Structural Basis of a Chemokine Heterodimer Binding to Glycosaminoglycans

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Abstract

Chemokines Cxcl1/KC and Cxcl2/MIP2 play a crucial role in coordinating neutrophil migration to the insult site. Chemokines’ recruitment activity is regulated by monomer-dimer equilibrium and binding to glycosaminoglycans (GAGs). GAG chains exist as covalently linked to core proteins of proteoglycans (PGs) and also as free chains due to cleavage by heparanases during the inflammatory response. Compared to free GAGs, binding to GAGs in a PG is influenced by their fixed directionality due to covalent linkage and restricted mobility. GAG interactions impact chemokine monomer/dimer levels, chemotactic and haptotactic gradients, life time, and presentation for receptor binding. Here, we show that Cxcl1 and Cxcl2 also form heterodimers. Using a disulfide-trapped Cxcl1-Cxcl2 heterodimer, we characterized its binding to free heparin and heparan sulfate (HS) GAGs using nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC), and to immobilized heparin and HS using surface plasmon resonance (SPR). These data, in conjunction with molecular docking, indicate that the binding characteristics such as geometry and stoichiometry of the heterodimer are different between free and immobilized GAGs and are also distinctly different from those of the homodimers. We propose that the intrinsic asymmetry of the heterodimer structure, along with differences in its binding to PG GAGs and free GAGs, regulate chemokine function.
Introduction

Chemokines, a family of small molecular weight proteins (MW \~10 kDa), mediate diverse roles from innate and adaptive arms of the host immune response to developmental biology, neurobiology, and tissue repair. Common to these functions is mobilizing different cell types from one site to another that also must be highly regulated to achieve the desired outcome. Chemokines reversibly exist as monomers and dimers and signal by activating receptors that belong to the GPCR (G protein-coupled receptor) class (1). Receptor interactions are regulated by binding to glycosaminoglycans (GAGs) (2). GAGs are a family of linear sulfated polysaccharides covalently attached to the core proteins of proteoglycans (PGs) (3, 4). PGs are located on cell surfaces, and also in extracellular space where they exist as macromolecular complexes with other PGs and proteins. In addition, GAGs and PG extracellular domains (ectodomains) are cleaved by heparanases and proteases (5-7). Therefore, chemokines can interact with GAGs associated with cell-surface and extracellular PGs, extracellular ectodomains, and those that are free. Mammals express five sulfated GAGs that differ in their backbone structure and sulfation pattern: heparin, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate, and keratan sulfate. A PG can carry a single GAG or hundreds of GAG chains, and one or two types of GAGs. Of the different GAGs, HS and CS are the most common. GAG interactions determine chemotactic and haptotactic gradients, presentation for receptor binding, monomer and dimer levels, life time, and protection against proteolysis (8-12).

Chemokines, on the basis of conserved N-terminal cysteines, are classified as CXC, CC, CX3C, and XC. Since their first report more than 3 decade ago, structures of a large fraction of chemokines have been determined using nuclear magnetic resonance (NMR) and X-ray methods. Despite amino acid sequence identity than can be less than 20%, chemokines share the same structural fold at the monomer level. However, their dimeric structures differ, with CXC-chemokines forming globular and CC-chemokines forming elongated dimers. This classification is not stringent, as some CC chemokines form CXC-type dimers. Further, dimerization constants can vary by several log units from nanomolar (nM) to millimolar (mM), highlighting the inherent plasticity of the dimer interface.

Microbial infection and tissue injury trigger release of multiple chemokines that can be described as a chemokine storm. For instance, several chemokines that recruit neutrophils are frequently observed together in human clinical samples and animal disease models (13-20). This suggests multiplicity of chemokines play non-redundant and synergetic roles in chemokine function. Several studies have shown chemokines including those that recruit neutrophils form heterodimers (21-28). Heterodimer formation has been characterized using NMR, surface plasmon resonance (SPR), chemical and disulfide trapping, modeling, and molecular dynamic (MD) measurements (22-28). These studies indicate CXC- and CC-chemokines form heterodimers within and across the sub-group. Functional studies have also shown that disrupting heterodimer formation alleviates disease symptoms in animal models (21, 24). However, less is known regarding the structural features of the heterodimers and even less regarding their GAG interactions, as characterizing a
heterodimer and its function are complicated by contributions from both monomers and homodimers.

In this study, we characterized GAG binding properties of the Cxcl1-Cxcl2 heterodimer. Cxcl1 (also known as mouse CXCL1/mCXCL1 and KC) and Cxcl2 (also known as mouse CXCL2/mCXCL2 and MIP2) are the major chemokines that orchestrate neutrophil recruitment in mice (18-20). We have previously shown that Cxcl1 and Cxcl2 dimers bind GAGs with higher affinity and that their GAG interactions and neutrophil recruitment profiles are different (29, 30). Our current study indicates that Cxcl1 and Cxcl2 form a heterodimer with a propensity comparable to homodimers. To characterize Cxcl1-Cxcl2 heterodimer function, we designed a non-dissociating heterodimer by introducing a disulfide across the dimer interface. NMR studies show that the disulfide-trapped heterodimer captures the structural features of the native heterodimer. We characterized binding of the trapped heterodimer to free HS and heparin using NMR and isothermal titration calorimetry (ITC) and to immobilized HS and heparin using surface plasmon resonance (SPR). These studies, in conjunction with molecular docking, indicate that binding characteristics of the heterodimer to free and immobilized GAGs are different and are also different from those of the homodimers. We conclude that the intrinsic asymmetry of the heterodimer structure and differences in its interactions between GAGs that are free and those that are constrained by a covalent link in PGs likely play important roles in function.

Materials and Methods

Expression of the disulfide-trapped Cxcl1-Cxcl2 heterodimer. A non-dissociating Cxcl1-Cxcl2 heterodimer was designed by introducing a disulfide across the dimer interface. The genes corresponding to Cxcl1 S26C and Cxcl2 T29C mutants were cloned in a pET32Xa vector, expressed in LB or enriched minimal media containing 15N-NH4Cl or 15N-NH4Cl and 13C-glucose, and purified using a combination of nickel column and reverse phase high-performance liquid chromatography (HPLC). Heterodimer formation was promoted by mixing Cxcl1 and Cxcl2 cysteine mutants overnight in a pH 7.0 buffer containing 20 mM sodium phosphate and 100 mM NaCl at 37 °C. The heterodimer was purified using HPLC, lyophilized, and stored at -20 °C until further use.

Chemical shift assignments. The chemical shifts of the trapped heterodimer were assigned using ~500 μM 13C/15N-Cxcl1-Cxcl2 and Cxcl1-13C/15N-Cxcl2 samples from HNCO, HNCA, CBCA(CO)NH, HCC(CO)NH, 15N-edited TOCSY-HSQC, and HCCH-TOCSY experiments. The NMR samples were prepared in 50 mM sodium phosphate buffer pH 6.0 containing 1 mM 2,2-dimethyl-2-silapentansesulfonic acid (DSS), 1 mM sodium azide, and 10% D2O. The NMR spectra were collected using Bruker Avance III 600 and 800 MHz spectrometers equipped with cryoprobe at 35 °C. All spectra were processed and analyzed using Bruker Topspin 3.2 or Sparky software.

Structural model of the heterodimer. A structural model of the Cxcl1-Cxcl2 heterodimer was generated using CS-Rosetta, a robust tool for generating de novo structures from NMR chemical...
shifts (31). The program uses the PDB database to select protein fragments based on the backbone $C_\alpha$, $C_\beta$, C, N, and NH chemical shifts and then assembles and relaxes these fragments into a converged structure using a ROSETTA Monte Carlo approach. A disulfide bond was introduced between Cxcl1 C26 and Cxcl2 C29 using PyMol. The disulfide-linked heterodimer was energy minimized to ensure that the disulfide adopts proper geometry and then to several cycles of global energy minimization to generate the final structure using the AMBER 12 suite of programs. The quality of the structure was assessed using PROCHECK.

**NMR characterization of GAG binding to the heterodimer.** We characterized the structural basis of heparin octasaccharide (Iduron, U.K.) binding to the trapped Cxcl1-Cxcl2 heterodimer in 50 mM phosphate pH 6.0 buffer at 35 °C using solution NMR spectroscopy. Heparin octasaccharide was prepared as a 10 mM stock in the same buffer. The NMR data were obtained using two heterodimer samples - one isotopically labeled on Cxcl1 and the other on Cxcl2. A series of $^1$H-$^15$N HSQC spectra were collected on titrating heparin until there were no spectral changes. The final molar ratio of heterodimer to GAG was ~1:4. Chemical shift perturbations were calculated as a weighted average of changes in the $^1$H and $^{15}$N chemical shifts (29).

**ITC characterization of GAG binding to the heterodimer.** We characterized the thermodynamics and stoichiometry of heparin octasaccharide binding to the trapped Cxcl1-Cxcl2 heterodimer using a Malvern PEAQ-ITC microcalorimeter at 25 °C. The protein and heparin solutions were centrifuged and degassed under vacuum before use. Titrations were performed by injecting 1×0.5 μl and 18 x 2 μl aliquots of 0.5 mM heparin to 20 μM chemokine in 50 mM sodium phosphate pH 6.0. The titrations were carried out twice that essentially gave the same results. The raw data were corrected using buffer and protein controls and analyzed using the software supplied by the manufacturer.

**SPR characterization of GAG binding to the heterodimer.** We characterized the kinetics, binding affinity, and accumulation of the heterodimer to immobilized heparin and HS chains using a Biacore T100 (GE Healthcare). We used a ~9 kDa HS (Iduron) and a ~15 kDa heparin (Calbiochem). The GAGs were biotinylated using the EZ-Link Biotin LC Hydrazide reagent, immobilized to a Sensor Chip SA, and binding measurements were carried out as described (30). We used two different chips – one containing ~80 RU of immobilized GAG defined as a low-density chip and the other ~220-250 RU of immobilized GAG defined as a high-density chip. Curves were analyzed and fitted using the Biacore T100 evaluation software. Measurements were repeated twice, and both runs provided similar kinetic and binding constants. Stoichiometry was calculated using the formula, $R_{max} \times MW_{L}/MW_{A} \times R_{L}$, where $R_{max}$ is the maximum response units (RU), $R_{L}$ is the density of the immobilized ligand (HS or heparin) in RU units, $MW_{L}$ is the MW of the ligand, and $MW_{A}$ is the MW of the chemokine heterodimer (analyte).

**Structural Model of the heparin-heterodimer Complex.** Molecular docking of heparin octasaccharide to the Cxcl1-Cxcl2 heterodimer was carried out using the High Ambiguity Driven biomolecular DOCKing (HADDOCK) (32). The Cxcl1-Cxcl2 heterodimer structure from
ROSETTA studies and the NMR structure of heparin (PDB ID: 1HPN) were used for docking. Ambiguous interaction restraints (AIRs) were selected based on NMR chemical shift perturbation data. The pair-wise “ligand interface RMSD matrix” over all structures was calculated and final structures were clustered using an RMSD cut-off value of 4 Å. The clusters were then prioritized using RMSD and a “HADDOCK score” (weighted sum of a combination of energy terms).

Results

Characterization of the Cxcl1-Cxcl2 heterodimer. Formation of native Cxcl1-Cxcl2 heterodimer was characterized using solution NMR spectroscopy. Cxcl1 and Cxcl2 reversibly exist as monomers and dimers and dimerize with similar µm affinities (29, 33). Chemical shifts of the backbone amide (¹H and ¹⁵N) are exquisitely sensitive to their environment, and therefore differences in the local environment of dimer-interface residues will result in distinct peaks for the monomer, homodimer, and heterodimer. The HSQC spectra of ¹⁵N-labeled wild type (WT) Cxcl2 show peaks of the dimer at 500µM and of both the monomer and dimer at 30µM (Figures 1A, 1B). A series of HSQC spectra were collected on titrating up to 2-fold excess of unlabeled WT Cxcl1 to 30µM ¹⁵N-labeled WT Cxcl2. On titration, new peaks appear that must correspond to the heterodimer (Figures 1C, 1D). The chemical shifts of most prominent new peaks correspond to dimer interface β₁-strand and C-terminal helical residues, indicating the same residues that promote homodimer also promote heterodimer formation.

Design and characterization of the trapped Cxcl1-Cxcl2 heterodimer. Structural and functional characterization of the native Cxcl1-Cxcl2 heterodimer is challenging due to contributions from Cxcl1 and Cxcl2 monomers and homodimers. Therefore, we designed a non-dissociating Cxcl1-Cxcl2 heterodimer by introducing a disulfide across the dimer interface (Figure 2). For introducing cysteine mutations, we selected residues S26 from Cxcl1 and T29 from Cxcl2. These residues were selected for the reason they are located away from the two-fold symmetry axis, which promotes heterodimer and disfavors homodimer formation. The individual cysteine mutants (Cxcl1 S26C and Cxcl2 T29C) were recombinantly expressed, purified, and the trapped heterodimer was allowed to form by simply mixing the proteins. Formation of the trapped heterodimer was confirmed from SDS-PAGE, mass spectrometry, and NMR spectroscopy. In SDS-PAGE gel, the band corresponding to the heterodimer was observed only under non-reducing conditions (Figure 2B). The NMR spectra of the singly ¹⁵N-labeled disulfide-trapped heterodimer (Figures 2C, 2D) show that both Cxcl1 and Cxcl2 are highly structured. Chemical shifts that could be assigned WT heterodimer were similar to those in the trapped heterodimer. The NMR data collectively indicate that the newly introduced disulfide does not disturb the native fold and that the trapped heterodimer captures the structural characteristics of the native heterodimer.

We assigned chemical shifts of the heterodimer using singly labeled proteins to minimize ambiguity in the assignment process. Around 95% of shifts of both Cxcl1 and Cxcl2 residues in the heterodimer were assigned except for a few terminal residues due to chemical shift degeneracy. The chemical shift index (CSI) analysis indicates that the secondary structural features
of the monomers in the heterodimer are similar to the homodimer. The Cβ chemical shifts of the introduced cysteines (C26 in Cxcl1 and C29 in Cxcl2) indicate that they are disulfide bonded (34). The characteristic downfield chemical shifts of Q11 in Cxcl1 at 11.9 ppm and Q10 in Cxcl2 at 11.8 ppm also indicate that the structural features of the heterodimer are not perturbed on disulfide bond formation.

**NMR characterization of the heterodimer-heparin interactions.** We characterized the binding of an heparin octasaccharide to the heterodimer by individual titrations to 15N-Cxcl1-Cxcl2 and Cxcl1-15N-Cxcl2 samples (Figure 3, Supp. Figures 1 and 2). Chemical shifts of the heterodimer are shown in supplementary table-I. For both titrations, we observed only one set of peaks, indicating that binding is in the fast exchange regime on the NMR time scale. The chemical shift perturbation (CSP) profile on heparin binding is shown in Figure 3. Previous studies have established that GAG binding to chemokines is driven by ionic and H-bonding interactions, and that several of the basic residues that mediate GAG interactions are conserved among both human and mouse neutrophil-activating chemokines (10, 29). Cxcl1 basic residues that show significant perturbation in the heterodimer are H20 and K22 from the N-loop, K46 and R49 from the 40s loop/β3-strand, and K62 and K66 from the C-helix (Figure 3C). Cxcl2 basic residues that are significantly perturbed in the heterodimer are R17 and K21 from the N-loop, and K61, K65, K69, and K71 from the C-helix (Figure 3D). Whereas Cxcl1 residues that are perturbed in the heterodimer are similar to those in the homodimer, Cxcl2 40s loop residues that are perturbed in the homodimer are not perturbed in the heterodimer (29). Several Cxcl1 and Cxcl2 hydrophobic and acidic residues are also perturbed, and the structure reveals that these residues are either in the proximity of basic residues and/or are buried suggesting that they are involved in packing and not in direct binding interactions. The NMR data unambiguously indicate that both Cxcl1 and Cxcl2 of the heterodimer are involved in binding. However, NMR data alone are not sufficient to define whether heparin binds with a unique geometry or adopts multiple binding geometries and whether one heparin or two heparin chains bind per chemokine dimer (29, 46, 49). Therefore, we determined stoichiometry from ITC measurements and used this and NMR data to generate a structural model using computational docking as described below.

**ITC characterization of the heterodimer-heparin interactions.** A complete understanding of heterodimer-GAG interactions requires knowledge of the thermodynamics of the binding process. ITC provides the thermodynamic parameters from a single experiment — enthalpy (ΔH), entropy (ΔS), free energy (ΔG) that is related to the binding affinity, and also stoichiometry (35). The isotherm of heparin binding to the heterodimer is shown in Figure 4. The data fit best to a single-binding site model, yielding a stoichiometry of one GAG binding per heterodimer with a $K_d$ of 8.9 μM. ITC studies of Cxcl1 and Cxcl2 dimers show a stoichiometry of 2 heparin chains bind per dimer with both binding with a $K_d$ of ~4 μM (29). The observed differences in stoichiometry between the homodimers and heterodimer must be due to differences in GAG-binding geometry. Binding of the heterodimer is enthalpically favored and entropically disfavored (Table-I), which is similar to the binding of the Cxcl1 homodimer but different from the Cxcl2 homodimer (29).
**Structural Model of the heterodimer-heparin complex.** We generated HADDOCK structural models of the heterodimer bound to free heparin to gain insight into heparin binding geometry and how each monomer of the heterodimer contributes to the binding process. We first generated a model of Cxcl1-Cxcl2 heterodimer using ROSETTA. Structural models of chemokine heterodimers have also been generated using modeling and molecular dynamics (MD) simulations (22, 24, 26, 27). As ITC indicated a 1:1 stoichiometry, we generated models of the heparin-heterodimer complex by providing restraints between one heparin chain and basic residues of Cxcl1 and Cxcl2 implicated in binding from NMR CSP measurements. HADDOCK runs resulted in one major and one minor cluster. In the major cluster, heparin engages all of Cxcl1 and Cxcl2 basic residues implicated from NMR studies (Figure 5). The model also indicates Cxcl1 K69 is involved in binding though this residue was minimally perturbed in the NMR titrations. Similar observations have been made for lysines in other chemokines and was attributed to cancellation of chemical shift changes from direct and indirect interactions (36, 46). In the minor cluster, heparin engages all of Cxcl1 N-loop and C-helical and only two of Cxcl2 basic residues, C-helical K65 and K69. These data suggest a plastic binding interface resulting in GAG adopting different geometries on the heterodimer.

**SPR characterization of the heterodimer-GAG interactions.** SPR measurements provide kinetic (k_on and k_off) and dissociation (K_D) constants, and maximum signal (R_max) is also an indicator of chemokine accumulation that can be related to stoichiometry. In this experiment, GAG chains are immobilized on a chip, which mimics binding to GAGs in a PG. We characterized binding of the heterodimer to HS and heparin at low and high densities. Measurement at different densities allows characterizing binding to a single GAG and binding to two or more GAGs that mimics binding to PG GAGs due to proximity. The SPR profiles are shown in Figure 6. The kinetic and dissociation constants of the heterodimer are distinctly different from the homodimer (Table-II). Higher accumulation and stoichiometry of the heterodimer are striking, suggesting more efficient packing that is precluded for the homodimer.

**Discussion**

A characteristic feature of host immune response to infection and injury is the release of multiple chemokines that mobilize blood neutrophils to the insult site. Neutrophils are the most abundant leukocyte and possess multiple arsenal for killing pathogens that includes cytotoxic peptides and proteases, superoxide, and NETs (37, 38). The killing response is robust but must be regulated to minimize collateral tissue damage. Chemokines exert their function by activating GPCRs that are regulated by binding to GAGs. During the course of neutrophil recruitment, chemokines encounter PG GAGs in different locations and forms — on the epithelium and endothelium, glycocalyx that lies in the proximity of the luminal surfaces of the epithelium and endothelium, and in the extracellular and pericellular matrices (2, 3). Cell surface PGs either span the membrane or anchored via glycosylphosphatidylinositol. Extracellular and pericellular PGs are secreted and exist as macromolecular complexes with other PGs and proteins. In addition, PG ectodomains and GAGs are cleaved by heparanases and proteases (7, 8). Tissue injury elicits an immediate immune
response, and animal models and clinical data show neutrophils migrate to the injury site within a few hours that lasts over a day or more. Chemokine levels during this time frame can vary by many log units at the injured site and in the vasculature. Animal model, cellular, and biophysical studies have shown that GAG interactions play multiple interconnected roles by regulating chemokine monomer and dimer levels, life time, stability, haptotactic (GAG-bound) and chemotactic (soluble) gradients, and presentation for receptor binding, and that these properties regulate neutrophil migration (9-14, 29, 30, 39-51). In particular, these studies indicate that the dimers bind GAG with higher affinity, monomers and dimers have differential receptor activity, and neutrophil recruitment profiles of monomers and dimers are different.

In this study, we show Cxcl1 and Cxcl2 form heterodimers. To understand the role of heterodimer in chemokine function, we characterized the binding of a disulfide-trapped Cxcl1-Cxcl2 heterodimer to free and immobilized GAGs using NMR, ITC, SPR, and molecular docking methods. We used an heparin octasaccharide for characterizing the structural basis of binding to a free GAG. Cxcl1 and Cxcl2 interact with HS and not heparin in the context of in vivo function. Both HS and heparin share a repeating disaccharide unit composed of glucosamine and hexuronic acid. HS has a modular structure with sulfated regions separated by non-sulfated regions and is also intrinsically heterogeneous due to differential N- and O-sulfation. Heparin is universally used for structural studies as it is more uniformly sulfated and size-defined oligosaccharides are commercially available. NMR studies have also shown that the binding interactions of chemokines CXCL1 and CXCL5 to heparin and HS polymers are similar (47), indicating heparin is a good surrogate for describing the structural features of binding to free HS. Our current NMR and ITC studies indicate that binding interactions, geometry, and stoichiometry of the Cxcl1-Cxcl2 heterodimer for free heparin are different from the homodimers.

Compared to free GAGs, gaining structural insights and the molecular features of binding to PG GAGs is challenging. GAG chains are covalently linked to serine residues in PGs, and so GAG chains have the same orientation and their mobility is also restricted compared to free GAGs due to its covalent linkage. SPR studies allow capturing structural features of PG GAGs, as they are immobilized on a sensor chip and so GAG chains have the same orientation. In principle, binding to the low density chip captures binding to a single GAG chain as it will be minimally influenced by neighboring GAGs, and binding to the high density chip allows capturing binding to two or more GAG chains due to proximity providing favorable interactions exist.

We characterized the binding of the heterodimer and also of the two homodimers to both heparin and HS immobilized at low density and high density. Our data show that the binding interactions vary between HS and heparin and between the low density and high density chips (Table-II). In particular, higher accumulation of the heterodimer under all experimental conditions stands out. Interestingly, heterodimer showed higher accumulation on the HS high density chip compared to heparin, suggesting structural heterogeneity in HS chains allows maximal packing and crosslinking of the HS chains. Much higher accumulation of the heterodimer compared to the homodimers on the high density HS chip also corroborates this observation. Homodimers also
show higher accumulation on the high density chip, but is less pronounced compared to the heterodimer, suggesting intrinsic asymmetry of the heterodimer allows interactions that are not possible for the symmetric homodimer. GAG binding sites in a chemokine homodimer are antiparallel due to two-fold symmetry, and so the basic residues in two monomers of the homodimer must bind non-equivalent acidic groups to promote crosslinking (Figure 7A). Binding will be less efficient if the distances and orientation between the chemokine basic residues and GAG acidic residues are not optimal. Efficient binding and crosslinking of GAG chains by the heterodimer can be attributed to non-equivalent binding surfaces of the Cxcl1 and Cxcl2 monomers (Figure 7B). It is unlikely that the binding mode and interactions to the free and immobilized GAGs are the same, as GAG binding across the heterodimer interface engages most of the chemokine basic residues. In principle, both the heterodimer and homodimer can also bind to GAG chains from two different PGs either on the cell surface, glycocalyx, or extracellular matrix. These binding interactions could reflect those observed for the free or immobilized GAGs due to a range of structural constraints imposed on the GAG chains at these locations (2).

To our knowledge, this is the first report of binding interactions of a chemokine heterodimer to immobilized GAGs. Binding-induced crosslinking of immobilized HS have been reported for several chemokines using fluorescence recovery after photobleaching measurements (52), suggesting differences in GAG binding between homodimers and heterodimer could play a regulatory role for the chemokine family at large, which is nevertheless tuned for any given chemokine pair. In summary, our studies indicate that the binding of the heterodimer to free and immobilized GAGs are different and are also different from those of the homodimers, suggesting its life time, receptor interactions, and recruitment activity, neutrophil phenotype for resolving infection and injury are likely to be different. We conclude that the ability to form heterodimers and their distinct GAG interactions play a role in tuning neutrophil response for successful resolution of inflammation.

Acknowledgements.

This work was supported in part by the NIH grants R21AI124681, R21AI135606, and S10OD023576, and a pilot grant from the Institute for Human Infections and Immunity. We thank Dr. Tianzi Wang for NMR technical support. The authors acknowledge the Sealy Center for Structural Biology and Molecular Biophysics at the University of Texas Medical Branch at Galveston for providing research resources.

Competing Interest

The authors declare no competing financial interest.

Abbreviations

KC, keratinocyte-derived chemokine; MIP2, macrophage inflammatory protein 2; Cxc11, CXC ligand 1; Cxcl2, CXC ligand 2; Cxcr2, CXC receptor 2; GAG, glycosaminoglycan; WT, wild-
type; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; HS, heparan sulfate; RU, response units

Data Availability Statement
All data are included in the main manuscript and in the supplementary data file
Table-I. Thermodynamic parameters of binding to an heparin octasaccharide as measured by isothermal titration calorimetry\textsuperscript{a,b}

|                  | n    | ΔH   | TΔS  | ΔG   | K\textsubscript{D} |
|------------------|------|------|------|------|------------------|
|                  | kcal/mol | kcal/mol | kcal/mol | (µM) |
| Cxcl1-Cxcl2 HD   | 1.0±0.1 | -14.7±0.1 | 7.8±0.1 | -6.9±0.1 | 8.8±0.1 |
| Cxcl1-Dimer      | 2.0±0.1 | -12.6±0.1 | 5.3±0.1 | -7.3±0.1 | 4.2±0.1 |
| Cxcl2-Dimer      | 2.0±0.1 | -5.4±0.1 | -1.9±0.1 | -7.4±0.1 | 3.9±0.1 |

\textsuperscript{a} reported values are a mean of two experiments

\textsuperscript{b} Data for the Cxcl1 and Cxcl2 dimers are from ref. 29
Table II. Kinetic and binding constants for binding to HS and heparin polymers using surface plasmon resonance\textsuperscript{a, b}

|                          | $k_{\text{on}}$ (M\textsuperscript{\text{-1}}s\textsuperscript{-1}) | $k_{\text{off}}$ (s\textsuperscript{-1}) | $K_D$ (nM) | $K_D$ (SS) (nM) | $R_{\text{max}}$ (RU) | n\textsuperscript{5} |
|-------------------------|--------------------|-----------------|-----------|----------------|-----------------|-----------|
| **LD-HS ($R_L = 78$)** |                    |                 |           |                |                 |           |
| Cxcl1-Cxcl2 HD          | 0.14 x 10\textsuperscript{4} | 0.5 x 10\textsuperscript{3} | 4.4       | 430            | 221             | 1.6       |
| Cxcl1 dimer             | 20 x 10\textsuperscript{4} | 0.6 x 10\textsuperscript{3} | 3         | 2300           | 153             | 1.1       |
| Cxcl2 dimer             | 7.5 x 10\textsuperscript{4} | 3.2 x 10\textsuperscript{3} | 43        | 4700           | 78              | 0.55      |
| **HD-HS ($R_L = 248$)** |                    |                 |           |                |                 |           |
| Cxcl1-Cxcl2 HD          | 24 x 10\textsuperscript{4} | 1.7 x 10\textsuperscript{3} | 7.4       | 98             | 2950            | 6.6       |
| Cxcl1 dimer             | 8.1 x 10\textsuperscript{4} | 1.6 x 10\textsuperscript{3} | 19        | 1300           | 893             | 2.0       |
| Cxcl2 dimer             | 1.6 x 10\textsuperscript{4} | 1.3 x 10\textsuperscript{3} | 77        | 2900           | 621             | 1.4       |
| **LD-heparin ($R_L = 81$)** |                    |                 |           |                |                 |           |
| Cxcl1-Cxcl2 HD          | 1.8 x 10\textsuperscript{4} | 0.6 x 10\textsuperscript{3} | 32        | 410            | 328             | 3.6       |
| Cxcl1 dimer             | 3.3 x 10\textsuperscript{4} | 0.4 x 10\textsuperscript{3} | 11        | 900            | 190             | 2.1       |
| Cxcl2 dimer             | 1.4 x 10\textsuperscript{4} | 1.1 x 10\textsuperscript{3} | 78        | 1200           | 152             | 1.8       |
| **HD-heparin ($R_L = 220$)** |                    |                 |           |                |                 |           |
| Cxcl1-Cxcl2 HD          | 1.2 x 10\textsuperscript{4} | 1.7 x 10\textsuperscript{3} | 144       | 851            | 1300            | 5.5       |
| Cxcl1 dimer             | 1.4 x 10\textsuperscript{4} | 1.2 x 10\textsuperscript{3} | 86        | 290            | 853             | 3.6       |
| Cxcl2 dimer             | 7.4 x 10\textsuperscript{3} | 1.1 x 10\textsuperscript{3} | 159       | 728            | 800             | 3.4       |

\textsuperscript{a}Data are representative of two independent experiments

\textsuperscript{b}Data for the Cxcl1 and Cxcl2 dimers are from ref. 30

\textsuperscript{5}Stoichiometry (n) is defined as number of dimers bound per GAG chain
Figure Legends.

Figure 1. Sections of the $^1$H-$^{15}$N HSQC spectra of Cxcl2 showing peaks at 500µM corresponding to the dimer (red; A), at 30 µM corresponding to both the monomer and dimer (green; B). On titrating Cxcl1, new peaks appear that must correspond to the heterodimer (blue; C). Overlay of the spectra B and C are shown in panel D.

Figure 2. (A) Heterodimer trapping strategy using cysteine mutations (shown in yellow). (B) SDS-PAGE gel showing the disulfide bond the formation. The higher molecular weight heterodimer band is observed only under non-reducing conditions. BME - β-mercaptoethanol. NMR structural features of the trapped heterodimer. $^1$H-$^{15}$N HSQC spectra of the (C) $^{15}$N-Cxcl1-Cxcl2 and (D) $^{13}$N-Cxcl2-Cxcl1 trapped heterodimers. The NMR spectra indicate a properly folded protein with no evidence of monomer or homodimer.

Figure 3. Sections of the $^1$H-$^{15}$N HSQC spectra showing the overlay of free (black) and heparin octasaccharide-bound Cxcl1-Cxcl2 heterodimer (red/blue). Panel A and B show movement of Cxcl1 and Cxcl2 peaks, respectively. Histogram plots of heparin octasaccharide binding-induced chemical shift changes in Cxcl1 (C) and Cxcl2 (D) of the Cxcl1-Cxcl2 trapped heterodimer. Residues that show CSP above the threshold (dashed line) are considered involved in binding. Basic residues arginine, lysine, and histidine are shown in blue.

Figure 4. ITC titration of heparin octasaccharide and the integrated data obtained after subtracting the heat of dilution are shown in the upper and lower panels, respectively. The titrations were performed in 50 mM sodium phosphate pH 6.0 at 25 °C.

Figure 5. (A, B) Two different orientations of the HADDOCK models of heparin bound Cxcl1-Cxcl2 heterodimer complex. (C) Electrostatic representation of the complex in the same orientation as shown in panel B. Cxcl1 and Cxcl2 are shown in pink and green and heparin chain as a stick.

Figure 6. Surface plasmon resonance (SPR) profiles of Cxcl1-Cxcl2 heterodimer binding to immobilized low density (LD) heparin (A), high density (HD) heparin (B), LD-HS (C), and HD-HS (D). The curves represent the signal (RU) minus the reference signal (no GAG).

Figure 7. A model of chemokine homodimer (A) and heterodimer (B) binding to immobilized GAGs. Monomers of the heterodimer are shown in different shades of green. GAG-binding residues in each monomer of the chemokine homodimer and heterodimer are arbitrarily labeled 1 to 4. GAG-binding residues are diametrically opposite in the homodimer because of two-fold symmetry about the dimer interface (shown by a black dot) but no such symmetry exists for the heterodimer. In the heterodimer, GAG binding residues in the two monomers and dimers are shown at different locations. For illustrative purposes, we use the schematic of an heparin octasaccharide, with iduronate and glucosamine shown as an oval and rectangle, and N-sulfate
(NS), 2-sulfate (2S), and 6-sulfate (6S) shown as spheres in different colors. Helical structure of heparin clusters the 3 sulfate groups on opposite faces of the helical axis. Direction of the GAG orientation is shown by arrows. The schematic shows interactions of the two monomers in the homodimer cannot be the same, and that the monomers of the heterodimer can effectively bind to both GAG chains.
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Figure 2

A. Schematic representation of Cxcl2 and Cxcl1 proteins with T29C and S26C mutations. The arrow indicates the direction of the reaction.

B. SDS-PAGE gel showing the heterodimer formation of Cxcl1-Cxcl2 with BME treatment.

C. NMR spectrum of Cxcl1-Cxcl2 heterodimer showing the chemical shift changes.

D. Another NMR spectrum showing similar changes in chemical shifts.
Figure 4

The figure shows a graph of μcal/s vs. Time (min) at the top and ΔH (kcal/mol) vs. Molar Ratio at the bottom. The graph depicts the calorimetric data over a time span of 40 minutes with a gradual increase in ΔH with respect to the molar ratio.
Figure 6

A
LD-heparin

B
HD-heparin

C
LD-HS

D
HD-HS

Time (seconds)
Figure 7

A  

Homodimer

core protein

B  

Heterodimer

core protein

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Figure 1
Figure 2

A

B

C

D

Cxcl2 + T29C + S26C → Cxcl1-Cxcl2 heterodimer

Heterodimer

BME - +

\[
\begin{array}{c}
\text{25 KDa} \\
\text{20 KDa} \\
\text{15 KDa} \\
\text{10 KDa}
\end{array}
\]

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\begin{bmatrix}
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\begin{bmatrix}
1 & 2 & 3 \\
4 & 5 & 6
\end{bmatrix}
Figure 4
Figure 6

(A) LD-heparin  (B) HD-heparin  (C) LD-HS  (D) HD-HS

Time (seconds)
Figure 7

A  core protein

Homodimer

B  core protein

Heterodimer