways, such as G proteins (by a G protein-biased agonist) or β -arrestins (by a β -arrestin-biased agonist), while not promoting signaling through the other pathways [183]. For many GPCRs, G-protein- and β -arrestin-mediated signaling events have been shown to have distinct biochemical and physiological actions from one another.

In recent years, there has been considerable interest in the role of biased agonism in the chemokine system, and several chemokines have been shown to act as biased agonists of their receptors [184,185]. For example, the chemokine receptor CXCR3, which is expressed on

activated T cells and plays significant roles in inflammation, vascular disease, and cancer, has four endogenous ligands: CXCL4, CXCL9, CXCL10, and CXCL11 [186–188]. Of these ligands, CXCL10 and CXCL11 signal through G_{ai} to inhibit cAMP generation with significantly higher potency and efficacy than CXCL4 and CXCL9 [189]. In contrast, CXCL11 is more potent and efficacious in recruiting β -arrestin to CXCR3 than the other three endogenous ligands [190]. Notably, in a mouse model of allergic contact dermatitis, a small-molecule β -arrestin-biased agonist promoted significantly more inflammation than a G protein-biased agonist by promoting the chemotaxis of activated T cells [191], suggesting that the endogenous β -arrestin-biased CXCL11 is more pro-inflammatory than other CXCR3 ligands. For the CXCL12-CXCR4 system, it has been shown that the monomeric CXCL12 activates both G proteins and β -arrestins and that dimeric CXCL12 acts as a G protein-biased ligand, demonstrating that chemokine dimerization can result in biased signaling [192]. These findings are consistent with biased receptor signaling as having a significant effect on inflammatory response and suggest that the ligand bias for other chemokine receptors is physiologically relevant [190,193–195]. However, at this time it is still largely unclear as to the specific contributions of G protein- and β -arrestin-mediated signaling to the inflammatory response mediated by different chemokine receptors.

How do these observations translate to ligand bias of NAC monomers and dimers for CXCR1 vs. CXCR2 and G protein vs. β -arrestin signaling pathways? There is evidence of ligand bias for CXCR2, although the physiological effects of such bias are not known. CXCL6 is relatively G-protein-biased and CXCL8 relatively β -arrestin-biased compared to other CXCR2 ligands [190]. Using trapped CXCL8 monomeric and dimeric variants, it has been shown in RBL cells that the monomer and dimer can have different activities for a given receptor and between receptors [17]. For CXCR1, the monomer compared to the dimer was more active for Ca^{2+} mobilization, PI hydrolysis, chemotaxis, and β -arrestin-1 recruitment. For CXCR2, both monomeric and dimeric forms mediate these activities to a similar extent [17]. There are also stark differences in CXCL8-mediated internalization rates between CXCR1 and CXCR2. Both monomer and dimer internalize CXCR2 efficiently and rapidly but internalize CXCR1 poorly, with the dimer being less efficient than the monomer [17]. CXCR1 is also recycled back more rapidly than CXCR2 [20]. Other NACs have not been systematically characterized in terms of monomer vs. dimer or G-protein vs. β -arrestin signaling activities. For CXCL1 WT and trapped dimer, CXCR2-mediated endocytosis occurs at similar rates [93]. CXCL1 internalization, compared to CXCL8, occurs at slower rates and is more prolonged. These findings suggest that biased agonism could play a role in signaling at CXCR1/2, but also highlight significant gaps in our knowledge of their signaling. Further, there is significant granularity within biased signaling; for example, different chemokines can exhibit bias towards different G-protein subunits, as endogenous chemokines for CCR5 and CCR7 signal through overlapping but distinct G-protein subtypes [195]. A careful dissection of these different pathways as a function of monomeric vs. dimeric variants for all seven NACs will be critical for identifying those signaling nodes that contribute to neutrophil recruitment and phenotype for microbial killing and repair activities.

What is the structural basis for differential activation of G-protein and β -arrestin signaling pathways? Studies to date suggest this information is encoded in the chemokine sequence. Ca^{2+} release and ERK phosphorylation activities of a panel of CXCL8 30s loop monomer mutants show large differences in Ca^{2+} release and ERK phosphorylation activities for both receptors that could not be correlated to the nature of the mutation, receptor type, and/or a specific signaling pathway [70]. NMR studies also show that the mutations do not perturb the global fold, and any local structural change could not be correlated to functional changes. Collectively, these observations suggest that 30s loop residues function as a conformational switch coupling Site-I and Site-II interactions, and control the distribution of conformational substates for G-protein vs. β -arrestin signaling pathways. Studies that

are more stringent and readouts that can be directly correlated to G-protein and β -arrestin signaling are essentially to fully define the molecular basis of how NAC monomers and dimers activate the various signaling pathways.

3. Missing Knowledge and Future Directions

Though much is known regarding the chemokine-CXCR1/CXCR2-G-protein/ β -arrestin axis and neutrophil functions, there is still much to be learnt. For instance, many of the insights regarding G-protein/ β -arrestin signaling pathways came from mouse model studies using the CXCR2 agonist KC (mouse CXCL1). There are fundamental differences between humans and mice. Mice do not express CXCL8, and further, evidence for a functional mouse CXCR1 was firmly established only recently. CXCR1 KO mouse studies indicate that CXCR1 and CXCR2 play non-redundant functions, LIX (mouse CXCL5) functions as a CXCR1 agonist, LIX/CXCR1 axis is essential for eliminating fungal and bacterial infection and survival in disease models, and CXCR1 is essential for neutrophil degranulation and ROS production [196,197]. Therefore, studies exploring how the mCXCL5/CXCR1/CXCR2 axis activates G-protein vs. β -arrestin signaling pathways will allow meaningful comparisons with human neutrophil function. Functional characterization of mouse chemokine monomers and dimers, similar to what has been done for human chemokines, is also essential to compare and contrast the insights gained from mouse models in the context of human neutrophil function.

Knowledge of the structures of binary and ternary complexes of NAC monomers and dimers bound to CXCR1 and CXCR2, and of CXCR1/CXCR2 bound to G proteins, β -arrestins, GRKs, and ternary complexes of chemokine/receptor/G-protein and chemokine/receptor/ β -arrestin are needed to fully appreciate the molecular basis of these signaling pathways. Further, complete activity profiles for several NACs that include potency and efficacy, using trapped monomers and dimers for both CXCR1 and CXCR2, in the same stably transfected cell lines for as many G-protein and β -arrestin signaling pathways and functional readouts as possible are needed. Such data will allow reliable comparison without mitigating factors of differences among cell lines and variability that arises from neutrophils isolated from human blood. Neutrophil activation during the process of isolation has always been a concern and remains a confounding factor. Emerging CRISPR technology will also allow answering questions that otherwise cannot be addressed with KO animal models alone. Currently, there are no FDA-approved drugs either targeting NACs or the receptors, though several small molecule inhibitors that target CXCR2 or both receptors are known and a few have gone through clinical trials [32,42]. Studies as described in this section should lead to potent inhibitors that are receptor-specific and G-protein/ β -arrestin pathway specific, and hence have high therapeutic potential in a clinical setting for neutrophil-related pathologies and diseases.

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