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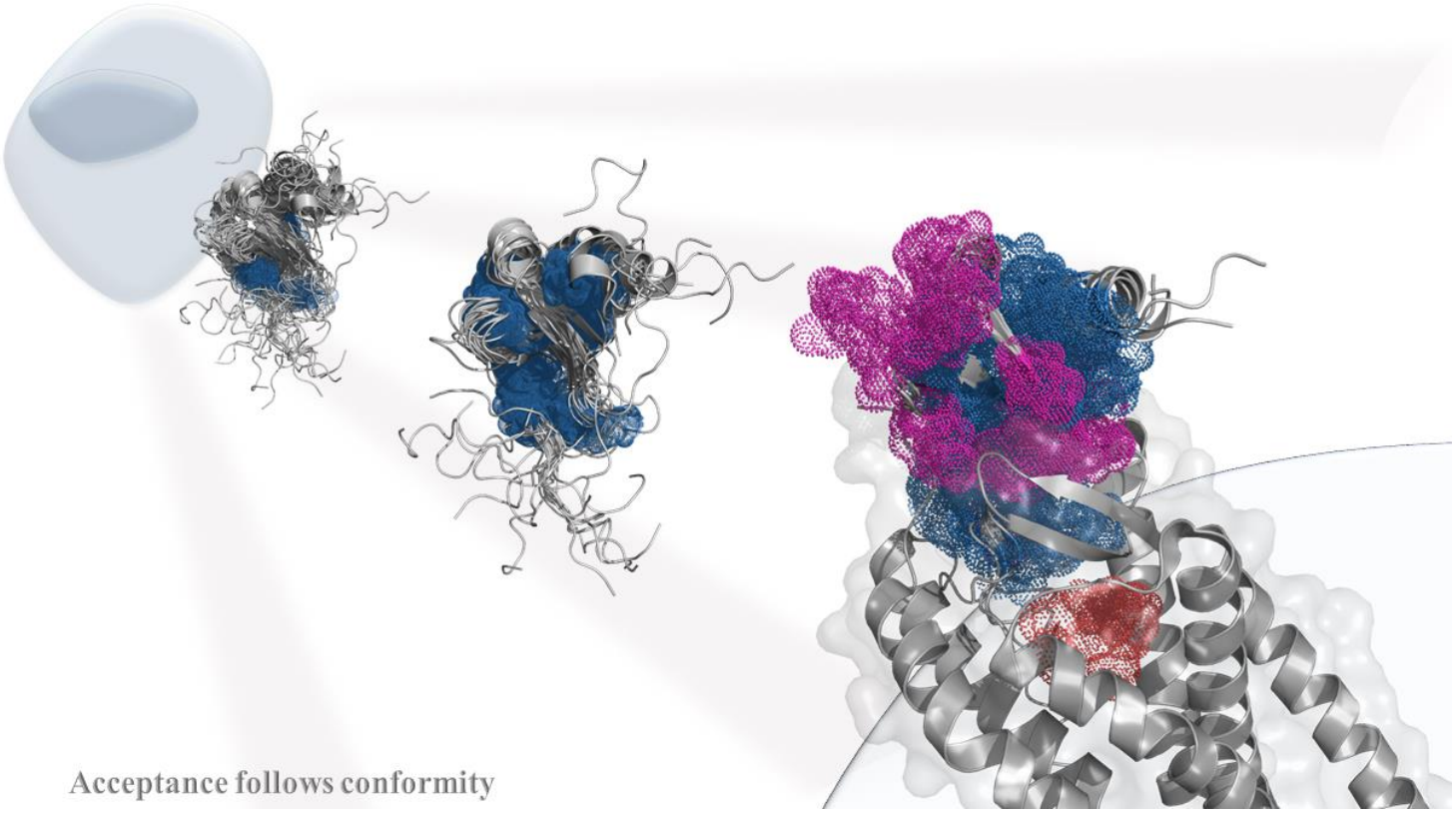

Impact of N-terminal Chemokine Modifications on Cooperativity with Small Molecule Ligands

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Preface

This thesis is being submitted for the purpose of obtaining the degree of *philosophiae doctor* (PhD) in Pharmaceutical Sciences at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, and for the purpose of obtaining the degree of Doctor of Biomedical Sciences at the Faculty of Medicine, KU Leuven, Belgium. The practical work involved has been carried out from December 2016 to December 2020 at the Laboratory of Molecular Pharmacology, University of Copenhagen and at the Laboratory of Molecular Immunology, KU Leuven. The project and employment was funded by the Faculty of Health and Medical Sciences and the Novo Nordisk Foundation at the University of Copenhagen, and a C1 grant at KU Leuven. The supervision of the project was undertaken by the following supervisors:

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A manuscript, a published article and an extended results section presents the main experimental work of this PhD thesis:

Manuscript 1: "**A conserved chemokine receptor motif confers selective engagement of MIP and MCP chemokines**", Submitted to Science Signaling, February 2021

Manuscript 2: "**Selective allosteric modulation of N-terminally cleaved, but not full length CCL3 in CCR1**", Published in ACS Pharmacology and Translational Science

Extended results: "**The minimalized contribution by chemokine N-termini to overall chemokine-receptor interactions is dictated by the receptor**", Preliminary results

Acknowledgements

I would like readers of this thesis to know that a single PhD student did not come up with this many ideas, or build this much data. I had a lot of help in order to plan projects, organize my time, perform experiments and digest the data. During the whole project, I was helped along immensely by Mette Rosenkilde, my primary supervisor, something everyone at the MolPharm lab has the privilege of experiencing. Mette has a talent to both lead and listen, and could therefore give me lots of encouragement, ideas, experience and patience in equal measure, and I was lucky that she guided my work.

It was also a great experience to work with my primary co-supervisor, Paul Proost. It is not common that the responsibilities of university professors allow them to play an active role with experiments in the lab, but Paul manages this and much more, with an impressive friendliness and humor. I hope to follow his good example in my years ahead.

A special thank you is also due for Jon Våbenø, who took an interest in my project and became somehow an oracle for my work and an essential collaborator to discuss my thoughts with. He paid attention to structural details of chemokines no one else had noticed, and predicted several of our key findings. I am also grateful to Stefanie Karlshøj, who was my mentor long before this PhD project, with me filling the role of student and helper to her chemokine projects, which I slowly inherited after she left for the green private sector pastures, and could no longer be my co-supervisor. I also want to thank Thomas Frimurer and Karen Martinez for making themselves available as my co-supervisors.

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I think the young students who come to help and learn in our labs deserve recognition as well, and in my case I was lucky that Sara Schuermans carried out her master's project working with me. She worked incredibly hard on the data presented at the end of my thesis, and I could not have managed without her help.

My family and my girlfriend were of course absolutely essential for me to be able to keep on going, and I hope they will always know that.

Summary

Migrating human immune cells carry out many essential roles that require them to relocate to appropriate tissues throughout the body and adjust their behavior depending on the local tissue situation. This involves instructions related to threats from pathogens, signals from peripheral cells that indicate stress or tumor-like characteristics, and from central immune organs controlling the further differentiation of the immune cells. Many of these instructions involve the chemokine system, a family of soluble signaling proteins (chemokines) secreted by cells contacting migrating immune cells through specific G protein-coupled receptors (GPCRs). A variety of common human diseases involve the immune system. As part of the immune network, the chemokine system plays an essential role in normal physiology and pathology. Therefore, it is important to better understand how the chemokine system could be triggered naturally and artificially.

In this thesis, we focus on two groups of chemokines, known as the macrophage inflammatory proteins (MIPs) and the monocyte chemotactic proteins (MCPs), that are characterized by seemingly redundant interactions with an overlapping set of receptors, in particular the CC chemokine receptors CCR1, CCR2 and CCR5. These are unusually difficult to target pharmacologically *in vivo*, because their role in the immune system is defined by nuances of chemokine recognition and receptor activation. We therefore study the molecular interface between the chemokines and their cognate receptors *in vitro* as well as features that allow the natural system to distinguish chemokines from one another.

In **manuscript 1**, we learn that there is a conserved cluster of aromatic residues in an exposed location of chemokine receptors. This cluster is central for the receptors to differentiate MIP from MCP chemokines. Furthermore, the same receptor cluster is important to define the role of the MIP chemokine N-termini, a chemokine region that is known to be central to the receptor activation mechanism. In **manuscript 2**, we describe how shortening the chemokine N-terminus leaves room for a small synthetic drug to occupy the same space, which allows us to boost chemokine-receptor binding beyond their natural affinity. This is important because only a selection of chemokines are naturally shortened this way, and drugs can therefore theoretically be designed for a matching selectivity. There is doubt about the relative importance of different regions of chemokines for binding and activation of receptors. This presents us with a problem to define when it is appropriate to target the chemokine N-terminal binding site with a drug. We address this in the **extended results** section, where we apply chemokine N-terminal peptides directly to the receptors at very high concentrations. This allowed us to make several conclusions. We found that chemokine N-termini can activate cognate receptors alone and with specificity, suggesting that they are not necessarily reserved for a specialized signaling role in the interaction. However, we also learned that this conclusion was only true for some interactions, meaning that it is likely futile to formulate a single model for chemokine interactions.

The lessons learned during this thesis will be valuable when more specific efforts are made by investigators attempting to target parts of the MIP/MCP interaction network either therapeutically or in further research.

Resumé (Danish summary)

Migrerende menneskelige immunceller varetager mange kritiske roller, hvor det er nødvendigt at de kan opsøge det passende væv i kroppen og tilpasse sin rolle til situationen i det lokale væv. Det involverer ikke blot instruktioner om direkte trusler fra pathogener, men også signaler fra perifære celletyper der kan indikere at de oplever stress eller at de udvikler tumor-lignende karakteristika, og fra centrale immunorganer der dirigerer den løbende differentiering af immunceller. Mange af disse instruktioner involverer kemokinsystemet, som er en familie af opløselige signalproteiner (kemokiner) der udskilles af celler for at skabe kontakt med migrerende immunceller, of deres receptorer udtrykt på overfladen af immunceller. En betydelig del af almindelige menneskelige sygdomme involverer aspekter af immunsystemet, og iblandt disse spiller kemokinsystemet ofte en betydelig rolle, selv hvis det fungerer korrekt. Derfor ville det være en væsentlig terapeutisk fordel hvis vi bedre kunne forstå hvordan systemet kan styres, både naturligt og med lægemidler.

I denne afhandling fokuserer vi på to grupper af kemokiner der grupperes under navnene MIP og MCP, og som kendetegnes ved tilsyneladende redundante interaktioner med en overlappende gruppe receptorer, især CCR1, CCR2 og CCR5. Disse receptorer er særligt udfordrende at påvirke farmakologisk *in vivo*, fordi deres rolle i immunsystemet defineres finere nuancer af kemokingenkendelse og receptoraktivering. Vi undersøger derfor den molekylære interaktionsoverflade mellem kemokinerne og deres genkendte receptorer *in vitro*, og hvilke kendetegn der tillader det naturlige system at skelne kemokiner fra hinanden.

I **manuskript 1** lærer vi om en konserveret gruppering af aromatiske aminosyrer i en udsat position på receptorerne som er central for at receptorerne kan differentiere mellem MIP og MCP kemokiner. Desuden er den samme aminosyregruppering vigtig for at definere rollen af MIP kemokiners N-terminus, som er en region der er kendt for at være vigtig for aktiveringsmekanismen af genkendte receptorer. I **manuskript 2** beskriver vi hvordan en forkortelse af kemokiners N-terminus skaber plads til at små syntetiske stoffer kan finde sig tilrette i den plads dette giver, hvilket tillader os at forstærke den naturlige kemokin-receptor binding. Dette er vigtigt fordi kun bestemte kemokiner naturligt forkortes således, og lægemidler kan derfor i teorien designes med en matchende selektivitet. Det er usikkert hvor meget receptorbinding og –aktivering varetages af forskellige kemokinregioner, hvilket gør det svært at forudsige hvornår det er passende at påvirke kemokiners N-terminus med et lægemiddel. Vi bearbejder dette i det udvidede resultatafsnit, hvor vi stimulerer receptorer direkte med kemokiners N-terminus ved høje koncentrationer. Dette førte til nye konklusioner, mest vigtigt at vi nu ved at kemokiners N-terminus kan aktivere receptorer alene og specifikt, som betyder at de ikke er begrænset til at varetage en stimulerende rolle. Vi lærte dog også at de opfører sig anderledes på visse receptorer, og derfor er det sandsynligvis ikke muligt at formulere en overordnet interaktionsmodel for kemokiner.

Afhandlingen beskriver derfor værdigfulde lektioner for forskere der for fremtiden forsøger at målrette nye lægemidler imod elementer af MIP/MCP interaktionsnetværket.

Abbreviations

7TM	7-transmembrane
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic adenosine monophosphate
CCL/CXCL/ CX_3CL/XCL	CC/CXC/CX_3C/XC -chemokine ligand
CCR/CXCR/ CX_3CR/XCR	CC/CXC/CX_3C/XC -chemokine receptor
CD	Cluster of differentiation
CREB	cAMP response element-binding protein
CS/CRS	Chemokine site/Chemokine recognition site
ECL	Extracellular loop
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
GAG	Glycosaminoglycan
GDP/GTP	Guanosine diphosphate/triphosphate
GPCR	G protein-coupled receptor
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
ICL	Intracellular loop
IP	Inositol phosphate
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PI	Protease inhibitor
TM	Transmembrane helix

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1. Introduction

This thesis revolves around members of the chemokine system with a focus on the molecular structure of chemokine ligands and receptors, their sequence variations and signaling capacity. Chemokines and their receptors play an essential role in a great number of activities of the human immune system, as well as during embryonic development and tissue remodeling/regeneration. Chemokines are named for their activity as chemotactic cytokines, which refers to induction of migration of leukocytes to tissue sites that require further interaction with the immune cells. The introduction will first describe the broader structural knowledge we have about the type of receptors we are referring to, then move on to the biological importance of the chemokine system, before finally diving into the finer details of the CC chemokine receptors CCR1, CCR2 and CCR5. These receptors have been the focus of this PhD project, and we point out some factors that make single ligand-receptor interactions "tick" in just the right way.

Chemokine receptors belong to the 7TM superfamily

There are 23 distinct endogenous chemokine receptors, all made up by a protein chain passing the cell membrane with 7 transmembrane (TM) helices. This leads to a structure with an extracellular N-terminus of about 30-50 amino acids, an intracellular C-terminus of about 50-70 amino acids, and a bundled "barrel"-structure formed by the 7 transmembrane helices (TM1-7). The helices are connected by loops, three of which are extracellular and three that are intracellular. The extracellular loops (ECLs) 1-3 are found in proximity to the N-terminus and the four segments are available for interactions with various components and soluble molecules found in the extracellular matrix (ECM). The intracellular loops (ICLs) 1-3 are in proximity to the C-terminus, and are available for interactions with components of the cell cytoplasm (figure 1)[1].

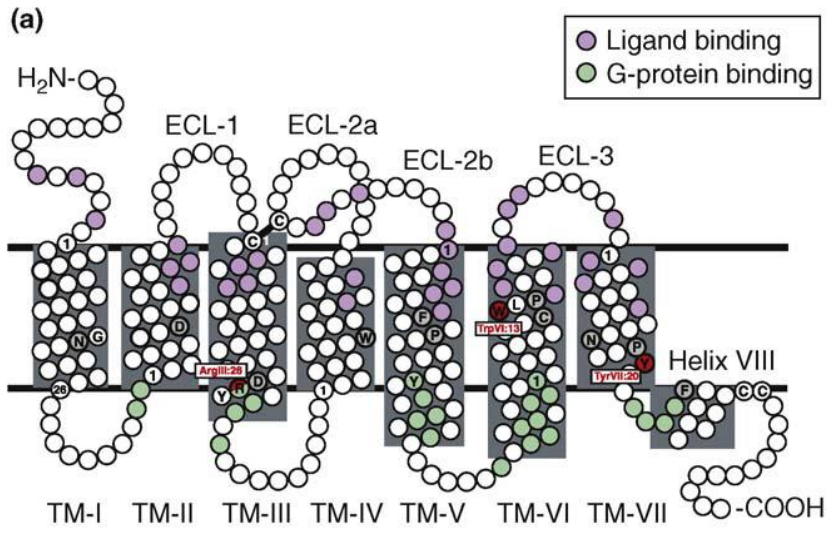

Figure 1. Serpentine model presenting common features of 7TM receptors. Black horizontal lines represent the cell membrane, with the receptor N-terminus and extracellular loops (ECLs) above the membrane and the C-terminus and intracellular loops (ICLs) below. A conserved residue in each transmembrane helix (TM) in grey is used as a reference in the Ballesteros-Weinstein 7TM residue numbering system[2]. 7TM positions associated with specific functions at the time of the original publication are highlighted in color[1].

In general terms, the root purpose of the 7 transmembrane (7TM) receptors is to allow binding to very specific elements in the extracellular matrix (ECM) by the extracellular loops and N-terminus, and binding to various signaling mediators expressed in the cytoplasm[3]. Binding partners will prefer interaction with recognized receptor domains when the receptor adopts a "resting" conformation that creates an optimal interaction interface with the binding partner. A series of connected discreet movements of amino acids along the transmembrane barrel structure (i.e. microswitches) means that when a binding partner, e.g. an extracellular ligand, disturbs the extracellular receptor regions, the shape-change can be transmitted through the membrane to change conformation of the intracellular regions[1, 4-6]. This can cause the release of bound intracellular signaling mediators from the receptor, which allows them to trigger the onset of signaling cascades appropriate to the receptor[7]. In ideal natural settings, cells will react to these signals with a suitable behavior based on the extracellular ligand they meet. Several signaling pathways are usually coupled to a single receptor activation event, but the so-called heterotrimeric G proteins are usually among those connected with 7TM receptors. Therefore, most 7TM receptors are commonly referred to as G protein-coupled receptors (GPCRs).

The varied cells and tissues of the body must be able to sample and respond to a great range of factors in the ECM to sense the situation in their immediate vicinity (paracrine factors) and at distant sites of the body (hormone factors)[8]. This is not a trivial task, and therefore the human genome encodes more than 700 different 7TM receptors, which is one among several receptor superfamilies (figure 2)[9-11]. Based on protein sequence homology, 7TM receptors are divided into the classes A (also known as the rhodopsin-like receptors), B (the secretin family), B2 (adhesion), C (metabotropic glutamate receptors) and F (Frizzled and Taste 2), where class A encompasses the majority of receptors, including chemokine receptors[10].

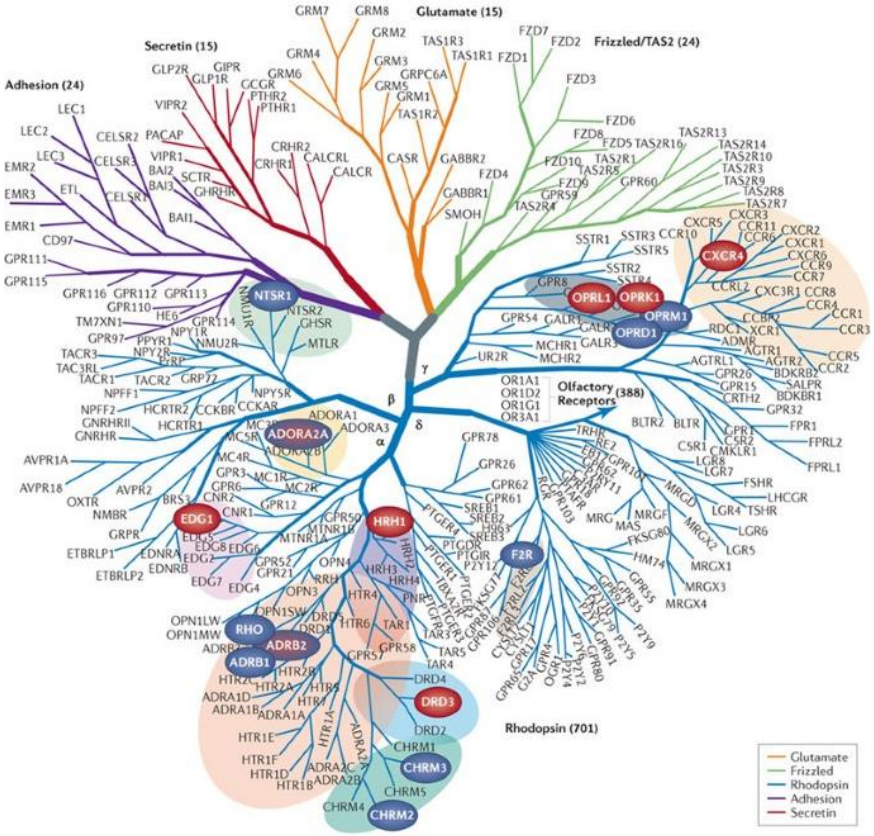

Figure 2. Phylogenetic tree of 7TM receptors based on sequence similarity of the transmembrane helices. The branches are colored according to class, with chemokines belonging to the Rhodopsins, or class A. Crystal structures of highlighted receptors had been solved at the time of publication. From Stevens et al. 2013[11].

GPCRs display complex signalosomes

GPCRs can interact with a range of signaling mediators or effector molecules, most notably combinations of heterotrimeric G proteins composed of different variants of the involved subunits, called the α, ß and γ subunits[12]. In its non-signaling state, a GPCR is bound to an inactive G protein trimer via recognition motifs in the intracellular loops (ICLs) and the C-terminus[13]. In this state the Gα subunit binds a GDP nucleotide, but upon activation, the receptor can act as a guanine nucleotide exchange factor and replace the GDP for GTP[14]. This triggers release of the G protein trimer from the receptor which separates into an active Gα subunit and a free Gßγ dimer. The Gα subunit can then transfer energy to the next signaling mediator by hydrolysis of GTP back to GDP, after which it can re-associate with the Gßγ dimer and the trimer can return to a receptor-bound state (figure 3).

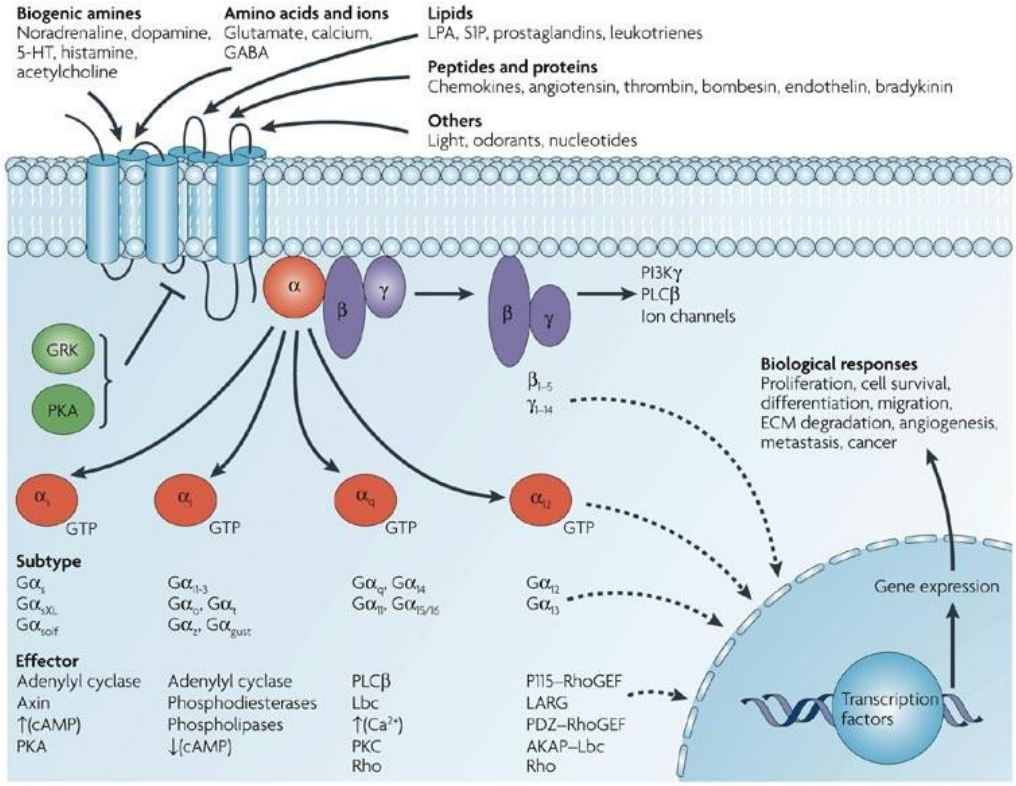

Figure 3. G protein heterotrimer subtype compositions that can be associated with GPCRs. The G protein subtypes each influence the activity of a distinct pattern of downstream signaling mediators. From Dorsam and Gutkind 2007[15].

The most thoroughly characterized outcome of GPCR activation depends on the Gα subtype. Gαs stimulates adenylate cyclase, which can generate cyclic AMP (cAMP) from ATP. cAMP acts as a second messenger that further triggers cascades of downstream effector activities, cAMP-binding ion channels, exchange proteins activated by cAMP (EPACs), protein kinase A (PKA) and the transcription factor cAMP response element-binding protein (CREB)[16]. Most chemokine receptors couple to Gαi, which inhibits adenylate cyclase, and thus blocks the cAMP signaling cascade. This too is a form of signaling, as many cells exhibit some level of constitutive (ongoing) Gαs signal which controls their behavior. Gαi also triggers the MAPK/ERK pathway which activates other transcription factors such as C-myc and C-fos and affects ribosomal protein synthesis[17, 18].

When G protein trimers leave their receptor upon activation, receptor sites that were otherwise blocked by the G protein become exposed to the cytoplasm. G protein-coupled receptor kinases (GRKs) phosphorylate patches of serine and threonine residues that are copiously present on GPCR C-termini. In its phosphorylated form, the receptor C-terminus can be recognized for binding by arrestins. The GPCR-arrestin complex blocks the binding of G proteins which desensitizes (arrests) the receptor to further ligand-induced stimulation[19, 20]. Next, the GPCR-arrestin complex interacts with clathrin and adaptor proteins that can form membrane pits capable of budding off as endosomes.

In early sorting endosomes, proton pumps generate a mildly acidic environment which is usually enough to dissociate ligand-receptor complexes[21]. From this type of endosome, receptors may either be sorted into compartments that bud off as recycling endosomes moving back to the cell membrane[19], or stay until the endosome develops into a "late" form, which fuses to lysosomes and degrades the contents. In this way, ligand-induced signaling is a time-limited event, followed by a desensitization that may be brief or long-lasting.

A special signaling feature in place for chemokine receptors is their mediation of integrin "inside-out" activation. Integrins are single-transmembrane heterodimers with a large adhesive extracellular domains that display an affinity to other cell surfaces and the extracellular matrix. They can be activated from within the cell cytoplasm which changes the conformation of the extracellular domains for increased interaction affinities. Many leukocyte subtypes need these interactions for the physical capacity to migrate, and it helps them to organize leading edge actin polymerization for movement along tissue surfaces, or to stop and extravasate through endothelial cell barriers where chemokines are expressed[22, 23].

Conserved structural and functional domains of chemokines

The chemokine ligands capable of triggering GPCR signaling cascades share a conserved and well-defined tertiary structure. They range in size from 8 – 12 kDa (roughly 65 – 110 amino acids), and are divided into a small N-terminal domain which is up to about 10 amino acids in mature chemokines, and a large core domain with a stable globular fold. Conserved cysteines are located at the border between the two domains and form disulfide bridges with other cysteines in the core domain, which stabilizes its conformation. Most chemokines have two cysteines between the two domains, either directly next to each other or separated by a single residue. This distinction defines chemokines as either CC or CXC chemokine ligands, respectively, and is important because the vast majority of chemokine receptors can only recognize chemokines from within one of these two groups[24, 25]. A few chemokines are outliers, having either three residues between the conserved cysteines, or only one conserved cysteine in this region, leaving us with four chemokine ligand groups (CCL, CXCL, CX_3CL and XCL) and their corresponding receptor groups (CCR, CXCR, CX_3CR and XCR)[26].

The two-site model is a generalized activation model of chemokine receptors that assigns a functional role of the aforementioned chemokine domains (figure 4)[27, 28]. According to this model, a large surface area of the chemokine core domain is recognized by the receptor N-terminus. This interaction interface makes up the major portion of the overall ligand-receptor interaction, and is therefore responsible for the majority of the binding affinity for correctly matched ligand-receptor pairings. The receptor N-terminus is designated Chemokine Recognition Site 1 (CRS1) and the involved part of the chemokine core domain is Chemokine Site 1 (CS1)[29]. A small segment of two residues where the receptor N-terminus transitions to the first transmembrane helix, known as Chemokine Recognition Site 1.5 (CRS1.5), binds to a small area next to the CC or CXC chemokine motif, known as Chemokine Site 1.5 (CS1.5)[30]. It is associated with the tilting of the chemokine core

domain to increase or decrease its distance from the receptor extracellular loops (ECLs) and transmembrane structures. An opening is found between the upper segments of the receptor transmembrane helices, covered by the ECLs, called the receptor pocket. It is also known as Chemokine Recognition Site 2 (CRS2) and can interact with the N-terminus of chemokines, known as Chemokine Site 2 (CS2) in this context. This interaction is often regarded as the interaction that stabilizes an active conformation of the receptor transmembrane structure.

Chemokine receptor activation has also long been reported as a two-step model, or two-step two-site model, adding that receptor N-terminus to chemokine core (CRS1-CS1) interactions are needed first in order to direct a subsequent interaction between the receptor pocket and chemokine N-terminus (CRS2-CS2)[31]. This model is based on cases where chemokine recognition depends critically on the receptor N-terminus, and others where chemokine agonism can be turned to antagonism by deleting parts of the receptor[32-36]. However, some data are difficult to reconcile when this model is broadly applied to the chemokine family[37].

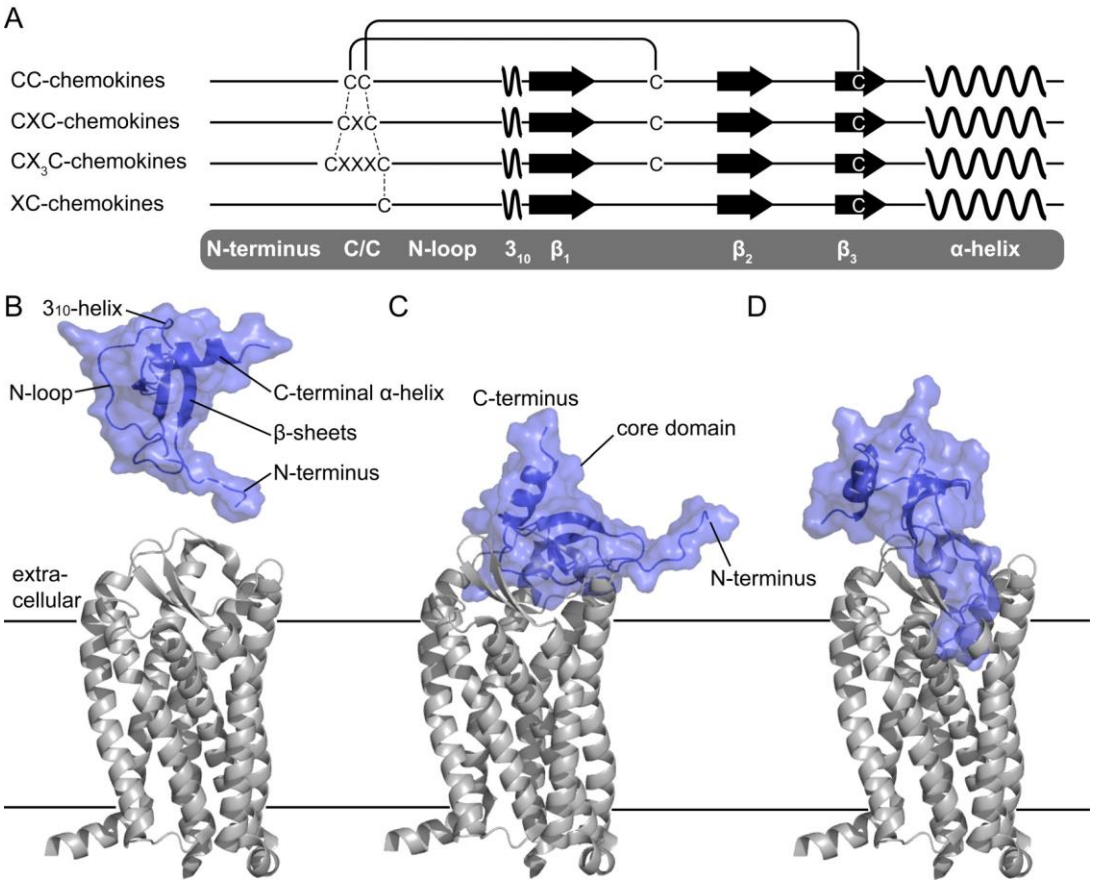

Figure 4. Chemokine domains and their involvement in receptor activation. The receptor core domain that comprises the N-loop, triple-stranded beta-sheet and a series of loop turns is believed responsible for initial recognition of the receptor, mainly at the receptor N-terminus (not visible). When the chemokine N-terminus subsequently interacts with the receptor binding pocket, an active receptor conformation can be stabilized. From Thiele and Rosenkilde 2014[27]

Variations of the interaction model can be brought together from experimental structures of the chemokine ligand-receptor complexes vMIP-II-CXCR4, CX_3CL1-US28, CCL20-CCR6, CXCL8-CXCR2 and [5P7]CCL5-CCR5[30, 38-41]. While all these complexes broadly conform to the general interaction model, the overall orientation of the chemokine ligand, particularly the depth and tilting of the core domain relative to the conserved CRS1.5 interaction, varies remarkably. For example, when binding to CCR5 the core domain of [5P7]CCL5 has settled in a deep position making contact with the receptor ECLs, whereas the core domain of vMIP-II only interacts with the CXCR4 N-terminus, with some distance to the ECLs. This means that, depending on the ligand-receptor interaction in question, a more detailed understanding of the composition of the chemokine core domains is likely needed, because parts of this region could participate in critical receptor activation steps.

Downstream of the CC/CXC motif, chemokines possess an N loop, which spans the core domain surface and is associated with recognition of the receptor N-terminus. This is followed by three ß-strands connected by loops: The $ß_1$-strand, 30s-loop, $ß_2$-strand, 40s-loop, $ß_3$-strand and 50s-loop. The 30s- and 50s-loops are connected by disulfide bridges to the CC/CXC motif and are essential to stabilize the folding of the core domain. Past the 50s-loop an α-helix makes up the end of the core domain in most cases[24]. In some chemokines an additional C-terminal tail with a net positive charge is present, and together with the α-helix, it is often associated with interactions with the extracellular matrix, which is rich in glycosaminoglycans (GAGs) – large polysaccharide structures with a strong negative charge[42].

Recently solved structures of chemokine-receptor complexes have built a detailed view of how these interactions can truly appear. At this point, there is a crystal structure of the viral chemokine vMIP-II in complex with CXCR4, a CCL5-based antagonist [5P7]CCL5 with CCR5, and cryo-EM structures of CCL20 with CCR6, monomeric CXCL8 with CXCR2 and dimeric CXCL8 with CXCR2[30, 39-41].

The experimental structure of [5P7]CCL5-CCR5 represents an interaction with a chemokine where the artificial N-terminus is antagonistic in nature, but the core domain is derived from the agonistic CCL5-CCR5 interaction. CCL20-CCR6 and CXCL8-CXCR2 are naturally active complexes and vMIP-II-CXCR4 and CX_3CL1-US28 do not induce G protein activity. Out of these structures, [5P7]CCL5-CCR5, CCL20-CCR6 and CXCL8-CXCR2 show a core domain where the 30s-loop and parts of the $ß_1$-strand make extensive contacts with the extracellular loops of the cognate receptors. In the [5P7]CCL5-CCR5 structure in particular, it is evident that the 30s-loop makes close contact within the receptor pocket itself, which significantly blurs the distinctions of the two-site model. It had not previously been reported that elements of the core domain can interact with the receptor activation-associated binding pocket.

Chemokine receptor activity can be "biased"

The various signaling cascades that follow activation of GPCRs can play out at different intensities, measured pharmacologically as potency or efficacy. Potency and efficacy refers to the ligand concentration needed for receptor activation and the amount of signal generated by activation, respectively. If the balance of the signaling through different pathways changes depending on for example the ligand, each ligand is said to be biased relative to the other[43]. Biased signaling may refer to a comparison of ligands for the same receptor, a comparison of receptors for a single ligand or a comparison of a single ligand-receptor interaction in different tissues (i.e. ligand-, receptor- or tissue bias)[44]. This sometimes allows cells to respond in a tailored manner to each interaction taking place at its surface even if very closely related interactions also occur[45].

Experimentally, biased signaling has most often been defined by the difference in coupling to the Gα subunit and arrestins, and this has developed into something of a benchmark for bias[46, 47]. However, much more thorough and complex comparisons may also be made to include a spectrum of pathways[48, 49].

The balance between these particular pathways has been shown to be important for the role of some of the most central members of the chemokine family. Dendritic cells and T cells express CCR7 to guide them to lymph nodes, or downregulate the receptor to migrate from lymph nodes to peripheral tissues, which is essential to many events in adaptive immunity, such as antigen presentation and T cell maturation[50]. CCR7 recognizes two chemokines, CCL19 and CCL21, which stimulate different patterns of signaling cascades. This allows CCL19 to mediate strong, acute signaling, while CCL21 mediates a weaker, but more lasting signal that can attract immune cells over large distances[51, 52].

Another important example of signaling bias involves CXCL12, which signals through CXCR4 to control several essential immune cell migration events across barriers from bone marrow to blood vessels, and from blood vessels to peripheral tissues[53]. CXCL12 is secreted by bone marrow stromal cells and in various peripheral tissues, and CXCR4 is expressed by myeloid progenitor cells, which means that the progenitor cells must first overcome the CXCL12 signal to migrate to the blood circulation, and then again become responsive to the CXCL12 signal to reach specific tissues from the circulation[54]. As the progenitor cells actively differentiate to more mature immune cell types, they manage their CXCL12-responsiveness by regulating their expression level of CXCR4, but blood vessel endothelial cells also interfere by expressing an alternative CXCL12 receptor, ACKR3. ACKR3 is an atypical chemokine receptor, defined by its lack of G protein signaling, but strong coupling to arrestins. This results in a circumvention of the migratory response, and an efficient internalization of the CXCL12-ACKR3 complex, after which CXCL12 is degraded and ACKR3 is recycled to the plasma membrane. This process is known as scavenging, as it allows endothelial cells to remove CXCL12 from their surroundings without responding in the manner many CXCR4-expressing immune cells would[55].

Chemokine signals rely on interactions with the extracellular matrix

Chemokines display a pleiotropic range of effects on their cognate chemokine receptors, but are primarily known for inducing immune cell migration. This mechanism relies on peripheral cells such as fibroblasts, epithelial and endothelial cells that may not be considered as primary immune cells but secrete soluble chemokine into the surrounding extracellular matrix. Many secreted chemokines will bind reversibly to glycosaminoglycan (GAG) components of the extracellular matrix, often using Base-Base-Xaa-Base (BBXB) motifs located at the C-terminal alpha helix or in an additional downstream "tail" region of the chemokine[42]. Chemokines thereby slowly diffuse away from the tissue of origin, which leads to lower concentrations at greater distances from the point of secretion. This creates a chemokine concentration gradient in equilibrium with the extracellular matrix. Patrolling leukocytes can respond to such a gradient via cognate chemokine receptors by migrating toward higher concentrations, allowing the leukocyte to reach the site of secretion[56].

Some chemokines are sequestered on cell surfaces, which allows more lasting signaling cues in the extracellular matrix, e.g. transmigration of myeloid-derived leukocytes from blood vessels to peripheral tissues via the chemokine CX_3CL1[57, 58]. Other chemokines are continuously secreted by resident cells at important sites of immune development to maintain a constant soluble gradient. CCL19, CCL21 and CXCL12 for example attract various immune cell subtypes to the lymph nodes and retain premature leukocytes in the bone marrow in this manner[59, 60].

In the extracellular matrix, the role of individual chemokines may change drastically due to post-translational modifications, with the most common modification being proteolytic cleavage at the N- or C-terminus of the chemokine[35]. Truncations at the N-terminus are of particular importance to chemokines, because it directly interferes with their activation of chemokine receptors. However, since the N-terminus is not necessarily involved with recognition by the receptor, chemokines may become antagonists by continuing to bind their receptors without signaling, and thereby block the recognition of active chemokines[34, 36, 61]. Tissues can thus regulate extracellular chemokine gradients by releasing specific proteases.

Much of the chemokine system is involved with a promiscuous interaction network

The highly conserved structure of chemokines and their conserved chemotactic activity also enables many chemokines to replace one another functionally. In fact, most receptors involved in migration of innate immune cells or activated T cells recognize several chemokines and many inflammatory chemokines activate several receptors (figure 5)[26, 44].

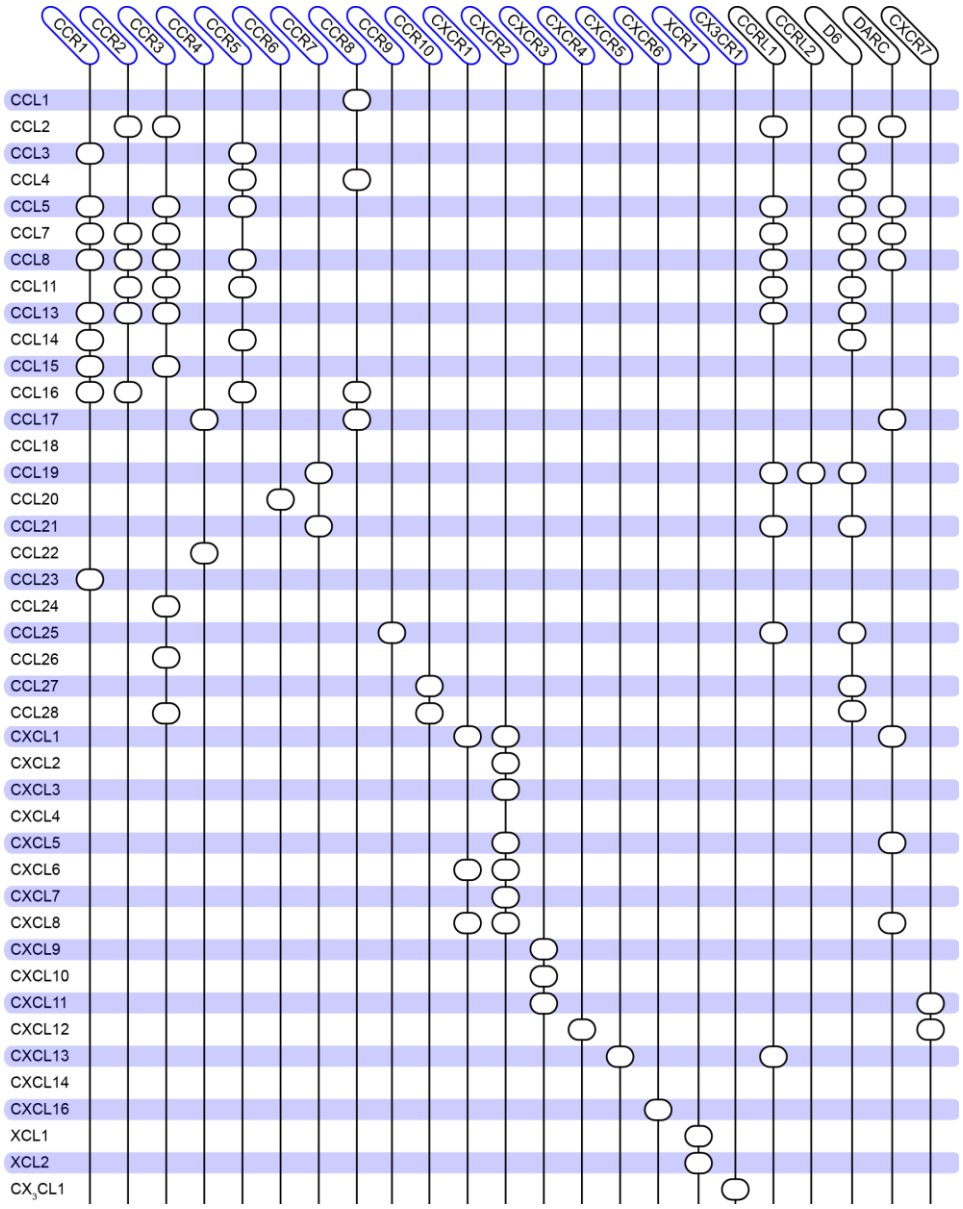

Figure 5. Overview of interactions (white circles) between chemokines to the left and their receptors on the top of the table. From Steen et al. 2014[44].

For some receptors, only a few chemokines are recognized by a single receptor, but there are also instances where large groups of chemokines and receptors form a complex network of selective interactions. The largest promiscuous interaction network involves the receptors CCR1, -2, -3 and -5, and the chemokines CCL2, -3, -3L1, -4, -5, -7, -8, -11, -13, -14, -15, -16, -23, -24 and -26. The intertwined interaction networks are biologically highly relevant because expression of both chemokine ligands and receptors from such interaction networks overlaps in various tissues and cells. In the case of CCR1, -2, -3 and -5, a combination of these receptors is expressed on monocytes, macrophages, activated dendritic cells and T cells and may appear on granulocytes[62-66]. CCR1, -2, and -5 are for example expressed simultaneously by different monocyte subtypes and Th1 cells, and all four receptors are expressed by dendritic cells and eosinophils[67]. Competing chemokines can also

be found together – CCL4, -5, -7 and -11 have for example all been detected simultaneously in murine blood plasma[68].

Chemokines of this interaction network can be further divided into subgroups based on both biological and biochemical characteristics. CCL3, -4, -5, -14, -15, -16 and -23 were originally known as Macrophage Inflammatory Proteins (MIPs) based on their ability to attract and induce inflammatory behavior of macrophages. They are furthermore genetically clustered together on human chromosome 17, and share a relatively high degree of sequence similarity[69]. Gene duplications have even occurred for two chemokines, CCL3 and -4, creating the "CCL-Like" chemokines CCL3L1, CCL3L3, CCL4L1 and CCL4L2, which are nearly identical in sequence but differ in receptor specificity[70, 71]. CCL2, -7, -8 and -13 belong to another subgroup, the Monocyte Chemoattractant Proteins (MCPs), and this subgroup is also genetically clustered on human chromosome 17, and displays high sequence similarity. CCL11, -24 and -26 are known as eotaxins and attract eosinophils in particular, but they are not fully clustered genetically.

The distinction of chemokines into subgroups is functionally significant, because their receptor recognition is somewhat systematic. Members from the MIP chemokines can activate CCR1, -3 and -5, but not CCR2, members from the MCP chemokines can activate primarily CCR2 but also all other receptors of the network, and eotaxin members can activate CCR2, -3 and -5, but are most effective on CCR3[72-74]. The chemokine subgroup distinction thus implies a mechanism for chemokine-secreting tissues that allow them to preferentially attract specific leukocyte subsets based on their pattern of receptor expression.

Complex regulation of MIP and MCP chemokines on CCR1, -2 and -5, indicating co-existence of specific and redundant interaction roles

Further implications of chemokine subgroup divisions become easier to grasp by considering the diverse signaling patterns of the involved interactions. Significant bias plays a role among ligands within and between chemokine subgroups and between receptors. For example, two thoroughly described MIP chemokines CCL3 and CCL5 are (ligand) biased on CCR1 and CCR5 as CCL3 produces a stronger relative arrestin over Gαi signal, whereas CCL5 produces a stronger relative Gαi signal[47, 49]. CCL8, an MCP, is also biased towards G protein over arrestin signaling on CCR2 and -5 relative to CCL2 and -3, respectively. How exactly the bias of these two particular benchmark pathways affects specific biological endpoint responses is vague, but at least in theory, biased signaling provides the possibility for cells to respond in a tailored manner to the combined pattern of chemokine-receptor complexes formed at any time. Given that chemokines can alter behaviors of leukocytes other than migration alone, such as their inflammatory state and polarization, the large interaction network could play a role in priming leukocyte activity before they arrive at a site of chemokine secretion[75-77].

An alternative interpretation on the role of large chemokine interaction networks is that they are important to prevent immune evasion by pathogens[78, 79]. Regardless of signaling bias, the outcome of chemokine responses largely overlaps, as they are all capable of directing leukocytes to the

secreting tissues. Therefore, chemokines of overlapping receptor recognition are said to be redundant. To pathogens, this poses a challenge because it is less likely that they can evolve a method to block a series of distinct molecular interactions at once.

One way to estimate when redundant or specific interactions of this chemokine network could play a role is to pay attention to their regulation of expression and activity. Among $CD14^+$ monocytes, it has been described that relatively minor changes to their polarization state will make them adjust receptor expression individually. The $CD14^+$ HLA-DR^{hi} subtype, which can present antigen to $CD4^+$ T cells, strongly upregulates CCR5 and somewhat lowers CCR2 expression[62, 67]. It has also been described that especially $CD14^+$ monocytes (of mixed subtypes) express cathepsin G to truncate the N-terminus of the CCL5 (1-68) form (a CCR1 and CCR5 chemokine) to a CCL5 (4-68) form[80, 81]. This variant is inactive on CCR1 but remains active on CCR5, which will be presented in manuscript 1. It is thus plausible that the specific pairing of chemokines and receptors matters to the response of specific immune cell subsets, since very specific mechanisms exist to modulate chemokine-receptor recognition.

Another intriguing example of regulation of specific chemokine interactions is found by considering proteolytic activity produced by neutrophils. Human blood-derived neutrophils not only express cathepsin G and efficiently convert CCL5 (1-68) to CCL5 (4-68), but also activate matrix metalloproteinase (MMP) -2 expressed on endothelial cells[82]. MMP-2 specifically cleaves CCL7 (1-76) into an antagonistic 5-76 form, but no other MCP chemokines[83]. Of the pertinent chemokine interaction network, only CCR5 does not recognize CCL7, and therefore this proteolytic activity conferred by neutrophils to endothelial cells directs chemokine activity towards CCR5 and its cognate MIP chemokines.

On the other hand, other post-translational proteolytic processing strongly influences the system on a larger scale by targeting several chemokines together, which in those cases would indicate that control over redundant characteristics is central in the chemokine system[84]:

At least 9 different MMPs, as well as cathepsins and CD26 (DPP-IV), can truncate specific subsets of chemokine N-termini[83]. In one example, MMP-3 is expressed in many types of fibroblasts and can cleave off 4 N-terminal residues of all 4 MCP chemokines, known to convert them into antagonists of intact MCPs[61, 85, 86]. At the same time, the N-termini of CCL15, and -23, inactive chemokines in their full length form, are targeted by all chemokine-specific MMPs[83]. They are members of the MIP subgroup, and can furthermore be found under normal non-inflammatory conditions and are therefore said to be homeostatic chemokines[26]. In contrast, the MIP chemokines CCL3 and -5, active in their full-length form, are not cleaved by MMP-3. MMP-3 thus orchestrates a bulk shift of chemokine activity away from the MCP subgroup and towards the MIP subgroup with little discrimination between individual chemokine variants.

Another famous example is the cell surface-expressed protease CD26, which recognizes the N-terminal sequences Xaa-Pro and Xaa-Ala, and cleaves off the first two residues of a wide range of soluble hormones to modulate their activity. This includes CCL3L1, CCL4 and CCL5, which in their full length form are potent members of the MIP chemokine subgroup, and they are only expressed

under inflammatory conditions[33, 69]. Interestingly, this changes the chemokine activity profile for each chemokine differently. CD26-mediated truncation of CCL3 confers increased activity on CCR1 and CCR5, while truncation of CCL5 does not decrease CCR5 activity but full loss of function on CCR1[36, 87]. CCL4 is normally a CCR5-agonist, but also becomes a CCR1-agonist after truncation. MCP chemokine sequences start with Gln-Pro, which would also be a target of CD26, but glutaminyl cyclase and iso-glutaminyl cyclase convert MCP N-termini into pyroglutamate which is protected from CD26 cleavage. Glutaminyl cyclase is ubiquitously expressed throughout organs and peripheral blood, resulting in efficient MCP protection, which has been shown to be essential to infiltration by primary blood monocytes[88-90]. On this immune cell subset and at this stage, CCR2 is strongly expressed, and is the dominant driver of tissue infiltration[62, 68].

There are thus clearly regulatory mechanisms in place that control chemokine ligand activity based on redundant subgroup characteristics and other mechanisms that control non-redundant characteristics. This is reflected in the importance of responsive receptors at distinct stages of monocyte recruitment to sites of inflammation. Dyer *et al.* compared monocyte behavior in mice where the genes for CCR1, -2, -3 and -5 had been knocked out either individually or simultaneously[68]. Loss of CCR2 leads to blood and spleen monocytopenia[91], while the additional knock-out of the remaining receptors (CCR1, -3 and -5) does not further affect the outcome, and thus CCR2 alone drives the egress of monocytes from bone marrow to blood. Monocytes from the blood may later be recruited to the skin, but at this stage CCR2 is no longer the main responsible receptor. Instead, every receptor knock-out negatively affects monocyte recruitment, and a simultaneous knock-out results in a stronger effect. This same role of chemokine receptors is observed when inflammation is induced. This hints that the receptors play a redundant role, somewhat in line with their recognition of promiscuous chemokines, in some peripheral tissues. The level of apparent redundancy seems to be strongly influenced by factors beyond a simple availability of chemokines or their receptors, as various myeloid-derived cell types and specific tissues are affected differently by individual receptor knock-outs (e.g. eosinophils rely strongly on CCR3 for correct tissue recruitment).

It is therefore reasonable to suppose that the actual leukocyte response elicited by chemokine signaling is an amalgam of signaling characteristics derived from very specific chemokine-receptor interactions, together with signaling characteristics of a more redundant nature, and cell- or tissue-based influences such as receptor regulation and chemokine proteolysis.

Immunological aspects of disease settings could benefit from a greater understanding of the chemokine system

The central mechanisms facilitated by the chemokine system gives it an inherent role in many biological contexts, but this also means that the chemokine system is involved in many common diseases or disease-related mechanisms. This involves cancer metastasis, autoimmune diseases and tissue remodeling.

Cancerous tissue originates by the acquisition of traits that allow it to grow, survive and spread beyond control. The required traits have been categorized into six central hallmarks along a few enabling characteristics, and the chemokine system may be centrally involved in multiple categories of cancer traits[92, 93]. Inflammation of the tumor environment in itself can contribute to tumor progression[94], but an unintentional expression of chemokine receptors on non-immune cells or incorrect immune cell types will further allow tumor cells to migrate to sites secreting chemokine gradients, in effect resulting in cancer metastasis. For the same reason, inappropriate chemokine secretion can contribute to cancer metastasis, as has been abundantly reported for many members of the CC chemokines and receptors[95]. Some key chemokines such as CCL2 and CCL5 are additionally reported to promote angiogenesis and resistance to apoptotic signals, also cancer hallmarks[95-97].

The chemokine system is also associated with chronic inflammation and autoimmunity, for example by continuing to attract and activate immune cells in local tissues that stress and break down an otherwise healthy environment. This is the way CCR6 and its exclusive chemokine ligand CCL20 has been shown to be involved in psoriasis, in that γδ-low T cells stimulate skin keratinocytes to secrete high levels of CCL20 to recruit inflammatory immune cell subsets[98-100]. Chemokines are similarly involved in rheumatoid arthritis, where joint synovial dendritic cells, macrophages and endothelial cells produce a host of chemokines, attracting neutrophils, monocytes, T cells and B cells[101-103]. These cells in turn intensify the local joint inflammation and damage which defines rheumatoid arthritis.

The highlighted connections of chemokines with disease could indicate that antagonizing the chemokine system therapeutically would resolve many symptoms, if not the underlying inflammatory damage of said diseases. Indeed, the chemokine inhibition strategy is actively being pursued to target cancers and rheumatoid arthritis, with some promise[101, 104]. However, many efforts to therapeutically target the chemokine system have failed in the past, which was by many perceived as a lesson that the nuanced activity of the system must also be taken into account before effective therapies can emerge[29, 105]. There are certainly examples where enhanced chemokine expression is associated with improved cancer prognosis, or where only specific interaction pairs of a promiscuous interaction network are implicated in a disease[95, 106].

This may also explain why successfully marketed drugs acting on the chemokine system are few in number and only target exclusive interaction partners. Maraviroc is a CCR5 antagonist which binds to the receptor pocket where chemokine N-termini are known to interact. However, it is marketed to treat HIV, rather than cancers or autoimmune diseases[107, 108]. The HIV envelope protein gp120 presents a protruding “V3-loop” which recognizes the CCR5 pocket, while other parts of gp120 recognize CD4, making HIV a pathogen specific to CD4 T cells[109, 110]. Other HIV strains, X4 tropic HIV, instead target CXCR4 as a CD4 coreceptor. The approved drug AMD3100 (also known as Mozobil or Plerixafor) inhibits this interaction although it is not used to treat HIV patients[111]. Maraviroc and AMD3100 moreover block chemokine ligand-receptor interactions, but in the case of AMD3100, the blocked interaction is a non-promiscuous pair, CXCL12-CXCR4, and interfering with this interaction has turned out to be an effective way to mobilize $CD34^+$ stem cells from the bone marrow, important to patients with non-Hodgkin’s lymphoma and multiple myeloma[112-115]. An antibody drug,

Mogamulizumab, targets CCR4, and is intended to be used as a treatment against T cell lymphomas, because T cells of this category are associated with increased CCR4 expression[116, 117]. The antibody Fc domain has been adapted to be highly recognizable by cytotoxic cells expressing Fcγ receptors, and thus cells with high CCR4 levels are more likely to be destroyed in the presence of Mogamulizumab[118]. Two chemokines, CCL17 and CCL22, recognize CCR4, but these interactions are not inhibited by Mogamulizumab binding[117]. Interestingly, drugs marketed for the chemokine system all rely on exclusive interaction partners in line with a traditional pharmacological approach[119, 120], but none so far combine with the delicate balance of redundancy or specificity intrinsic to large parts of the chemokine system, despite their central involvement in many biological- and disease settings.

2. Aims of study

We have focused this PhD project on studying the interaction network of the receptors CCR1, CCR2 and CCR5 with a range of cognate chemokine ligands from the MIP and MCP subgroups. Our goal was to learn on a molecular level if chemokine subgrouping is a meaningful distinction, as well as ligand redundancy. In the last decade, the concept of biased signaling has been introduced by specialists of GPCR pharmacology, and their findings of bias in the chemokine system has sometimes been at odds with the idea of redundancy and "backup actors" in the system. We also wanted to know if there are systematically preserved molecular determinants on receptors that recognize either redundant or biased signaling properties of chemokines. Focusing on the accessible interaction surface/interface in this regard was important, because we could thereby learn if such properties in theory can be targeted pharmacologically, and open the door to a practice of drug development that better accounts for the complexity of chemokine biology.

I initially presumed that we would probably be able to connect pathway-selective signaling to variations of the interaction between chemokine N-termini and receptor binding pockets, but I since learned through the work on **manuscript 1** that this was truly oversimplified. In the end, we did observe that chemokines and receptors display certain molecular patterns that are conserved and recognized on a subgroup basis. However, conserved subgroup motifs among MIP and MCP chemokines, while essential to signaling in their own way, appeared to coexist with extensive signaling bias. Moreover, we found that among MIP chemokines naturally occurring N-terminal truncations critically influence the role of biased and redundant signaling patterns, indicating that the balance is subject to regulatory mechanisms. Since this project extended slightly beyond the scope of a single article, it will receive a dedicated introduction and a few extended results will follow it.

Manuscript 2 was in fact completed before manuscript 1, and at this point we attempted to focus on a single interaction pair, CCL3-CCR1. We mapped the contribution of the chemokine N-terminus to overall signaling and to selectivity between the G protein and arrestin pathways, which was consistent with our expectations based on literature. Although it had been described before, these

results made us appreciate that regions other than the chemokine N-terminus must have a major influence on signaling, but that N-terminal proteolysis is a strong regulatory mechanism. We also showed that this regulatory mechanism can be exploited pharmacologically with small molecule drugs occupying the receptor pocket, although careful understanding of the literature would be required, since such drugs may be both allosteric and orthosteric in different tissue contexts.

Extended results will present results from an ongoing study of the activity of chemokine N-terminal peptides alone. A dogma of chemokine structure-function is that the chemokine core domain and N-terminus carry out distinct functions in terms of receptor binding and signaling, but as we had been accumulating conflicting data in that regard, we started to ask if chemokine N-termini should sometimes simply be regarded as an addition to the same role carried out by the core domain. We knew that if an N-terminus exhibits its own binding and signaling, it would be severely weakened, as the missing core domain constitutes about 85% of the original protein, as will be presented in the extended results. The biggest surprise however, was that we could also identify a positive modulatory effect by N-terminal peptides on intact chemokine-receptor interactions, in combinations that do not necessarily reflect the type of allosteric activity observed from small molecule modulators. The interpretation of these results is therefore still very open to new data and input.

3. Manuscripts

Manuscript 1:

A conserved chemokine receptor motif confers selective engagement of MIP and MCP chemokines

Authors:

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Under review in Science Signaling

Introducing remarks:

As many others working with chemokine research, we suspected that in cases where a cell expresses multiple receptors for one chemokine, or a receptor for multiple chemokines, there would likely be interaction features that are variable in a way that allows cells to distinguish between chemokine-receptor combinations. At the same time, we know that chemokine structures and interactions are highly similar, and so we know that many interaction features must also be conserved, especially among chemokines displaying redundant interaction patterns. Both the redundant and selective interaction features are often regarded as underlying reasons for many failed attempts at targeting the chemokine system therapeutically, and we thus reason that chemokine drug development would be aided by identifying such interaction sites. This is particularly true for the MIP and MCP chemokine subfamilies, given their complicated interaction network with the central inflammatory monocyte receptors CCR1, CCR2 and CCR5. Data presented with this manuscript are obtained from experiments designed to answer a central scientific question in the chemokine field: Do chemokine receptors contain a pattern recognition motif that can be targeted therapeutically to overcome previous roadblocks of redundant and selective chemokine engagement?

We answered this question by structurally analyzing the ligand interaction surface of chemokine receptors CCR1, CCR2 and CCR5. By applying thorough pharmacological testing, mutagenesis and computational analysis to a dozen receptors combined with a broad range of chemokines, we identify an exposed receptor motif that is conserved and essential in six chemokine receptors. Importantly, we show that this motif is required for recognition of the large MIP chemokine subfamily, but not the closely related MCP chemokines. We further identify two MIP chemokine domains that dictate the importance of the receptor-recognition motif: The stable ß1-strand including a conserved sequence motif, and the flexible N-terminus which is not conserved. These two chemokine regions physically approach the receptor motif from different directions, allowing for drugs to be designed that block receptor interactions with either the conserved or variable domains of MIP chemokines.

Due to the many structural similarities between the MIP and MCP subgroups, they are difficult to distinguish pharmacologically, and it was recently shown that the N-terminus, which is central for receptor signaling, can sometimes be exchanged between some members from the two groups to control nuanced signaling properties[48]. Still, an N-terminal exchange is not always possible within a single subgroup, indicating that N-terminal interactions can be highly variable[121]. Even so, it had been demonstrated that a redundant role shared by a broader range of chemokines for CCR1, CCR2 and CCR5 is critically important for many biological roles carried out by monocytes, which we speculated could be due to the presence of some less selective signaling features[68]. We believe that understanding these features is of interest in the field, particularly to drug development efforts, as the topic has continuously been discussed for at least a decade now[105, 122, 123]. In theory, drugs targeting selective or redundant properties would also be useful to study the biology of the chemokine system, as it would be possible to investigate if redundancy drives various immune cell recruitment events. Finally, this is to our knowledge the first example clearly showing ligand domains with both redundant and specific signaling properties interacting with a single receptor motif.

While the manuscript discusses motifs found on MIP and MCP chemokines, we were not able to include how we found these sites, although this could be useful to readers interested in expanding on the presented work. After some trial and error, we found that an effective approach to build an overview was to focus on receptor domains that are frequently, but not universally, associated with ligand interactions. For example, the conserved (Pro-Cys) receptor CRS1.5 site could likely be essential to almost all chemokine interactions[124], but therefore pharmacological targeting of this motif would be very unspecific. Instead by organizing chemokines into functional subgroups and testing the pharmacological relationship between receptor domains and specified chemokine features, we can determine what allows receptors to discriminate between even closely related chemokines, and the same concept should be true for the chemokine ligands.

We performed sequence alignments of all chemokines and in parallel structural alignments (combinatorial extension alignment, see methods appendix) of one example of every chemokine structure solved thus far, this in total representing 32 distinct chemokines[125]. *Sequence* conservations higher than 90% were highlighted in the *structural* alignment (figure 6A). Widely conserved residues are buried and relate to the classical chemokine fold, and structural and chemical variation is possible on most of the chemokine surface, shown in grey. When only the CC-chemokines are included in the alignments, the conserved core region grows and the variable domains shrink (figure 6B, shown in cyan). This is furthermore associated with a diminishing structural variation in regions of high sequence variation (grey). When only MIP chemokines are left in the alignments (here including CCL3, -4, -5, -14 and -15), structural variation is minimal, and conserved residues cover much of the surfaces of the chemokines (figure 6C). For these chemokines, we grouped the conserved residues into those that are conserved in both MIP- and MCP chemokines (figure 6C, blue), and those that are exclusively conserved in MIP chemokines and not MCPs (purple). We reasoned that blue residues, those shared by the two chemokine groups, cannot be the foundation used by interaction partners (e.g. receptors) to distinguish between the groups. On the other hand, MIP conserved residues (in purple) can give rise to systematic MIP/MCP distinctions,

and are found before the 30s-loop, and at numerous sites along the CRS1 binding grove (CS1) and the 40s-loop. N-terminal truncation sites used in this study (marked in red) are not conserved among MIP chemokines, and may as a consequence confer unique signaling properties to individual chemokine:receptor pairs. We could similarly focus on MCP chemokines and highlight the residues conserved in both MCP- and MIP residues (figure 6D, blue), or the residues that are specifically conserved for MCP chemokines (purple). By visually comparing the residues conserved only on a subgroup level (purple residues in figure 6C and 6D), we found that many of these residues are in fact located and exposed in the same surface regions, relative to the other subgroup. To us, this added to the probability that there are likely distinct receptor regions that recognize the subgroup division or character of the interacting chemokine. The visual comparison also shows us that the same line of thinking can hypothetically be applied to the fully conserved (blue) and the fully variable (grey) regions of chemokines. It is thus possible to order interaction areas of overlapping chemokine:receptor complexes into tiers: i) Common interactions, ii) those that are specific to chemokine subgroups and iii) those that confer a more unique character to single chemokines (figure 6E).

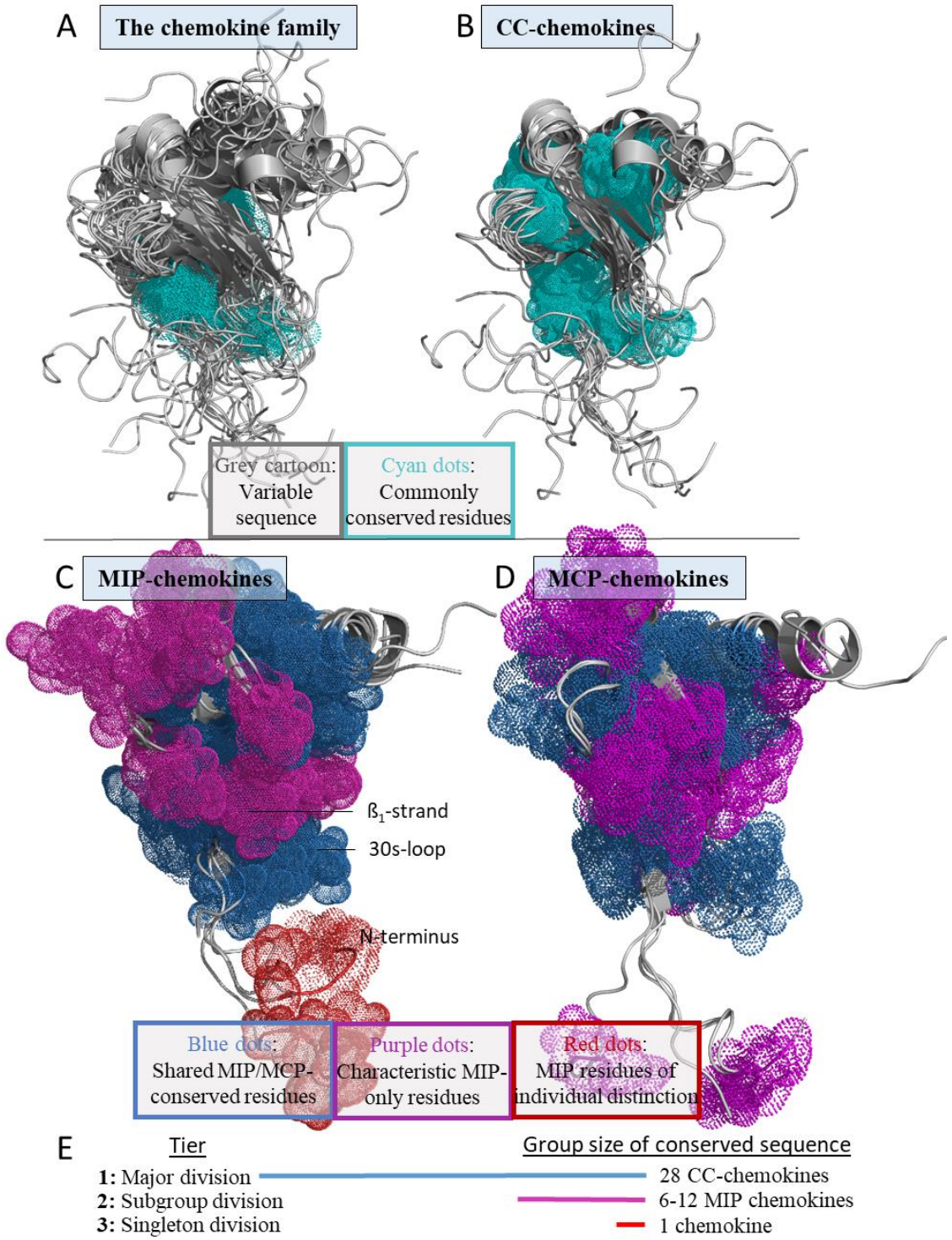

Figure 6 (A) One structure of every available chemokine, aligned and shown in grey cartoon. Low-density dot representation of the surface area of conserved residues shown in blue. (B) Alignment of one of every available CC-chemokine in grey cartoon, with dot representation of conserved residues in blue. (C) Alignment of MIP chemokines (CCL3, -4, -5, -14 and -15) in grey cartoon. Dot representations shown for residues conserved for both MIP and MCP chemokines (blue), residues conserved only among MIP chemokines (purple) and residues not conserved or functionally similar (red). Rough distinction of chemokine site 1, -1.5 and -2 (CS1, -1.5 and -2) and secondary structure elements examined in this study are denoted. (D) Alignment of MCP chemokines (CCL2, -7, -8 and -13) in grey cartoon, with a similar coloring scheme as for MIP chemokines. (E) Simplified diagram of how a chemokine sequence can be sorted into levels according to the conservation status of each residue.

A conserved chemokine receptor motif confers selective engagement of MIP and MCP chemokines

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Abstract

Chemokine receptors control migration of leukocytes and are intimately associated with inflammatory diseases and cancers. An extensive ligand-receptor promiscuity has been viewed as both a redundancy and specificity feature, and a better understanding of these features on a molecular level is required for effective pharmacological modulation of the chemokine system. Here we describe a conserved cluster of three aromatic receptor residues that anchors the second extracellular loop (ECL2) to the top of receptor transmembrane helix 4 and 5 and enables recognition of both redundant and specific characteristics of interacting chemokines. We show that this cluster is essential for activation of the majority of CC chemokine receptors. Furthermore, we describe how characteristic ß$_1$-strand/30s-loop motifs make the two main CC-chemokine subgroups, the Macrophage Inflammatory Proteins (MIPs) and Monocyte Chemoattractant Proteins (MCPs), depend differently on this cluster in the promiscuous receptors CCR1, CCR2 and CCR5. The cluster additionally allows CCR1 and CCR5 to recognize unique MIP patterns via the chemokine N-terminus. Consequently, this extracellular site could play a key role in the future development of chemokine therapeutics.

Introduction

A vital property of the immune system is the capacity to mobilize leukocytes to sites where they are needed under changing circumstances. Migrating cells are guided by chemokines, which are small soluble proteins that are secreted by tissues that require recruitment of subsets of immune cells (1). Chemokine receptors are G protein-coupled receptors (GPCRs) of the class A family that typically activate $G_{\alpha i}$-signaling pathways and recruit arrestins upon stimulation. The cognate chemokines are named according to the organization of the first two cysteines (yielding a CC, CXC, XC or CX_3C motif), where the CC- and CXC ligands (CCL1-28 and CXCL1-17) are the major groups, recognized by distinct subgroups of receptors (CCR1-10 and CXCR1-6, respectively, Fig 1A). An important and complex feature of the chemokine system is that it includes both chemokines that are monogamously paired to specific receptors, and chemokines that are highly promiscuous to several receptors. The chemokine system includes numerous promising therapeutic targets, but a low success rate in drug trials has led to a widely held view that investigators must take a more nuanced approach to effectively modulate this system (2, 3).

A group of chemokine receptors, CCR1, CCR2, and CCR5, are connected functionally by mediating chemotaxis of monocyte-derived leukocytes to inflamed sites in both appropriate and autoimmune circumstances (4-7). They are recognized by a tangled network of promiscuous cognate chemokines, the majority of which are inflammatory (8, 9), and their co-expression by single cells is by now well established (10-13). Activity of these receptors is reflected in the nature of their cognate chemokines, most of which were originally named monocyte chemoattractant proteins (MCPs) or macrophage inflammatory proteins (MIPs). The MCPs and MIPs are chromosomally clustered into 4 MCP genes and 12 MIP- and MIP-like genes (9, 14), and their large number may in part be explained by redundancy (10, 15). Furthermore, the receptors that primarily recognize MCP chemokines (CCR2) or MIP chemokines (CCR1 and CCR5) have been shown to control chemotaxis in distinct tissues for the same cell types (10), and at different stages in disease models for experimental autoimmune encephalomyelitis, ischemia-reperfusion injury and acutely induced inflammatory conditions (6, 10, 16).

However, it is also clear that there are chemokine distinctions beyond the MCP and MIP classifications, underscored by individualized chemokine receptor recognition patterns and extensive ligand- and receptor-mediated signaling bias, which is the preferential activation of one signaling pathway over another (17-19). This can become important when comparing promiscuous or seemingly redundant chemokines (8, 10, 20-22). Post-translational ligand and receptor modifications further amplify the diversity in the system (23). It is therefore certain that variations to the chemokine ligand-receptor interaction interface are important

features to distinguish related chemokines that share a degree of receptor recognition, because these interaction differences are the root of how a cell distinguishes related chemokine encounters.

All chemokines possess a characteristic fold with a globular core domain and a flexible N-terminus, and this potentially allows them to engage their receptors according to the same overall interaction mechanism, i.e. the two-step, two-site model (24-27). This model states that the chemokine core domain binds at the receptor site 1 during step 1, after which the chemokine N-terminus activates the receptor at site 2 during step 2 (Fig 1A). While the conventional model is simplified and does not apply to all receptor-chemokine pairs (28, 29), it aids the organization of the two essential interaction properties of binding affinity and receptor activation. The chemokine core domain interacts extensively with the receptor proximal and distal N-terminus, which is designated into the two segments chemokine recognition site 1 and 0.5, respectively (CRS1 and CRS0.5, Fig 1B) (30). A majority of the interaction affinity is conferred as the receptor N-terminus wraps along the chemokine CRS1 binding groove. If these interactions occur, the chemokine N-terminus may interact within the upper transmembrane region formed by the receptor helices (known as CRS1.5 above the minor binding pocket in transmembrane helix (TM) 1 and CRS2 in the deeper binding pocket), thus stabilizing receptor conformations responsible for receptor activation.

Extracellular loop 2 (ECL2) is essential for chemokine interactions, and is the largest single region covering the activation-associated receptor binding pocket (CRS2). This likens CCR5 to many members of the broader class A GPCRs that employ ECL2 for ligand interactions (31). ECL2 is divided into the segment before and after the 7TM-conserved disulfide bridge (ECL2a and ECL2b, respectively) and there is precedence that chemokine signaling selectivity can rely on residues from both parts of ECL2 in tailoring their pharmacological activity (32-35).

In this study, we investigate how promiscuous chemokines activating CCR1, CCR2 and CCR5 rely differentially on ECL2, how this region enables receptors to differentiate between MIP and MCP chemokines, as well as add a degree of individual diversification amongst MIP chemokines. Our data suggest a novel receptor site as a promising pharmacological target in future drug development.

Results

A cluster of aromatic residues in TM4, TM5 and ECL2 is highly conserved in chemokine receptors

Chemokine recognition ranges from exclusive receptor interactions to highly promiscuous interaction networks (Fig 1A). Comparison of experimental structures of CCR5, CXCR2, and CXCR4 with and without a bound chemokine ligand shows that the second extracellular loop (ECL2) reorients over the ligand binding pocket when these receptors adapt to [5P7]CCL5, CXCL8, or vMIP-II, respectively (Fig 1A and Fig S1) (36-38). Importantly, the recently published structures of active and inactive CXCR2 also show that receptor activation is associated with a substantial inward shift of TM5 along with reorganization of ECL2b (38). In CCR5, ECL2 is in close proximity to both the chemokine core ($ß_1$-strand and 30s-loop) and the chemokine N-terminus in the transmembrane pocket below. However, the ECL2 structure, including the antiparallel beta-hairpin, remains largely intact during this movement, allowing the needed rearrangements to hinge on the ends of ECL2 (top of TM4 and TM5) and the 7TM disulfide bridge. Interestingly, a relatively undisturbed conserved triplet of aromatic residues, here termed *the aromatic cluster*, is found in this hinge region (Fig 1C and Fig S1): a Phe/Tyr residue located in position Cys+4 of ECL2 (4 residues downstream of the conserved $Cys^{45.50}$ residue in class A GPCRs, i.e. position 45.54 using the Ballesteros/Weinstein nomenclature (39)), is oriented to form aromatic stacking interactions with residues at the top of TM4 (4.63) and TM5 (5.34). Due to the organization of ECL2b, which in this segment is a beta-strand anchored at the 7TM bridge, the exact 4-residue distance is important to allow the formation of this aromatic cluster. In other peptidergic GPCR structures, we observe that when the Cys+4 position is aromatic, the side chain points down toward the major receptor binding pocket (Fig 1D), as also recognized previously (33). A sequence analysis of chemokine receptors reveals that the TM part of the aromatic cluster is highly conserved, as 14 receptors (78%) have a Trp or – rarely – Phe at $TM5^{5.34}$, and a Phe or Tyr at $TM4^{4.63}$ is 100% conserved (Fig 1E). At the Cys+4 position on ECL2, 67% of the chemokine receptors, mostly CCRs, present an aromatic residue, allowing formation of an aromatic cluster and suggesting that the aromatic Cys+4 residue may stabilize ECL2b and nearby residues, and thereby be functionally important for chemokine receptors.

The aromatic side chain of Cys+4 is essential for chemokine, but not small molecule -mediated G protein signaling

The aromatic residue at the Cys+4 position was replaced with Ala in each chemokine receptor, and the ability of the mutant receptors to signal via $G_{\alpha i}$ was probed by selected endogenous chemokine agonists (8). The impact of the mutations could be divided into two groups, where half of the receptors were severely or entirely impaired, and the remaining receptors signaled close to wild type levels (less than 10-fold EC_{50}

change, Fig 2A). While there was no obvious connection among receptors where the Cys+4 aromatic residue was partly (CCR2, CCR4) or fully dispensable (CCR8, CXCR3, CXCR4 and CX_3CR1), the Cys+4-dependent receptors were all CC-chemokine receptors (CCR1, CCR3, CCR5, CCR6, CCR9 and CCR10). Chemokines with a CC motif share common interaction properties with CRS1.5 at the top of the minor binding pocket (36).

Clustering of the chemokine receptor family based on overall protein sequence showed that the receptors do not group by Cys+4-dependency (Fig 2B), and thus the mutagenesis outcome cannot be explained by a general receptor homology. We therefore set out to make a pharmacological comparison of chemokines activating three highly similar receptors, CCR1, CCR2 and CCR5, where the Cys+4 mutagenesis outcome was different. ELISA was used to verify that the effect on CCR1 and CCR5 signaling was not caused by a lack of surface expression of these mutants, although the expression of the corresponding CCR5 mutant was decreased to 38% (Table S1). However, the small molecule metal ion chelator Zn-phe (zinc phenanthroline), which activates CCR1 and CCR5 by binding deep within the ligand binding pocket (40, 41), reached the same efficacy as on WT receptors with a minor decrease in potency (3.5-fold on CCR1 F187A and 1.7-fold on CCR5 F182A, Fig S2) on the Cys+4 mutant, compared to the complete loss of chemokine-induced activity. Therefore, the lower expression of CCR5 F182A could not account for a loss of chemokine signaling, and instead suggests that the aromatic residue in Cys+4 is specifically important for chemokine-mediated activation in CCR1 and CCR5.

CCR1 and CCR5 require the aromatic Cys+4 residue for activation by MIP-chemokines, but not MCP-chemokines

CCR1, CCR2 and CCR5 respond to two groups of chemokines: the MIP (Macrophage Inflammatory Protein) and the MCP (Monocyte Chemoattractant Protein) chemokines in a semi-redundant manner. The two groups are encoded from distinct gene clusters (9), and display a greater degree of protein sequence conservation within their own group than with other chemokines (Fig 3A). This conservation is especially prominent among MCP chemokines. CCR1 and CCR5 respond to members of both chemokine subgroups, allowing us to test if chemokine features conserved within a subgroup play a deciding role in Cys+4-dependency.

We compared the effect of the Cys+4 mutation on the potent MIP chemokines CCL3, CCL4 and CCL5, and the potent MCP chemokines CCL2, CCL7 and CCL8, on their cognate receptors among CCR1, CCR2 and CCR5. On CCR1, the Cys+4 mutation (F187A) led to complete loss of $G_{\alpha i}$ activity of the MIP chemokines CCL3 and CCL5 (Fig 3B), while potency of the MCP CCL7 was partially diminished (12.7-fold) and CCL8 efficacy had slightly improved (Fig 3C). On CCR5, signaling of the MIP chemokines CCL3, CCL4 and CCL5 was strongly diminished by the Cys+4 mutation (F182A, Fig 3D), though some activity remained for CCL4 and CCL5 (up to 42% at 100

nM). CCL8 was affected by the CCR5 mutation, though less severely than the MIP chemokines (4.6-fold potency loss, Fig 3E). On CCR2, the MCP chemokines CCL2, CCL7 and CCL8 were unaffected by the Cys+4 mutation (F194A, Fig 3F).

This pattern of signaling outcomes consistently highlighted that MIP chemokines were very sensitive to the Cys+4 mutation, while MCP chemokines were partially or completely insensitive (Fig 3G), suggesting that MIP chemokines contain a conserved feature that leads to their dependence of an aromatic Cys+4 residue. However, the specific receptor also appeared to play a supplementary role in defining the MIP/MCP relationship with the Cys+4 residue. On CCR5, which on a protein sequence level is more similar to CCR2 than to CCR1 (Fig 2B), the outcome of Cys+4 mutagenesis mostly resembled that of CCR1, although the distinctive MIP and MCP effect was less prominent.

The Cys+4 residue is part of a structural configuration connecting ECL2 to TM4 and TM5

In current structures of chemokine receptors, ECL2 is typically positioned along the top of the main binding pocket. However, there are two exceptions, CCR7 and CCR9, where ECL2 is either displaced over the opening of the major binding pocket (CCR7, Fig 4A) or unresolved (CCR9) (36, 37, 42-45). In both cases, the highly conserved aromatic cluster is incomplete (Fig 1E), with CCR7 missing the aromatic residue at position Cys+4, and CCR9 missing the aromatic residue in TM5 (5.34). These observations suggest that interactions within the aromatic cluster anchor ECL2 to the top of TM4 and TM5, meaning that its main role in CCR1 and CCR5 could be loop stabilization, and not mediation of direct interactions between Cys+4 and MIP chemokines. A loss of the aromatic ECL2 anchor could conceivably lead to a displacement of several nearby ECL2 residues, making them unavailable for chemokine interactions, or obstructing the major binding pocket as seen for CCR7, thereby preventing CRS2 interactions specifically required by MIP chemokines.

We therefore analyzed the effect of Ala mutations of the two transmembrane residues in the aromatic cluster, which are located at the top of TM4 (F4.63) and TM5 (W5.34, Fig 4B). They are both within interaction distance of the Cys+4 position in the [5P7]CCL5:CCR5 structure (37), but any path for direct interactions between F4.63 or W5.34 and the chemokine ligand is obstructed in all receptor structures where the aromatic cluster is present (see Fig 1C, Fig S1 and Fig 4B insert). Therefore, if Ala mutation of either one or both of these residues mirrors the effect of the Cys+4 mutation on MIP chemokines, it likely relates to an indirect role, rather than direct chemokine binding.

In CCR1, Ala substitution of W5.34 (W195A) resulted in a strong loss of CCL3 potency (50-fold), while F4.63 (F171A) was dispensable (Fig 4C), and receptor expression was not markedly affected by the mutations (see

Table S1). For CCL5, both residues of the aromatic cluster were required for proper G protein signaling (potency loss of 47- and 162-fold, respectively, Fig 4D). On CCR5, removing either the aromatic F4.63 (F166A) or W5.34 (W190A) abolished the activity of CCL3 (Fig 4E), while CCL5 did not significantly depend on either of the underlying residues (Fig 4F). Mutating the corresponding F4.63 and W5.34 residues on CCR2 (F178A and W197A) did not affect the potency of CCL2 (Fig 4G) or CCL8 (Fig 4H).

Overall, a significant role of the underlying residues of the aromatic cluster was observed for MIP chemokines on CCR1 or CCR5, while no effect was observed when the corresponding residues were mutated on CCR2 for MCP interactions. This suggests that the aromatic Cys+4 residue of CCR1 and CCR5 is needed to correctly stabilize the position of a segment of ECL2 over the major binding pocket via interactions within the aromatic cluster. However, the individual MIP ligand-receptor interactions rely on the underlying aromatic residues in a manner and extent specified by each chemokine ligand-receptor pair.

Two chemokine regions come within reach of ECL2b, with the 30s-loop directing the MIP/MCP distinction

Analysis of the [5P7]CCL5-CCR5 structure shows that the two regions of [5P7]CCL5 that are in contact with ECL2b are the N-terminus and the 30s-loop, and that very little space remains for movement between these regions (Fig 5A) (37). To compare if the corresponding regions of other chemokines could be found in an analogous proximity to ECL2b in CCR1 and CCR2, and thereby be involved with the differential role of Cys+4 described for MIP and MCP chemokines, we first created consensus sequences for the MIP and MCP chemokines (from sequence alignments of the two chemokine groups, Fig S3). For the two regions of interest, we thereafter compared the consensus sequences to the MIP and MCP sequence logos (Fig 5B), which showed the internal MIP or MCP sequence variation behind the consensus for the group.

The N-terminus of MCP chemokines contains a few highly conserved elements, most notably the pyroglutamate-proline (pQP) motif of the extreme N-terminus, which is uncharged and protease-resistant. Conversely, the N-terminus of MIP chemokines displays almost no conserved traits (Fig 5B), and therefore, if it is responsible for the important role of the Cys+4 residue, the mechanism must be highly individual among MIP subtypes.

The 30s-loop contains a highly conserved TSS motif that is common for both MIP and MCP chemokines (Fig 5B); this motif is predicted to reach the top of the major binding pocket. However, upstream of the 30s-loop we detected a short segment in the $ß_1$-strand that is well conserved on a MIP to MCP basis, with the consensus motif FE in MIP chemokines and RRI in MCP chemokines (Fig 5B). This region makes contacts closer to the tip of ECL2 and is itself less likely to interact with residues in the aromatic cluster, but in MCPs the

longer tripeptide motif does correlate with a greater protrusion of the 30s-loop for unbound chemokines (Fig 5C). This is additionally associated with increased steric bulk due to the orientation of the side chains of MCP 30s-loops (Fig 5C, insert 1). For CCL5, the 30s-loop retains a stable position when comparing the CCR5-bound and solution structures (Fig 5C, insert 2).

To estimate how preserved the structural features of the ß$_1$-strand/30s-loop region are in a broader sense among MIP and MCP chemokines, we created a structure-based sequence alignment of representative MCP and MIP chemokine structures based on the position of the alpha carbons in this region (26-EYFYTSGKCSNP-37 in CCL5, Fig 5D). Interestingly, this structural alignment grouped the chemokines reliably into MIP and MCP backgrounds; in fact, the similarity was highest among MIP chemokines, although their overall sequence similarity is moderate (see Fig 3A).

Since the FE- (in MIP-chemokines) or RRI- (in MCP-chemokines) motif in the ß$_1$-strand immediately precedes the 30s-loop, these motifs could influence how the 30s-loop relates to ECL2 near the aromatic cluster. CCL13 contains some interesting features in this regard, as it is an MCP chemokine in sequence similarity and nomenclature (MCP-4) but has a unique VI-sequence before the 30s-loop. It is thus distinctly hydrophobic, but short akin to the MIP FE-motif. CCL13 also activates CCR1 and CCR5, albeit with only 48 and 56% G protein efficacy at 100 nM, respectively (relative to CCL3, see Fig 3A), which made it possible to test its relationship to the aromatic cluster of CCR1 and CCR5. Indeed, CCL13 fully depended on all residues of this cluster on CCR1 and -5 (Cys+4, F4.63 and W5.34, Fig 5E-F); however, CCL13 was unaffected by the corresponding mutations in CCR2 (Fig 5G). Thus, this is a case where an MCP chemokine with a MIP-like pre-30s-loop motif behaves like a MIP chemokine on CCR1 and CCR5 and like an MCP chemokine on CCR2 in terms of its relationship to the aromatic cluster. In other words, we find that the composition of the 30s-loop correlates well with the MIP- versus MCP-dependent relationship to ECL2b, and in this context, CCR1 and CCR5 are both predisposed to recognize CCL13 as a MIP chemokine.

Interactions between CCR1 and CCR5 and MIP chemokines can be distinguished by the relationship between the aromatic cluster and chemokine N-termini

Structural analysis suggested that the role of the MIP N-termini could be functionally connected to ECL2b near the Cys+4 position of CCR1, but the sequence variation of MIP N-termini implied that any such connection would vary according to the individual MIP chemokine (Fig 5A-B). We therefore tested the truncation variants CCL3 (5-70) and CCL5 (4-68) for activity on CCR1 and CCR5.

On CCR1, the shorter CCL3 (5-70) variant exhibited increased potency (by a factor 5.4, shown before (40)), and was less dependent upon the Cys+4 aromatic residue (6.9-fold decreased potency) compared to the complete Cys+4 dependency for the full length CCL3 (1-70) (Fig 6A, left). Thus, a part of the CCL3 dependency on the aromatic Cys+4 must derive from residues 1-4 of the chemokine N-terminus. The diminished role of the Cys+4 residue extended to the underlying aromatic cluster, as the importance of the W5.34 residue was greatly reduced (from a 50- to 3.2-fold potency loss on the mutant). On CCR5, the truncated CCL3 (5-70) was also more potent, but the impact of mutations within the aromatic cluster was similar to what was observed for the full length CCL3 (1-70), indicating functional independence (Fig 6A, right).

Consistent with previous data (46), CCL5 (4-68) was incapable of signaling through CCR1, and this effect was unchanged by either of the aromatic mutations (Cys+4, F4.63 and W5.34, Fig 6A, left). On CCR5, the CCL5 (4-68) truncation was tolerated, and the aromatic Cys+4 residue remained essential (Fig 6A, right). However, in contrast to other tested MIP chemokine ligand-receptor interactions, truncation of CCL5 led CCR5 to depend on the underlying aromatic cluster residues (F4.63 and W5.34), which had otherwise been dispensable for the full length CCL5 (1-68).

These data imply that both the N-terminus and 30s-loop of MIP chemokines are functionally connected to the aromatic cluster, but that the N-terminus confers a degree of nuance that allows us to distinguish individual MIP-receptor interactions. The N-terminus thereby contrasts the invariable 30s-loop, which is associated with a MIP-conserved role of the aromatic Cys+4 residue.

The four CCL3 N-terminal residues (1-ASLA-4) imparted a negative effect on signaling on CCR1 and CCR5 (Fig 6B, top), but on CCR1 they additionally created a dependency on the aromatic cluster. The corresponding peptide segment of CCL5 (1-SPY-3) contributed positively to CCR1 and CCR5 signaling (Fig 6B, bottom), but on CCR5 they additionally provided CCL5 with independence from the underlying aromatic residues (F4.63 and W5.34). We thus found that the variable sequence of MIP N-termini was connected to a variable functional role, and in the context of the aromatic cluster, this role was receptor-specific.

The diverging role of the aromatic cluster on a CCR1 and CCR5 level was further underscored by employing receptor binding studies using CCL3 (1-70) and CCL3 (5-70) radioligands. On CCR1, we observed that the Cys+4 mutation (F187A) caused a full loss of binding of CCL3 (1-70) (Fig S4A). CCL3 (5-70), which in terms of signaling was less sensitive to aromatic cluster mutations, had recovered partial binding on the Cys+4 mutation, implying that the signaling effects of mutagenesis was a reflection of a binding effect. On CCR5 however, the corresponding mutagenesis did not have a negative effect on the binding of CCL3 (1-70) in spite of the loss of signal (Fig S4B). The truncated CCL3 (5-70) on the other hand, did lose its ability to bind to the

Cys+4 mutant of CCR5, showing that the N-terminus did contribute to binding, but not signaling, via the aromatic Cys+4 for CCL3.

The degree of arrestin-coupling does not influence the role of the Cys+4 residue

To clarify if the role of the Cys+4 residue was chemokine-specific and indispensable beyond G protein-coupling, we investigated the arrestin recruitment, a commonly described intracellular mediator coupling to CCR1, CCR2 and CCR5 (20, 47-49). MIP chemokines and CCL7 were equally potent in G protein signaling on CCR1 (Fig S5A), but a more variable pattern emerged from arrestin recruitment (Fig S5B). On CCR5, MIP chemokines and CCL8 were potent agonists of G protein signaling (Fig S5C), and arrestin recruitment did not vary to the same extent as for CCR1 (Fig S5D). Truncation of CCL3 (1-70) to CCL3 (5-70) selectively enhanced arrestin recruitment on CCR1 and CCR5, highlighting the importance of this N-terminus in specifying the signaling pattern. On CCR2, the signaling pattern of MCP chemokines varied greatly (Fig S5E-F). By balancing area under the curve (AUC) for the two pathways, we determined that chemokines interact in a manner that produces highly variable active states for CCR1 and CCR2, whereas CCR5 activation is more similar when comparing different chemokines (Fig S5G-H). The role of the Cys+4 residue on CCR1 and CCR5 was thus not associated with a specific signaling pattern, unless the CCL3 N-terminal truncation was introduced, in which case the decreased CCR1 aromatic cluster-dependency was associated with increased arrestin recruitment.

The Cys+4 Ala mutation abolished all MIP chemokine-induced recruitment of arrestin on CCR1 and CCR5 (Fig S6 A and C), and the truncation of CCL3 (5-70) made the chemokine less sensitive to the Cys+4 mutation on CCR1, but not on CCR5. This outcome mirrored what we observed for G protein activity, and thus the connection between MIP chemokines and the aromatic cluster appeared unaffected by chemokine pathway selectivity, unless N-terminal truncations were involved. Conversely, the MCP chemokines that were able to induce significant arrestin recruitment on CCR1 and CCR5 had lost this activity on the Cys+4 mutants (Fig S6 B and D), indicating that to a certain extent, the aromatic cluster influences MCP chemokines. This effect was not seen on CCR2, however (Fig S6E).

Discussion

The overlapping leukocyte expression pattern of CCR1, CCR2 and CCR5 coinciding with their shared recognition of numerous MIP and MCP chemokines led us to search for a receptor interaction site that might allow these receptors to recognize chemokines according to a systematic pattern (8).

We show that targeting of receptor domains that are frequently, but not universally, associated with ligand interactions can be an effective approach to identify such sites. By organizing chemokines into functional subgroups and testing the pharmacological relationship between receptor domains and specified chemokine features, we describe what allows receptors to discriminate between closely related chemokines. It is thus possible to order interaction points of overlapping/redundant chemokine ligand-receptor complexes into tiers: those that are specific to chemokine subgroups and those that confer a more unique character to single chemokines.

Specifically, we show that two MIP chemokines, CCL3 and CCL5, make unique contact points with cognate receptors compared to each other, but at the same time, all MIP chemokines share additional common contact points that differentiate them as a group to MCP chemokines (Fig 7) (50, 51).

The almost binary nature of the outcome of removing the aromatic Cys+4 residue across all endogenous chemokine receptors (Fig 2A) showed that interactions depending on this residue are of a highly selective nature. For example, the requirement of an aromatic Cys+4 residue grouped strongly along MIP versus MCP lines. We observed that chemokines typically stimulated a greater $G_{\alpha i}$-response than ß-arrestin2 recruitment, but the balance of these pathways were not systematically connected to the involvement of the aromatic receptor cluster. The fact that MCP chemokines are less- or insensitive to the Cys+4 mutation on any cognate receptor indicates that the receptors allow chemokines to bind in distinctive MIP or MCP “modes”. This modality is more apparent for CCR1, where MIP chemokines are profoundly affected by the Cys+4 mutation, and where the sequence is less similar to CCR2 than is the case for CCR5. Accordingly, on CCR1 the Cys+4 residue is required for CCL3 binding to occur, which was not the case in CCR5. We observed that the responsible receptor aromatic cluster may be structurally approached by the broadly conserved TSS motif, but is functionally connected based on the MIP-specific FY motif.

Furthermore, we found that a comparable N-terminal truncation potentiates the activity of CCL3 on CCR1 relative to CCR5, while it instead potentiates CCL5 on CCR5 relative to CCR1, due to a complete loss of CCR1 activity. The CCL3 truncation reduces sensitivity to mutations in the aromatic cluster of CCR1, while the CCL5 truncation increases sensitivity to mutations in the aromatic cluster of CCR5. As such, the variable N-terminal sequence in question is functionally connected to the aromatic cluster in a manner that is more nuanced

than the overall MIP subgroup, contrasting it to the MIP-conserved sequence and function found in and near the 30s-loop.

This is in line with three past studies: One showed that CCL5 cannot afford an exchange of its N-terminus with that of CCL3 for CCR1 activity, while it is tolerated by CCR5 (52), and another showed that specific interactions take place between the CCL3 N-terminus and the CCR1 ECL2 (53). Finally, it was found that the $ß_1$-strand and most of the 30s-loop of CCL5 and murine CCL3 can be exchanged without loss of activity (54), illustrating an important functional distinction between variable and conserved MIP regions. Present and previous data therefore point to a receptor-specific and selective role of MIP chemokine N-termini, but a conserved and redundant role of the $ß_1$-strand/30s-loop. Selectivity and redundancy in the signaling induced by chemokine subgroups, and interpretation of their importance, has been a topic of much debate, but we here show that signaling aspects of a receptor can be characterized in both ways simultaneously.

Many examples in the literature highlight the importance of considering the broader differences between the MIP and MCP subgroups. Most notably, the work of Dyer *et al*. on knock-out mouse models demonstrated that the main MCP receptor CCR2 is responsible for $Ly6C^{hi}$ monocyte trafficking from bone marrow to blood, while monocyte trafficking to several peripheral tissues additionally relied on CCR1, CCR3 and CCR5 (10). For example, monocytic infiltration of inflamed air pouches was most affected by combined knock-out of receptors, was not blocked by a CCR1-specific inhibitor, and led to increased tissue levels of CCL3 and CCL5, but not CCL12 (mouse CCR2-binding chemokine) (10).

A MIP and MCP division of chemokines is also being made by proteases targeting the chemokine N-termini. DPP4 cleaves CCL3(L1), CCL4 and CCL5, which make up the main inflammatory MIP chemokines, and this leads to a diversification of their activities on CCR1, -5 and on CCR2 (55). Several matrix metalloproteinases (MMPs) cleave all MCP chemokines (CCL2, CCL7, CCL8 and CCL13) indiscriminately, converting them into antagonists of their cognate receptors and granting them anti-inflammatory activity in vivo (14, 56). CCL14, CCL15 and CCL23 are homeostatic MIP chemokines that become active when they are truncated at the N-terminus by some of the same MMPs that convert MCP chemokines into antagonists (57, 58), allowing local tissues to control the balance of chemokine activity on a MIP/MCP level. Enzymatic cleavage processes thereby demonstrate that coordinated mechanisms naturally interfere with chemokines along the MIP/MCP division.

Among CCR1, CCR2, and CCR5 and, in theory, among the atypical chemokine receptors, chemokine subgroup distinction would facilitate a more rational approach to interfering with promiscuous interaction networks. In essence, our study indicates that the extracellular face of CCR1 and CCR5 could be a promising candidate for pharmacological targeting of MIP activity over MCP activity. In a study that compared antibodies targeting

CCR5, it was found that those targeting ECL2 were the most effective at blocking CCL3 and CCL5 binding (59). It is feasible to develop small drugs targeting ECL2b, as shown by the antagonist Aplaviroc that specifically requires this part of CCR5 (60), and LMD-559 that relies on the aromatic Cys+4 residue in CCR1 (61). Zn-phe targets the major binding pocket of CCR1 and CCR5, and can thus modulate truncated, but not full length MIP variants (40, 41).

Our data suggests that targeting the aromatic cluster is interesting and therapeutically promising for several reasons: disturbing the aromatic cluster overcomes the potential redundancy that MIP chemokines may elicit on a receptor, ligand- and receptor-induced selective signaling does not influence the role of the cluster and – importantly – pharmacological efficacy of MIP chemokines depends heavily on this receptor site.

Despite the central role of the chemokine family in cell migration and inflammation, and its involvement in a range of autoimmune disorders and cancer metastasis, none of these connections have been successfully exploited therapeutically (62-64). The only successful drugs in the chemokine field target either a chemokine (Mogalizumab against CCL4), a viral interaction (Maraviroc inhibiting HIV gp120 interaction with CCR5) or a monogamous chemokine ligand-receptor pair (Mozobil targeting CXCR4 for stem cell mobilization). Attempts have been made to target the inflammatory CCRs, but multiple clinical drug trials have failed, citing lack of efficacy and interchangeable chemokine ligand and receptor roles as main causes (65, 66). Identifying target sites where drugs will be capable of overcoming both redundancy and bias, while remaining efficacious, has proven successful in other GPCRs, and it is therefore a priority to develop a similar approach to chemokine receptor targeting (67, 68).

Methods

Mammalian cell culture and transfection

COS-7 cells for competition binding and IP3 assays were grown in DMEM 1885 supplemented with 10 % FBS, 2 mM glutamine, 180 units/mL penicillin and 45 µg/mL streptomycin at 10 % CO_2, 90 % air humidity and 37 ^{o}C. They were transfected with receptor plasmid DNA for binding assays, or co-transfected with receptor plasmid DNA and G_{qi4myr} (kindly provided by Evi Kostenis, University of Bonn) in a 2:3 ratio for IP3 assays, approximately 40 h before the assays, using the calcium phosphate precipitation method (40).

HEK 293 cells (ATTC; Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM), containing 1% GlutaMAX^{TM}, and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (180 U/mL)/streptomycin (45 µg/mL), and incubated at 37 °C, 10% CO_2 and 95% air humidity. For transfection, HEK 293 cells (8 x 10^5 – 1 x 10^6) were seeded in tissue culture-treated 6 wells plates, after which a polyethylenimine (PEI) transfection was performed (69). Here, 0.8 µg of mem-citrine-SH3, 0.8 µg G protein-coupled receptor kinase 2 (GRK2), 0.042 µg Rluc-Arrestin3-SP1 and 0.33 µg of wild type or mutant chemokine receptor were mixed with PEI in non-supplemented DMEM in a ratio of 1:1.5 (DNA:PEI). After a 15-minute incubation, the sample was added dropwise to the cells. Approximately after 24 hours, the transfection was terminated by replacing the medium with fresh supplemented DMEM.

Molecular biology, mutagenesis and cell surface ELISA

Wild type chemokine receptors were incorporated into pcDNA3.1(+) (Invitrogen) downstream of an N-terminal FLAG-tag. Point mutations were introduced by applying the QuikChange PCR technique (Agilent Technologies), using Pfu polymerase (Stratagene) and primers synthesized by TAG Copenhagen. Mutant plasmids were verified by DNA sequencing (GATC Biotech) and amplified in XL1-blue cells.

Cell surface ELISA was used to verify receptor expression. Cells were washed in Tris-buffered saline (TBS) approximately 40 h after transfection to match cell used in signaling assays. The cells were then fixed in 3.7 % formaldehyde, washed three times in TBS and left in TBS with 2 % BSA for 30 min. Fixed cells were incubated with anti-FLAG M1 antibody (Sigma-Aldrich) for 90 min, washed in TBS with 1 mM $CaCl_2$ and incubated with goat anti-mouse HRP-conjugated antibody (Thermo Fischer Scientific) for 1 h. The cells were finally washed with TBS, incubated with TMB Plus substrate (Kem-En-Tec Diagnostics) for 5 min and the reaction was stopped with 0.2 M H_2SO_4. The TMB substrate was then transferred from the cells to a clear 96-well plate, and a Wallac EnVision 2104 Multilabel Reader (PerkinElmer) was used to measure absorbance at 450 nm.

Chemical synthesis of N-terminally truncated CCL5 (4-68)

The N-terminally truncated CCL5 variant CCL5 (4-68) was chemically synthesized using fluorenylmethoxycarbonyl (Fmoc) chemistry on an Activo-P11 automated peptide synthesizer (Activotec) as described previously (70). Deprotection with 20% (v/v) piperidine in N-methyl-2-pyrrolidone (NMP; Biosolve) was performed to remove the N-terminal Fmoc protecting group, allowing coupling of the next amino acid. The deprotection efficiency of the piperidine treatment was monitored by measuring the UV absorbance of the released Fmoc protecting groups, and additional deprotection steps were included where needed. In case three or more piperidine treatments were needed, a double instead of single coupling was performed to increase the coupling yield. In case of single coupling, the next amino acid was activated by 2-1H-benzotriazol-1yl-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU, Activotec) / 1-hydroxybenzotriazole (HOBt, Acros Organics, Thermo Fisher Scientific, Waltham, MA, USA) [both 0.45 M in dimethylformamide (Acros Organics)]. In case of double coupling, the next amino acid was activated by 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, Activotec) and HOBt in dimethylformamide, and the duration of both coupling steps was extended from 30 minutes to one hour.

After chemical synthesis, additional deprotection was performed to cleave CCL5 (4-68) from the resin and to remove side chain protecting groups present on the amino acids of the synthesized chemokine. CCL5 (4-68) was incubated with a mixture consisting of 0.75 g crystalline phenol (Merck KGaA) 0.5 ml thioanisole (Acros Organics), 10 ml trifluoroacetic acid (TFA, Biosolve), 0.25 ml 1,2-ethanedithiol (Merck) and 0.25 ml Milli-Q water for 1.5 hour under continuous shaking at room temperature. Afterwards, the chemokine was separated from the resins by filtration and washed using diethyl ether (Acros Organics).

After deprotection, the chemokine was dissolved in Milli-Q water and loaded on a 250x8.0-mm PepMap C18 column (VDS optilab) for purification using reversed-phase high-performance liquid chromatography (RP-HPLC; Waters 600 pump and Waters 600 controller, Millipore Corporation). CCL5 (4-68) was eluted by using a gradient with decreasing polarity ranging from 0% to 80% (v/v) acetonitrile (Biosolve) in 0.1% (v/v) TFA, as described previously (70). The eluting peptide was detected and collected by the UV detector and fraction collector, respectively. The eluting molecules were identified based on their molecular mass by sending 2% of the mobile phase to the electrospray ionization ion trap mass spectrometer (Esquire LC, Bruker Daltonics) coupled to the RP-HPLC system. After deconvolution of the averaged spectra, fractions containing the synthetic chemokine with the correct relative molecular mass (M_r) were folded by incubating them overnight at room temperature in folding buffer containing 1 M guanidium chloride, 0.3 mM reduced glutathione, 3

mM oxidized glutathione, 3 mM ethylenediaminetetraacetic acid (EDTA) and 0.01 % (v/v) p-nitrophenyl-β-D-glucuronide in 150 mM Tris (pH 8.6). Finally, folded CCL5 (4-68) was purified analogous to the unfolded CCL5 (4-68).

Inositol triphosphate (IP3) assay

IP3 is a second messenger generated as part of the $G_{\alpha q}$ signaling cascade. Chemokine receptors are $G_{\alpha i}$-coupled, but their G protein activity can reliably be measured by circumventing their activity to $G_{\alpha q}$ in cells expressing G_{qi4myr}, which is a chimeric $G_{\alpha q}$ protein that recognizes $G_{\alpha i}$-coupled receptors (71). COS-7 cells expressing G_{qi4myr} and a chemokine receptor were seeded at 35.000 cells per well into 96-well culture plates and kept overnight in growth medium supplemented with 5µL/mL, 2 µCi/mL [^{3}H]myo-inositol (PerkinElmer). Cells were then washed in HBSS buffer (Invitrogen) and kept in HBSS buffer with 10 mM LiCl, and incubated with ligand for 90 min at 37°C. Stimulation was terminated by the addition of 40 µL 10 mM formic acid, and the lysate was incubated with a solution of 12.5 µg/µL YSi-poly-D-Lys-coated SPA beads (PerkinElmer) for 30 min while shaking. After another 8 h equilibration, signal of the lysate was measured on a Packard Top Count NXT counter.

BRET beta-arrestin2 recruitment assay

One day after cell transfection, the HEK 293 cells were resuspended in PBS with 1% glucose (5 mM). Subsequently, the cells were distributed over a white CulturPlate-96 (85 µL, Perkin Elmer), after which Coelenterazine h (Nanolight Technologies) was added in a final concentration of 5 µM. The reaction was started after the addition of ligands (concentrations were ranging from 0.01 nM to 0.1 µM). Following a 20-minute incubation at room temperature, the luminescence (Rluc 485/40 nm and YFP 530/25 nm) was measured with a LB 940 Mithras Multimode Microplate Reader (Berthold Technologies GmbH & CO), as previously described (40).

CCL3 iodination and competition binding assay

2 nmol of CCL3 (1-70) (PeproTech) was labelled with ^{125}I (NEX033A, PerkinElmer) using the oxidative iodination technique and ChloramineT, and the labelled CCL3 was purified by reverse-phase HPLC on a C8 column as described previously (72).

COS-7 cells expressing CCR1 or CCR5 were seeded in duplicates in 24- or 12-well plates one day before the assay. For the assay, the cells were kept on ice as they were washed in 50mM Hepes buffer supplemented with 5 g/L BSA, followed by addition of tracer and unlabeled chemokine. The number of cells per well and tracer amount was adjusted to work with 5-10 % specific binding after cell washing. After 3 h incubation at 4 ^{o}C, the cells were washed with 50 mM Hepes buffer supplemented with 5 g/L BSA and 29.22 g/L NaCl to remove unbound tracer, after which the cells were lysed and radioactivity was measured on a Wallac 1470 Wizard Automatic Gamma Counter (Perkin Elmer).

Structural analysis

Maestro v12.1 or Pymol 2.3 (Schrödinger LLC(73)) was used to visualize X-ray, NMR, and cryo-EM structures from the Protein Data Bank (PDB). The [5P7]CCL5:CCR5 structure (pdb: 5UIW) was used as a reference during pharmacological data acquisition. The Maraviroc:CCR5 (pdb: 4MBS), IT1t:CXCR4 (pdb: 3OE9) and vMIP-II:CXCR4 (pdb: 4RWS) structures were also used for generation of hypotheses.

Receptor alignment (Fig. 1d): The PDB was manually searched for experimental structures of human peptide/protein-activated class A GPCRs (here referred to as peptidergic GPCRs) with an aromatic residue in Cys+4 position, which resulted in the identification of 16 receptors: Type-1 angiotensin II receptor (AGTR1), Type-2 angiotensin II receptor (AGTR2), Apelin receptor (APJ), C5a anaphylatoxin chemotactic receptor 1 (C5AR1), C-C chemokine receptor type 2 (CCR2), C-C chemokine receptor type 5 (CCR5), C-C chemokine receptor type 6 (CCR6), C-X-C chemokine receptor type 4 (CXCR4), N-formyl peptide receptor 2 (FPR2), Substance-P receptor (NK1R), Neuropeptide Y receptor type 1 (NPY1R), Delta-type opioid receptor (OPRD), Kappa-type opioid receptor (OPRK), Mu-type opioid receptor (OPRM), Orexin receptor type 1 (OX1R), and Orexin receptor type 2 (OX2R). The following PDB structures were imported into Maestro and structurally aligned to the seven transmembrane helices of the reference receptor structure (CCR5, PDB: 5UIW): AGTR1: 4ZUD, AGTR2: 5UNF, APJ: 5VBL, C5AR1: 5O9H, CCR2: 5T1A, CCR5: 4MBS, CCR6: 6WWZ, CXCR4: 3ODU, FPR2: 6OMM, NK1R: 6HLL, NPY1R: 5ZBH, OPRD: 4N6H, OPRK: 4DJH, OPRM: 4DKL, OX1R: 4ZJ8, OX2R: 4S0V.

Geneious software version 10.2 was used to perform and organize sequence alignments and sequence logos. Multiple sequence alignments were performed with the ClustalW tool separately on ECL2a and ECL2b of chemokine receptors, to account for the invariable positioning of the 7TM disulfide bridge, and on whole receptor sequences to build a dendrogram.

30s-loop alignments (Fig. 5d): Representative experimental structures of MIP chemokines (CCL3: 2X69, CCL4: 1HUM, CCL5: 5UIW, CCL14: 2Q8R, CCL15: 2HCC, CCL16: 5LTL, CCL23: 1G91) and MCP chemokines (CCL2: 1DOK, CCL7: 1BO0, CCL8: 1ESR, CCL11: 1EOT, CCL13: 2RA4) were imported from the PDB into Maestro, and then structurally aligned to the secondary structure elements (sheets and helices) of the reference chemokine structure ([5P7]CCL5, PDB: 5UIW). Next, the "Align sequences according to structure superposition" function (found in the Multiple Sequence Viewer) was used to generate a sequence alignment based on the spatial proximity of the α-carbons in the chemokine structures. Finally, the alignment columns spanning from E26 to P37 in [5P7]CCL5 (EYFYTSGKCSNP) were selected, and the percent similarity in this region calculated using the "Compare sequences" tool.

Data analysis

All binding and signaling data is presented as mean ± SEM. GraphPad Prism software version 8 was used to determine EC_{50}, efficacy and binding by non-linear regression where appropriate. Unpaired two-sided Students *t* test was applied to determine the significance of mean differences.

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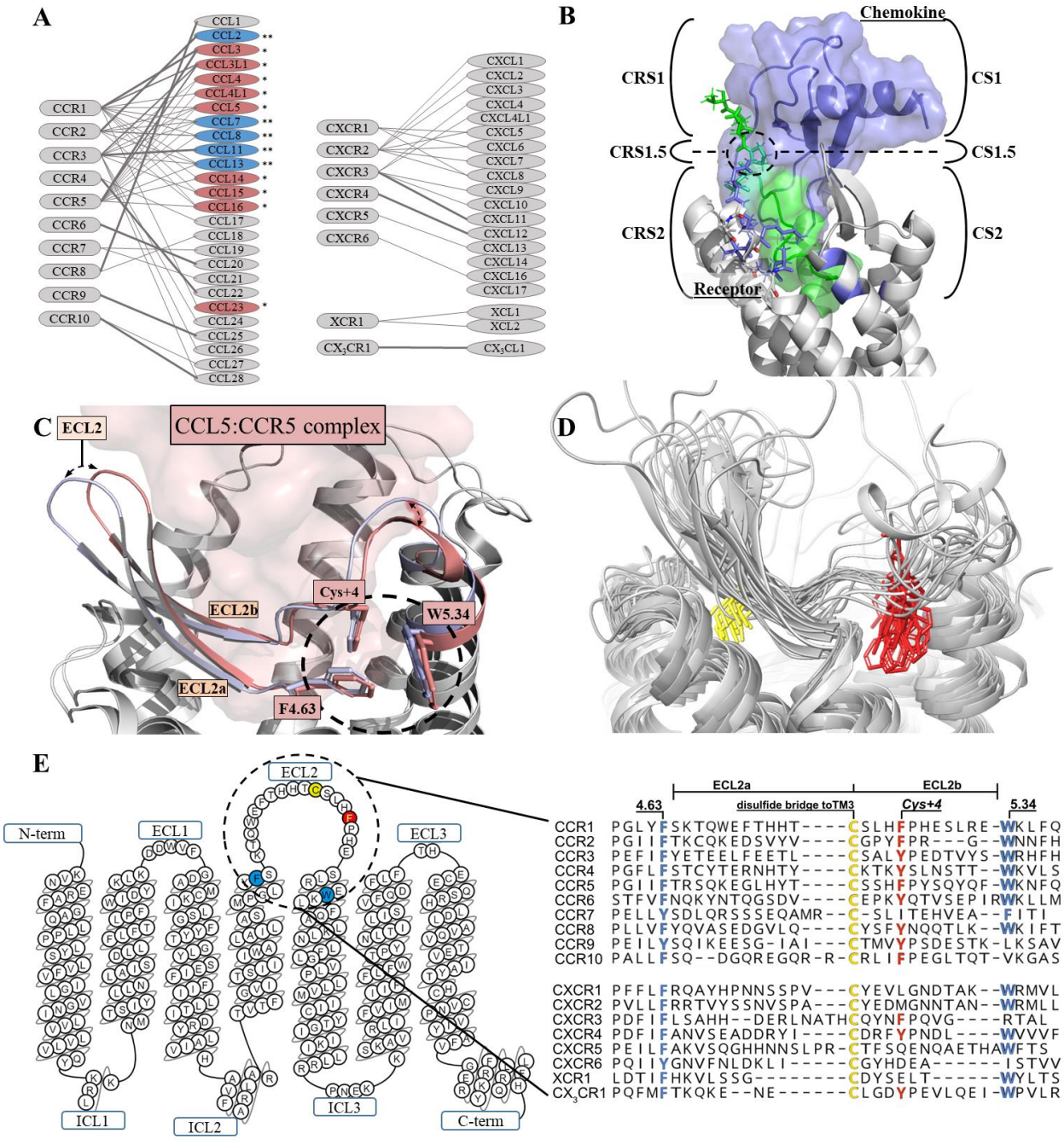
Contributions from authors

O.L., W.J.C.v.d.V., S.S., M.M., and P.C.R. conducted *in vitro* experiments and analyzed data. O.L., S.S. and P.P. synthesized CCL5 (4-68). O.L., M.G., J.V. and M.M.R. performed *in silico* structure- and sequence analysis. O.L. and J.V. produced graphical presentations of experimental data and structural context. O.L. wrote the first draft of the manuscript. All authors read and contributed to the manuscript. Technical supervision was provided by O.L., W.J.C.v.d.V., S.K. and P.P.. Hypotheses were generated in concert between O.L., S.K., M.G., P.P., J.V. and M.R.. M.R. provided overall supervision of the project.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Figures


A
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CCR9
CCR10
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CXCR6
XCR1
CX3CR1
B
Chemokine
CRS1
CS1
CRS1.5
CS1.5
CRS2
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ECL2b
W5.34
ECL2a
F4.63
D
E
ECL2
N-term
ECL1
ECL3
ICL1
ICL2
ICL3
C-term
ECL2a
ECL2b
4.63
disulfide bridge toTM3
Cys+4
5.34
CCR1 PGLYFSKTQWEFTHHT----CSLHFPHESLRE-WKLFQ
CCR2 PGIIFTKCQKEDSVYV----CGPYFPR---G--WNNFH
CCR3 PEFIFYETEELFEETL----CSALYPEDTVYS-WRHFH
CCR4 PGFLFSTCYTERNHTY----CKTKYSLNSTT--WKVLS
CCR5 PGIIFTRSQKEGLHYT----CSSHFPYSQYQF-WKNFQ
CCR6 STFVFNQKYNTQGSDV----CEPKYQTVSEPIRWKLLM
CCR7 PELLYSDLQRSSSEQAMR--C-SLITEHVEA--FITI
CCR8 PLLVFYQVASEDGVLQ----CYSFYNQQTLK--WKIFT
CCR9 PEILYSQIKEESG-IAI---CTMVYPSDESTK-LKSAV
CCR10 PALLFSQ--DGQREGQR-R-CRLIFPEGLTQT-VKGAS
CXCR1 PFFLFRQAYHPNNSSPV---CYEVLGNDTAK--WRMVL
CXCR2 PVLLFRRTVYSSNVSPA---CYEDMGNNTAN--WRMLL
CXCR3 PDFIFLSAHH--DERLNATHCQYNFPQVG----RTAL
CXCR4 PDFIFANVSEADDRYI----CDRFYPNDL----WVVVF
CXCR5 PEILFAKVSQGHHNNSLPR-CTFSQENQAETHAWFTS
CXCR6 PQIIYGNVFNLDKLI-----CGYHDEA------ISTVV
XCR1 LDTIFHKVLSSG---------CDYSELT------WYLTS
CX3CR1 PQFMFTKQKE--NE------CLGDYPEVLQEI-WPVLR

Figure 1. An aromatic cluster in chemokine receptors. A Chemokine receptors often recognize multiple overlapping chemokines, represented by grey lines, according to Bachelerie *et al* (8). MIP chemokines are marked with one star and red coloration, and MCP chemokines are marked with two stars and blue coloration. **B** General theme of chemokine recognition. Chemokine Recognition Site (CRS) 1 (i.e. the receptor N-terminus, green) recognizes Chemokine Site (CS) 1 (i.e. the chemokine core domain, blue) at the top. CRS1.5 recognizes CS1.5 in the middle (small regions, both in cyan). CRS2 (i.e. the receptor TM binding pocket, blue) recognizes CS2 below (i.e. the chemokine N-terminus, green). **C** Comparison of the extracellular domains of CCR5 in a state recognizing the chemokine [5P7]CCL5 (pdb 5UIW) with red highlights, and in a state recognizing the small molecule antagonist Maraviroc (pdb 4MBS) with blue highlights. Reorientation of ECL2 backbone is marked with arrows, the space occupied by [5P7]CCL5 presented as a red hue, and a cluster of three aromatic residues (F166/TM4.63, F182/Cys+4, W190/TM5.34) is shown. **D** Alignment of peptidergic GPCR class A receptors where the structure has been solved and an aromatic Cys+4 residue is present, showing the extracellular domain with ECL2 in front. The 7TM-conserved disulfide bridge is shown in yellow and the Cys+4 residue shown in red. **E** Left; serpentine diagram of CCR1, outlining the long ECL2 with the conserved cysteine in yellow, the Cys+4 residue in red, and the two transmembrane residues of the aromatic cluster in blue. Right; ClustalW alignment of the ECL2 segment in chemokine receptors. The structural location of the conserved cysteine is presumed to be immobile, and the upstream and downstream sequences are thus aligned separately.

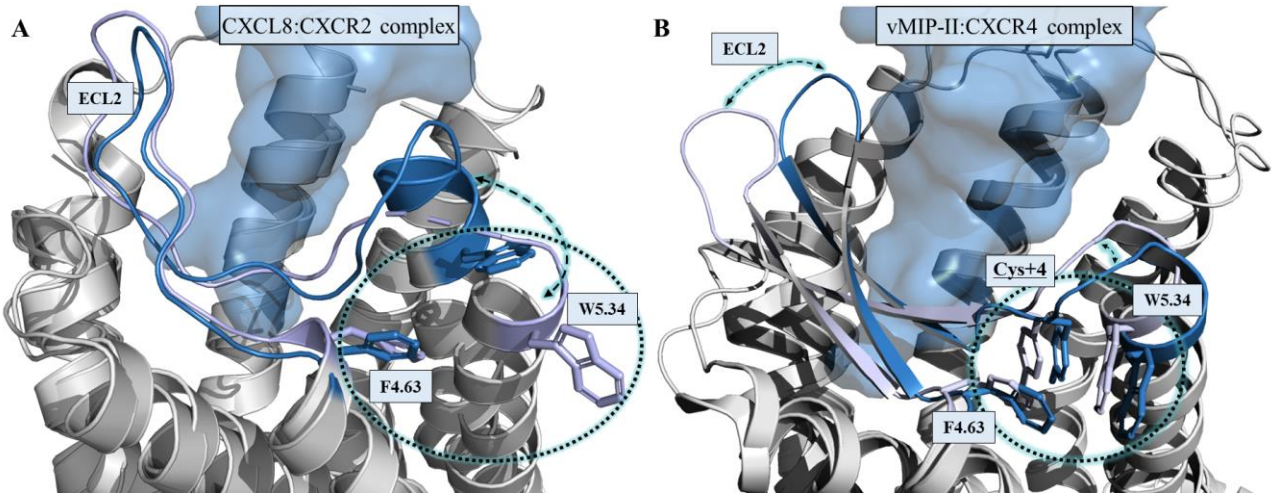


Figure S1. ECL2 movement in CXCR2 binding to CXCL8 and CXCR4 binding to vMIP-II. A Comparison of the extracellular domains of CXCR2 in a state recognizing CXCL8 (pdb 6LFO) with deep blue highlights, and in a state recognizing the intracellular antagonist 00767013 with pale blue highlights. Reorientation of ECL2b/TM5 is marked with arrows and the space occupied by CXCL8 is presented as a blue hue. Residues otherwise associated with the aromatic cluster (TM4.63 and TM5.34) are shown. **B** Comparison of the extracellular domains of CXCR4 in a state recognizing the viral chemokine vMIP-II (pdb 4RWS) with deep blue highlights, and in a state recognizing the small molecule antagonist IT1t (pdb 3OE9) with pale blue highlights. Reorientation of ECL2 backbone is marked with arrows and the space occupied by vMIP-II is presented as a blue hue. The aromatic cluster of residues (TM4.63, Cys+4, TM5.34) is shown within the dotted circle.

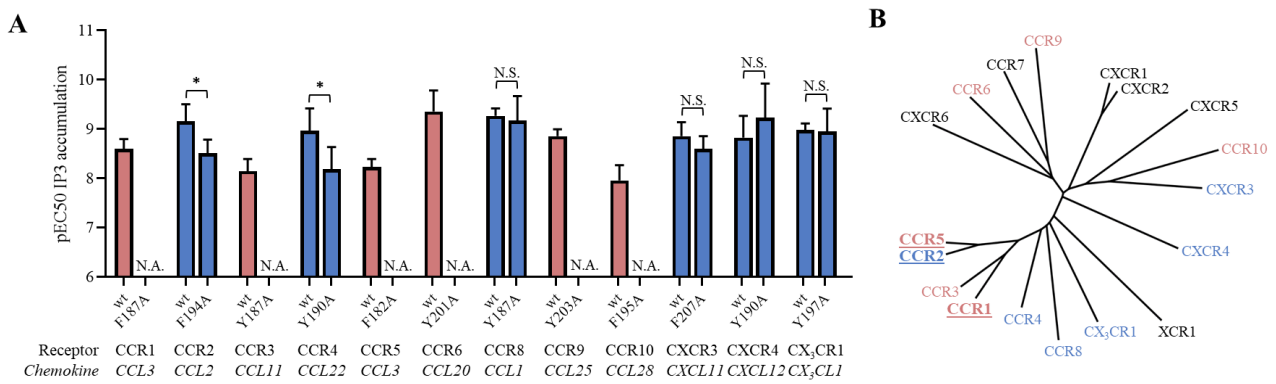


Figure 2. The aromatic Cys+4 residue is required by most CC-chemokine receptors. A The potency of $G_{\alpha i}$ signaling by chemokine receptors, presented as pEC_{50} values (i.e. (-log)EC_{50}), determined by IP3 assays. On each receptor with an aromatic residue at position Cys+4, one classical chemokine agonist was tested on the wild type and the alanine mutant of said position. N.A. (not active) denotes ≤20% activity at a chemokine concentration of 100 nM and the corresponding wild type bars are red. Blue bars mark receptors where removal of the aromatic Cys+4 residue caused less than 10-fold loss of potency. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM). Statistical significance was determined using an unpaired t-test (*: P value < 0.05). **B** Dendrogram presenting the overall sequence similarity of chemokine receptors as distance, showing a lack of reliable clustering according to sequence similarity can be seen for receptors without an aromatic Cys+4 residue (black), receptors with a dispensable aromatic Cys+4 residue (blue) and receptors that depend on an aromatic Cys+4 residue (red).

Receptor	CKR mutant / position	% of WT expression ± SEM
CCR1	F171A / F4.63	92 ± 2.7
	F187A / Cys+4	86 ± 5.2
	W195A / W5.34	82 ± 5.0
CCR2	F178A / F4.63	59 ± 1.0
	F194A / Cys+4	102 ± 5.9
	W197A / W5.34	81 ± 0.91
CCR5	F166A / F4.63	58 ± 7.9
	F182A / Cys+4	38 ± 8.4
	W190A / W5.34	76 ± 3.4

Table S1. ELISA expression of chemokine receptor mutants. Surface expression of receptor mutants presented as average percent of wild type expression ± SEM. N-terminally Flag-tagged receptors were detected with an anti-Flag (M1) antibody. Experiments were performed independently and in duplicate at least 3 times.

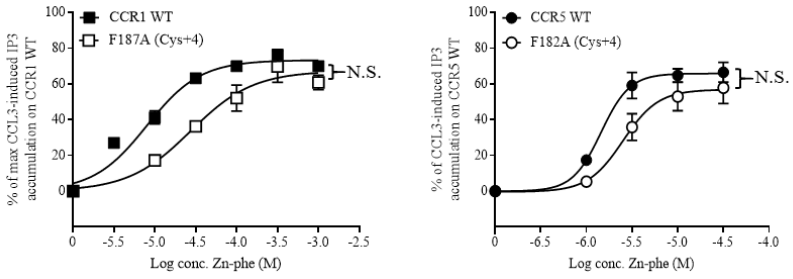


Figure S2. Activation of G protein signaling on CCR1 and CCR5 by zinc phenantroline (Zn-phe). Left: IP3 assay of CCR1 wild type and CCR1 Cys+4 mutant F187A. Right: IP3 assay of CCR5 wild type and CCR5 Cys+4 mutant F182A. N.S.: No significant difference of efficacy was determined in duplicates from at least 3 independent experiments using an unpaired t-test.

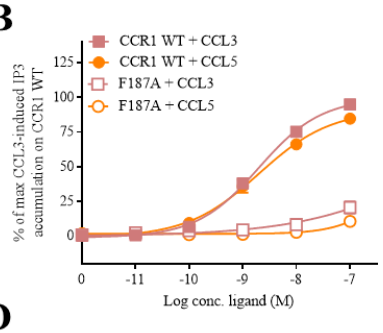
A

% Primary sequence similarity, MIP and MCP chemokines

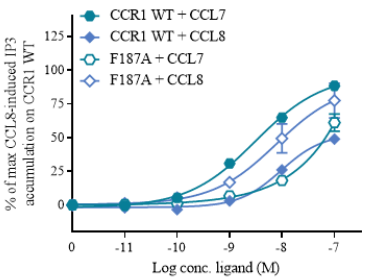
		CCL4	CCL5	CCL14	CCL15	CCL23	CCL16	CCL2	CCL7	CCL8	CCL11	CCL13
MIP gene cluster	CCL3	68.1	45.6	49.3	48.6	48.6	34.3	33.8	32.4	36.6	33.8	35.2
	CCL4		48.5	49.3	37.7	37.7	40.6	32.9	35.7	37.1	38.6	35.7
	CCL5			36.8	36.8	33.8	35.3	24.6	27.5	30.4	30.4	26.1
	CCL14				38.6	41.1	38.9	30.0	32.9	35.7	32.9	34.3
	CCL15					64.4	26.0	28.8	32.9	32.9	31.5	30.1
	CCL23						26.3	31.6	35.5	30.3	31.6	27.6
	CCL16							25.0	30.3	28.9	27.6	30.3
MCP gene cluster	CCL2								71.1	61.8	64.5	60.5
	CCL7									57.9	63.2	57.9
	CCL8										64.5	55.3
	CCL11											57.9
	CCL13											

%:10 25 40 55 70

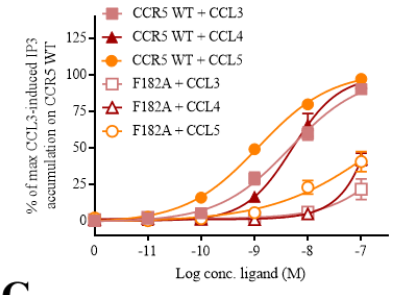
B



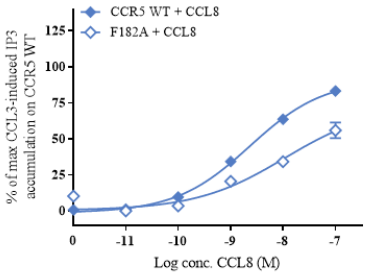
C



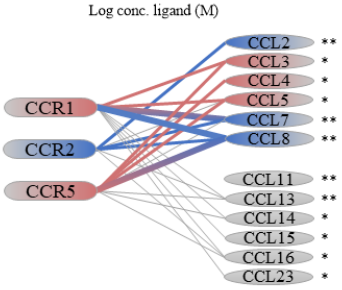
D



E



G



*) Macrophage Inflammatory Protein (MIP)
**) Monocyte Chemoattractant Protein (MCP)

F

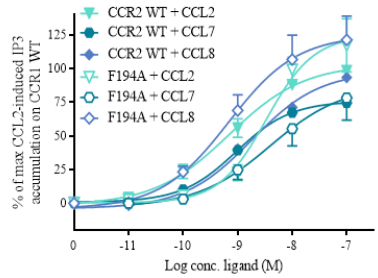


Figure 3. Distinct role of aromatic Cys+4 residue based on the MIP or MCP denomination of a chemokine. **A** Matrix of primary protein sequence similarity of chemokines genetically defined as MIP or MCP chemokines. Inconsistent receptor activities have been reported for CCL16, and it is therefore presented between the MIP and MCP subgroups (57, 74). **B**, **D** MIP chemokine-induced G protein activation of CCR1 and CCR5 tested by IP3 assay. **B** CCL3 and CCL5 on CCR1 wt and F187A. **D** CCL3, CCL4 and CCL5 on CCR5 wt and F182A. **C**, **E**, **F** MCP chemokine-induced G protein activation of CCR1, CCR2 and CCR5 tested by IP3 assay. **C** CCL7 and CCL8 on CCR1 wt and F187A. **E** CCL8 on CCR5 wt and F182A. **F** CCL2, CCL7 and CCL8 on CCR2 wt and F194A. **G** Interactions between chemokines belonging to the MIP or MCP subgroups and their cognate receptors. Colored lines represent interactions that have been tested to show dependency (red) or lack of dependency (blue) of an aromatic Cys+4 residue, while untested interactions are presented in grey. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM).

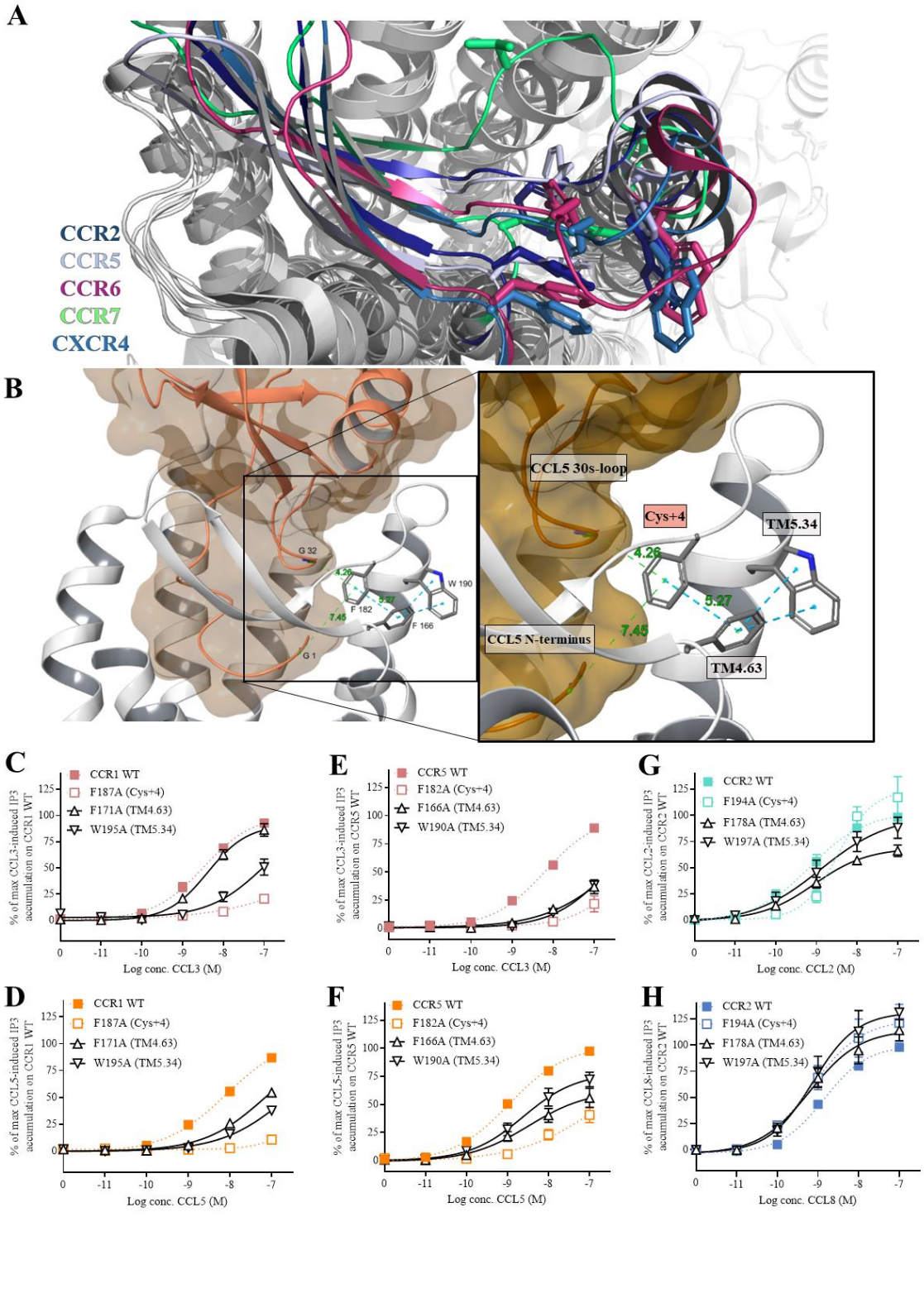

A
CCR2
CCR5
CCR6
CCR7
CXCR4
B
CCL5 30s-loop
Cys+4
TM5.34
CCL5 N-terminus
TM4.63
4.26
5.27
7.45
G 32
W 190
F 182
F 166
G 1
C
CCR1 WT
F187A (Cys+4)
F171A (TM4.63)
W195A (TM5.34)
% of max CCL3-induced IP3 accumulation on CCR1 WT
Log conc. CCL3 (M)
D
% of max CCL5-induced IP3 accumulation on CCR1 WT
Log conc. CCL5 (M)
E
CCR5 WT
F182A (Cys+4)
F166A (TM4.63)
W190A (TM5.34)
% of max CCL3-induced IP3 accumulation on CCR5 WT
Log conc. CCL3 (M)
F
% of max CCL5-induced IP3 accumulation on CCR5 WT
Log conc. CCL5 (M)
G
CCR2 WT
F194A (Cys+4)
F178A (TM4.63)
W197A (TM5.34)
% of max CCL2-induced IP3 accumulation on CCR2 WT
Log conc. CCL2 (M)
H
% of max CCL8-induced IP3 accumulation on CCR2 WT
Log conc. CCL8 (M)

Figure 4. Association of the Cys+4 residue with F4.63 and W5.34 in aromatic cluster. A Top-down view of the ECL2 section proximal to Cys+4, from an alignment of X-ray/cryo-EM structures of CCR7 (green), CCR2 (dark blue), CCR5 (light blue), CCR6 (magenta) and CXCR4 (teal). **B** The organization of the aromatic cluster in [5P7]CCL5:CCR5 (pdb 5UIW). Distances are marked in Angstrom from the central Cys+4 residue (F182, center of phenyl ring) to the closest potential stacking interaction with the aromatic cluster (F166) and the alpha carbon of the closest [5P7]CCL5 residues (G1 and G32). **C-H** Chemokine-induced G protein activation tested by the IP3 assay of F4.63A and W5.34A mutants (up- and downward-facing open triangles, respectively) relative to wt and Cys+4 in colored dotted lines for reference (closed and open squares, respectively). **C** CCL3 (MIP) activation of CCR1 F171A and W195A. **D** CCL5 (MIP) activation of CCR1 F171A and W195A. **E** CCL3 (MIP) activation of CCR5 F166A and W190A. **F** CCL5 (MIP) activation of CCR5 F166A and W190A. **G** CCL2 (MCP) activation of CCR2 F178A and W197A. **H** CCL8 (MCP) activation of CCR2 F178A and W197A. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM).

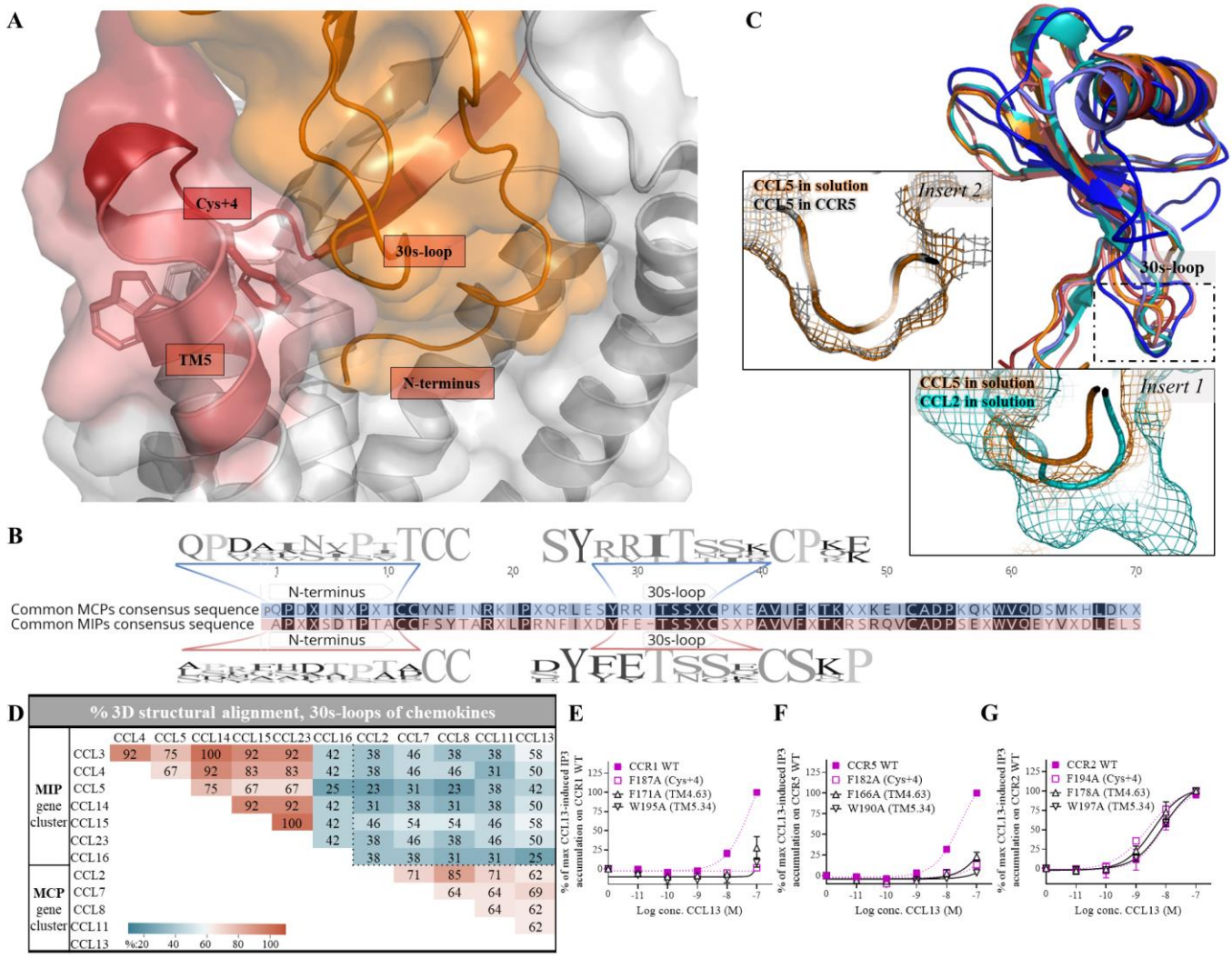

A
Cys+4
30s-loop
TM5
N-terminus
C
CCL5 in solution
CCL5 in CCR5
Insert 2
30s-loop
CCL5 in solution
CCL2 in solution
Insert 1
B
N-terminus
30s-loop
Common MCPs consensus sequence
Common MIPs consensus sequence
N-terminus
30s-loop
D
% 3D structural alignment, 30s-loops of chemokines
CCL4 CCL5 CCL14 CCL15 CCL23 CCL16 CCL2 CCL7 CCL8 CCL11 CCL13
MIP gene cluster
CCL3 92 75 100 92 92 42 38 46 38 38 58
CCL4 67 92 83 83 42 38 46 46 31 50
CCL5 75 67 67 25 23 31 23 38 42
CCL14 92 92 42 31 38 31 38 50
CCL15 100 42 46 54 54 46 58
CCL23 42 38 46 38 46 50
CCL16 38 38 31 31 25
MCP gene cluster
CCL2 71 85 71 62
CCL7 64 64 69
CCL8 64 62
CCL11 62
CCL13
%:20 40 60 80 100
E
CCR1 WT
F187A (Cys+4)
F171A (TM4.63)
W195A (TM5.34)
% of max CCL13-induced IP3 accumulation on CCR1 WT
Log conc. CCL13 (M)
F
CCR5 WT
F182A (Cys+4)
F166A (TM4.63)
W190A (TM5.34)
% of max CCL13-induced IP3 accumulation on CCR5 WT
Log conc. CCL13 (M)
G
CCR2 WT
F194A (Cys+4)
F178A (TM4.63)
W197A (TM5.34)
% of max CCL13-induced IP3 accumulation on CCR2 WT
Log conc. CCL13 (M)

Figure 5. Chemokines are functionally associated with the aromatic cluster via the 30s-loop in a subgroup-specific manner. A Cut-out view of the surface contact area of [5P7]CCL5 adjacent to the aromatic cluster of CCR5. C178-S185 is colored deep red, and represents the section of ECL2b potentially affected by the removal of the aromatic Cys+4 (F182). The receptor-interacting surface area projected by the [5P7]CCL5 30s-loop and N-terminus is shown in an orange mesh. **B** Alignment of the consensus sequences of MCP chemokines (blue) and MIP chemokines (red), derived from primary alignments in Fig S3. For the N-terminus and a segment that spans the 30s-loop, the sequence variability from individual chemokines is shown as sequence logos. **C** Structural alignment of CCL2, CCL7 and CCL8 (MCPs, shown in cyan, deep blue and light blue, respectively) and CCL3, CCL4 and CCL5 (MIPs, shown in red, salmon and orange, respectively) based on unbound structures. Insert 1 shows steric bulk of the 30s-loop of CCL5 and CCL2. Insert 2 shows overlap of the 30s-loop of CCL5 in unbound and CCR5-bound form. **D** Structure-based sequence alignment of unbound chemokine structures based on proximity of α-carbons, showing percent similarity in the $ß_1$-strand/30s-loop region (26-EYFYTSGKCSNP-37 in CCL5). **E** CCL13-induced G protein activation of CCR1 wt (purple closed squares), F187A (Cys+4, purple open squares), F171A (TM4.63, upward-facing triangles) and W195A (TM5.34, downward-facing triangles). **F** CCL13-induced G protein activation of CCR5 wt (purple closed squares), F182A (Cys+4, purple open squares), F166A (TM4.63, upward-facing triangles) and W190A (TM5.34, downward-facing triangles). **G** CCL13-induced G protein activation of CCR2 wt (purple closed squares), F194A (Cys+4, purple open squares), F178A (TM4.63, upward-facing triangles) and W197A (TM5.34, downward-facing triangles). Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM).

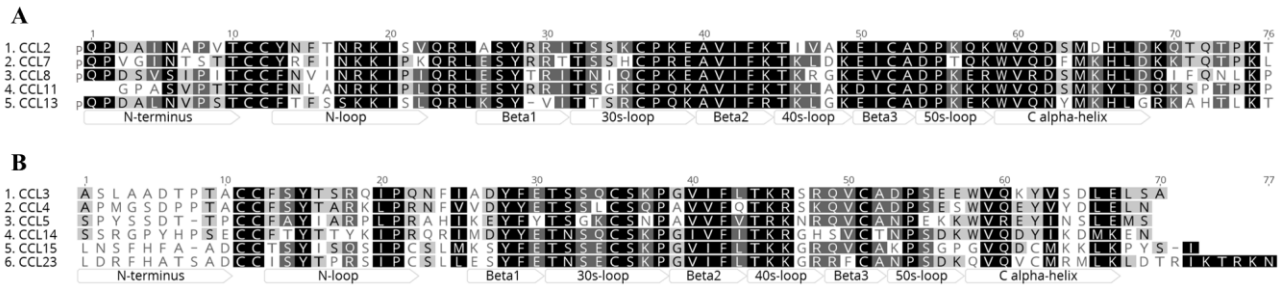


Figure S3. Primary sequence alignment of MCP and MIP chemokines. A Alignment of MCP chemokines with secondary structure annotations. **B** Alignment of most MIP chemokines with secondary structure annotations. The extended N-termini of CCL14, CCL15 and CCL23 are omitted.

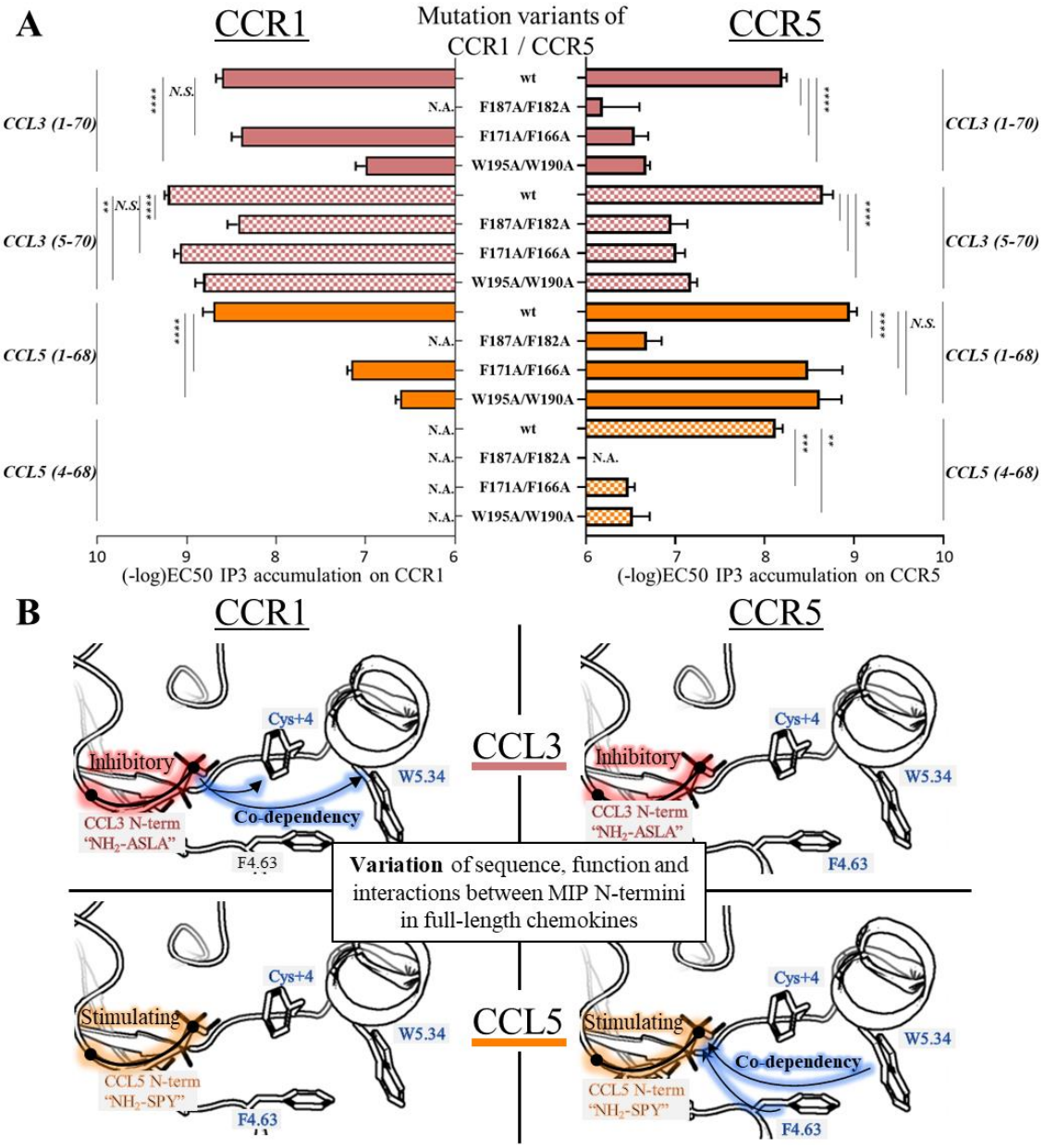

A
CCR1
Mutation variants of
CCR1 / CCR5
CCR5
wt
F187A/F182A
F171A/F166A
W195A/W190A
CCL3 (1-70)
CCL3 (5-70)
CCL5 (1-68)
CCL5 (4-68)
N.S.
N.A.
10 9 8 7 6
(-log)EC50 IP3 accumulation on CCR1
6 7 8 9 10
(-log)EC50 IP3 accumulation on CCR5
B
CCR1
CCR5
CCL3
CCL5
Cys+4
W5.34
F4.63
Inhibitory
Co-dependency
CCL3 N-term
"NH2-ASLA"
Stimulating
CCL5 N-term
"NH2-SPY"
Variation of sequence, function and
interactions between MIP N-termini
in full-length chemokines

Figure 6. Effect of N-terminal truncation on activity of MIP chemokines is affected by the aromatic cluster. **A** Potencies of G protein activation of CCR1 and CCR5 determined by chemokine dose-response curves in IP3 assays, and presented as (-log)EC_{50} values. Activities of CCR1 wt, F187A (Cys+4), F171A (TM4.63) and W195A (TM5.34) are shown on the left, and activities of CCR5 wt, F182A (Cys+4), F166A (TM4.63) and W190A (TM5.34) are shown on the right. CCR1 and CCR5 variants were stimulated with CCL3 (1-70) (red bars), CCL3 (5-70) (checkered red), CCL5 (1-68) (orange bars) or CCL5 (4-68) checkered orange). N.A.: no activity, N.S.: not significant, **: P≤0.01, ***: P≤0.01, ****: P≤0.0001. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM). **B** Sketched representation of the aromatic clusters of CCR1 (left) and CCR5 (right). The inhibitory role of the CCL3 N-terminus (top) and the stimulating role of the CCL5 N-terminus (bottom) is deduced from pharmacological data. The involvement of aromatic residues in MIP activity and co-dependency with chemokine N-terminal truncations is indicated.

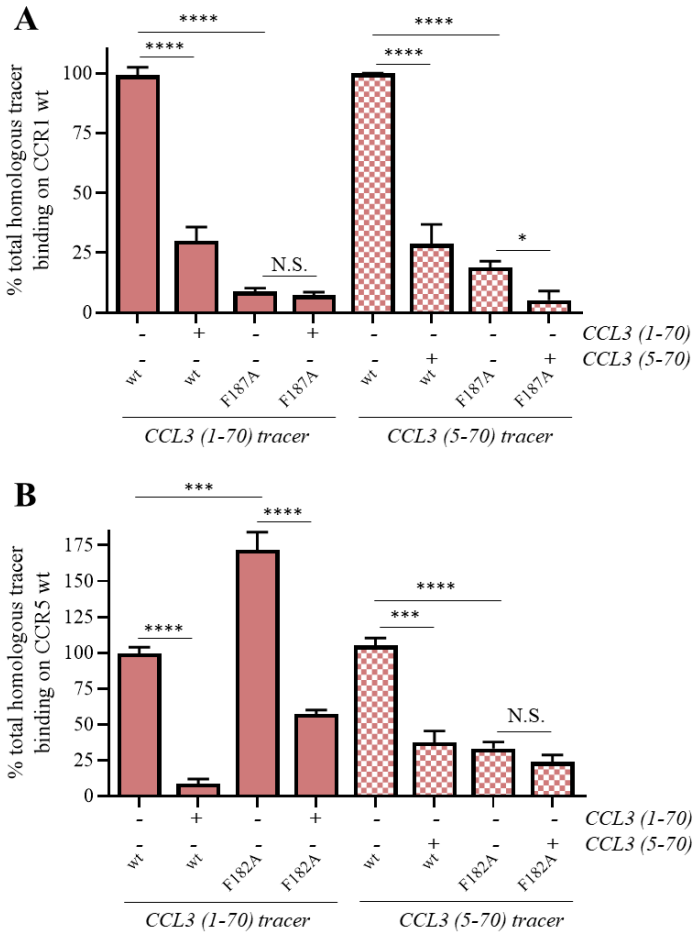


Figure S4. Effect of the Cys+4 mutation on the binding of CCL3. A, B Total binding of ^{125}I-CCL3 (1-70) or ^{125}I-CCL3 (5-70) on COS-7 cells transfected to express CCR1 or CCR5, where binding to corresponding wt receptor is normalized to 100% and binding to mock-transfected cells is set to 0%. **A** Binding of ^{125}I-CCL3 tracers on CCR1 wt or F187A (Cys+4) ± competition with 100 nM unlabeled chemokine of matching N-terminal length. **B** Binding of ^{125}I-CCL3 tracers on CCR5 wt or F182A (Cys+4) ± competition with 100 nM unlabeled chemokine of matching N-terminal length. N.S.: not significant, *: P≤0.05, **: P≤0.01, ***: P≤0.01, ****: P≤0.0001. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM).

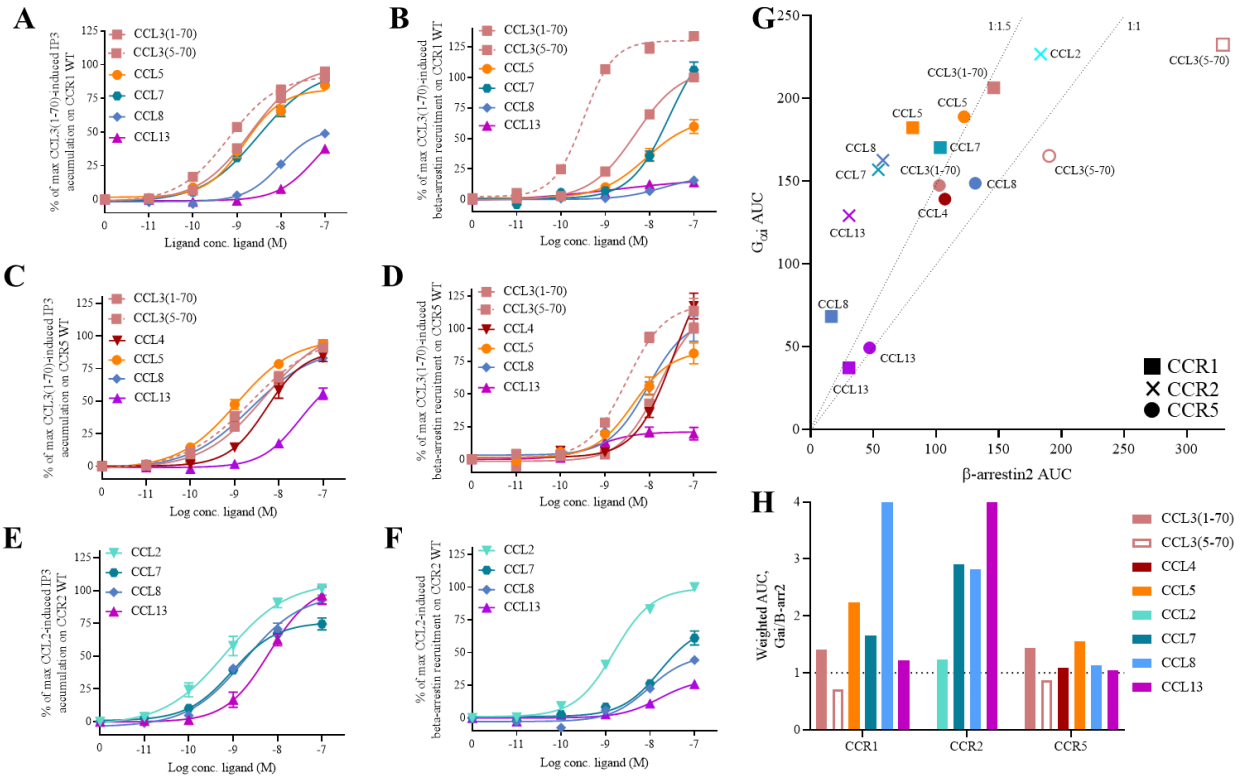


Figure S5. Comparison of G protein and beta-arrestin2 activity of chemokines on CCR1, CCR2 and CCR5. G protein activation **A**, **C** and **E** tested by IP3 assay and beta-arrestin2 recruitment **B**, **D** and **F** tested by Bioluminescence Resonance Energy Transfer (BRET) of CCR1 (**A**-**B**), CCR5 (**C**-**D**) and CCR2 (**E**-**F**). Activity of selected pathways tested for the MIP chemokines CCL3 (1-70), CCL3 (5-70), CCL4 and CCL5 and the MCP chemokines CCL2, CCL7, CCL8 and CCL13 on their cognate receptors. **G** Area under curve (AUC) quantified from IP3 and beta-arrestin2 recruitment assays over equal dose-ranges (up to 100 nM chemokine). Chemokines placed between the dotted lines are close to balanced between G protein and beta-arrestin2 activity. Closed squares are chemokines tested on CCR1, crosses are chemokines tested on CCR2, and closed circles are chemokines tested on CCR5. **H** G protein AUC over beta-arrestin2 AUC as a quantification of the relative AUC. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM).

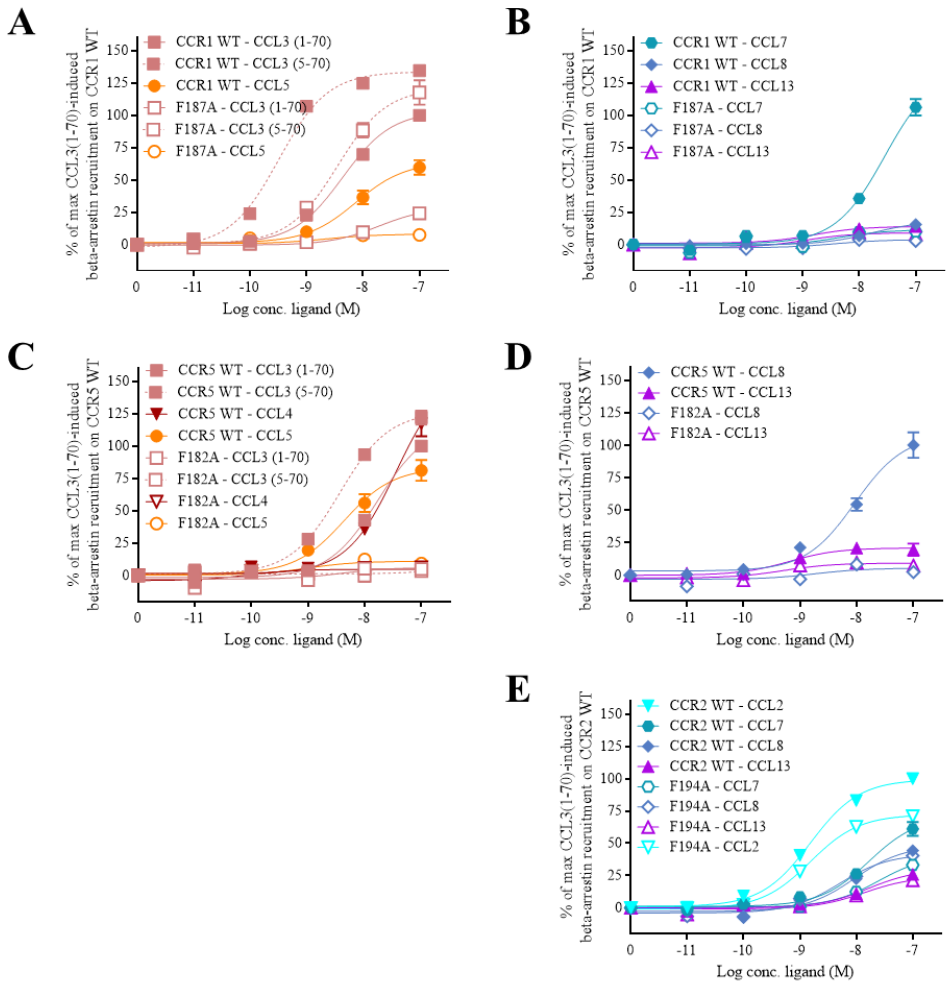


Figure S6. Effect of the Cys+4 mutation on beta-arrestin2 recruitment. Arrestin recruitment to CCR1, CCR2 and CCR5 induced by MIP chemokines (left) and MCP chemokines (right). **A**-**B** Activation of CCR1 wt and F187A (Cys+4) by MIPs CCL3 (1-70), CCL3 (5-70) and CCL5 (**A**) and MCPs CCL7, CCL8 and CCL13 (**B**). **C-D** Activation of CCR5 wt and F182A (Cys+4) by MIPs CCL3 (1-70), CCL3 (5-70), CCL4 and CCL5 (**C**) and MCPs CCL8 and CCL13. **E** Activation of CCR2 wt and F194A (Cys+4) by MCPs CCL2, CCL7, CCL8 and CCL13. Presented data are a mean of at least 3 independent experiments performed in duplicate ± standard error mean (SEM).

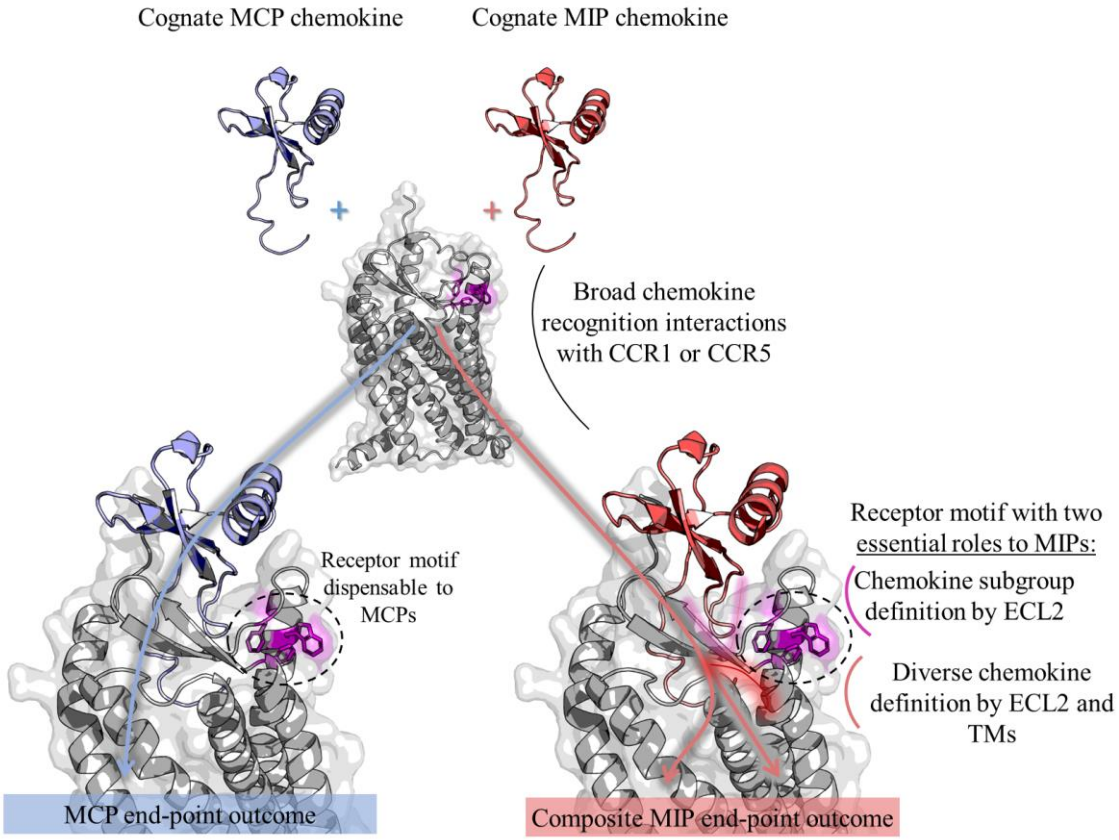


Figure 7. Cognition beyond recognition of chemokine subgroups. Many chemokines belonging to the MCP or MIP subgroups (represented in light blue and –red, respectively) can be recognized by the same receptors, e.g. CCR1 and CCR5. While some receptor recognition sites may be commonly employed by all cognate chemokines, others, such as the aromatic cluster (purple), can lead to a distinctive recognition pattern of the MIP subgroup members (right, purple highlight). Additionally, our data show that individual MIP chemokines can make fine adjustments to this recognition pattern via their N-termini (red highlight).

References

1. J. W. Griffith, C. L. Sokol, A. D. Luster, Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev Immunol* **32**, 659-702 (2014).
2. C. A. Anderson, R. Solari, J. E. Pease, Biased agonism at chemokine receptors: obstacles or opportunities for drug discovery? *J Leukoc Biol* **99**, 901-909 (2016).
3. I. Roy, A. E. Getschman, B. F. Volkman, M. B. Dwinell, Exploiting agonist biased signaling of chemokines to target cancer. *Mol Carcinog* **56**, 804-813 (2017).
4. C. Shi, E. G. Pamer, Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* **11**, 762-774 (2011).
5. M. Ruster, H. Sperschneider, R. Funfstuck, G. Stein, H. J. Grone, Differential expression of beta-chemokines MCP-1 and RANTES and their receptors CCR1, CCR2, CCR5 in acute rejection and chronic allograft nephropathy of human renal allografts. *Clin Nephrol* **61**, 30-39 (2004).
6. C. A. Reichel *et al.*, Chemokine receptors Ccr1, Ccr2, and Ccr5 mediate neutrophil migration to postischemic tissue. *J Leukoc Biol* **79**, 114-122 (2006).
7. M. Gschwandtner, R. Derler, K. S. Midwood, More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front Immunol* **10**, 2759 (2019).
8. F. Bachelerie *et al.*, International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* **66**, 1-79 (2014).
9. A. Zlotnik, O. Yoshie, H. Nomiyama, The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol* **7**, 243 (2006).
10. D. P. Dyer *et al.*, Chemokine Receptor Redundancy and Specificity Are Context Dependent. *Immunity* **50**, 378-389 e375 (2019).
11. K. G. Sandblad *et al.*, Chemokine receptor expression on monocytes from healthy individuals. *Clin Immunol* **161**, 348-353 (2015).
12. L. Skelton, M. Cooper, M. Murphy, A. Platt, Human immature monocyte-derived dendritic cells express the G protein-coupled receptor GPR105 (KIAA0001, P2Y14) and increase intracellular calcium in response to its agonist, uridine diphosphoglucose. *J Immunol* **171**, 1941-1949 (2003).
13. M. Gouwy *et al.*, Chemokines and other GPCR ligands synergize in receptor-mediated migration of monocyte-derived immature and mature dendritic cells. *Immunobiology* **219**, 218-229 (2014).
14. G. A. McQuibban *et al.*, Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* **100**, 1160-1167 (2002).
15. A. Mantovani, The chemokine system: redundancy for robust outputs. *Immunol Today* **20**, 254-257 (1999).
16. S. Eltayeb *et al.*, Temporal expression and cellular origin of CC chemokine receptors CCR1, CCR2 and CCR5 in the central nervous system: insight into mechanisms of MOG-induced EAE. *J Neuroinflammation* **4**, 14 (2007).
17. A. Steen, O. Larsen, S. Thiele, M. M. Rosenkilde, Biased and g protein-independent signaling of chemokine receptors. *Front Immunol* **5**, 277 (2014).
18. G. Xanthou, T. J. Williams, J. E. Pease, Molecular characterization of the chemokine receptor CXCR3: evidence for the involvement of distinct extracellular domains in a multi-step model of ligand binding and receptor activation. *Eur J Immunol* **33**, 2927-2936 (2003).
19. T. Kenakin, Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**, 296-302 (2011).

20. J. Corbisier, C. Gales, A. Huszagh, M. Parmentier, J. Y. Springael, Biased signaling at chemokine receptors. *J Biol Chem* **290**, 9542-9554 (2015).
21. S. Rajagopal *et al.*, Biased agonism as a mechanism for differential signaling by chemokine receptors. *J Biol Chem* **288**, 35039-35048 (2013).
22. G. M. Hjorto *et al.*, Differential CCR7 Targeting in Dendritic Cells by Three Naturally Occurring CC-Chemokines. *Front Immunol* **7**, 568 (2016).
23. P. Proost *et al.*, Chemokine isoforms and processing in inflammation and immunity. *J Autoimmun* **85**, 45-57 (2017).
24. F. S. Monteclaro, I. F. Charo, The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1alpha receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *J Biol Chem* **271**, 19084-19092 (1996).
25. D. J. Scholten *et al.*, Pharmacological modulation of chemokine receptor function. *Br J Pharmacol* **165**, 1617-1643 (2012).
26. I. Kufareva, M. Gustavsson, Y. Zheng, B. S. Stephens, T. M. Handel, What Do Structures Tell Us About Chemokine Receptor Function and Antagonism? *Annu Rev Biophys* **46**, 175-198 (2017).
27. S. Thiele, M. M. Rosenkilde, Interaction of chemokines with their receptors--from initial chemokine binding to receptor activating steps. *Curr Med Chem* **21**, 3594-3614 (2014).
28. A. B. Kleist *et al.*, New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. *Biochem Pharmacol* **114**, 53-68 (2016).
29. M. Gustavsson, D. P. Dyer, C. Zhao, T. M. Handel, Kinetics of CXCL12 binding to atypical chemokine receptor 3 reveal a role for the receptor N terminus in chemokine binding. *Sci Signal* **12** (2019).
30. M. Gustavsson *et al.*, Structural basis of ligand interaction with atypical chemokine receptor 3. *Nat Commun* **8**, 14135 (2017).
31. M. C. Peeters, G. J. van Westen, Q. Li, I. J. AP, Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends Pharmacol Sci* **32**, 35-42 (2011).
32. A. S. Jorgensen *et al.*, Biased Signaling of CCL21 and CCL19 Does Not Rely on N-Terminal Differences, but Markedly on the Chemokine Core Domains and Extracellular Loop 2 of CCR7. *Front Immunol* **10**, 2156 (2019).
33. L. Barington *et al.*, Role of Conserved Disulfide Bridges and Aromatic Residues in Extracellular Loop 2 of Chemokine Receptor CCR8 for Chemokine and Small Molecule Binding. *J Biol Chem* **291**, 16208-16220 (2016).
34. C. Blanpain *et al.*, The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle. *J Biol Chem* **278**, 5179-5187 (2003).
35. P. C. Rummel *et al.*, Extracellular disulfide bridges serve different purposes in two homologous chemokine receptors, CCR1 and CCR5. *Mol Pharmacol* **84**, 335-345 (2013).
36. L. Qin *et al.*, Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. *Science* **347**, 1117-1122 (2015).
37. Y. Zheng *et al.*, Structure of CC Chemokine Receptor 5 with a Potent Chemokine Antagonist Reveals Mechanisms of Chemokine Recognition and Molecular Mimicry by HIV. *Immunity* **46**, 1005-1017 e1005 (2017).
38. K. Liu *et al.*, Structural basis of CXC chemokine receptor 2 activation and signalling. *Nature* **585**, 135-140 (2020).
39. J. A. Ballesteros, H. Weinstein, "[19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors" in Methods in Neurosciences*,* C. S. Stuart, Ed. (Academic Press, 1995), vol. Volume 25, pp. 366-428.
40. O. Larsen *et al.*, Selective Allosteric Modulation of N-Terminally Cleaved, but Not Full Length CCL3 in CCR1. *ACS Pharmacology & Translational Science* **2**, 429-441 (2019).

41. S. Karlshoj *et al.*, Molecular Mechanism of Action for Allosteric Modulators and Agonists in CC-chemokine Receptor 5 (CCR5). *J Biol Chem* **291**, 26860-26874 (2016).
42. D. J. Wasilko *et al.*, Structural basis for chemokine receptor CCR6 activation by the endogenous protein ligand CCL20. *Nat Commun* **11**, 3031 (2020).
43. A. K. Apel *et al.*, Crystal Structure of CC Chemokine Receptor 2A in Complex with an Orthosteric Antagonist Provides Insights for the Design of Selective Antagonists. *Structure* **27**, 427-438 e425 (2019).
44. K. Jaeger *et al.*, Structural Basis for Allosteric Ligand Recognition in the Human CC Chemokine Receptor 7. *Cell* **178**, 1222-1230 e1210 (2019).
45. C. Oswald *et al.*, Intracellular allosteric antagonism of the CCR9 receptor. *Nature* **540**, 462-465 (2016).
46. J. K. Lim, J. M. Burns, W. Lu, A. L. DeVico, Multiple pathways of amino terminal processing produce two truncated variants of RANTES/CCL5. *J Leukoc Biol* **78**, 442-452 (2005).
47. A. M. Aragay *et al.*, Monocyte chemoattractant protein-1-induced CCR2B receptor desensitization mediated by the G protein-coupled receptor kinase 2. *Proc Natl Acad Sci U S A* **95**, 2985-2990 (1998).
48. C. T. Gilliland, C. L. Salanga, T. Kawamura, J. Trejo, T. M. Handel, The chemokine receptor CCR1 is constitutively active, which leads to G protein-independent, beta-arrestin-mediated internalization. *J Biol Chem* **288**, 32194-32210 (2013).
49. Y. A. Berchiche, S. Gravel, M. E. Pelletier, G. St-Onge, N. Heveker, Different effects of the different natural CC chemokine receptor 2b ligands on beta-arrestin recruitment, Galphai signaling, and receptor internalization. *Mol Pharmacol* **79**, 488-498 (2011).
50. R. Nygaard, T. M. Frimurer, B. Holst, M. M. Rosenkilde, T. W. Schwartz, Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci* **30**, 249-259 (2009).
51. Q. Zhou *et al.*, Common activation mechanism of class A GPCRs. *Elife* **8** (2019).
52. C. Blanpain *et al.*, A chimeric MIP-1alpha/RANTES protein demonstrates the use of different regions of the RANTES protein to bind and activate its receptors. *J Leukoc Biol* **69**, 977-985 (2001).
53. S. Zoffmann, A. Chollet, J. L. Galzi, Identification of the extracellular loop 2 as the point of interaction between the N terminus of the chemokine MIP-1alpha and its CCR1 receptor. *Mol Pharmacol* **62**, 729-736 (2002).
54. K. Ottersbach, J. McLean, N. W. Isaacs, G. J. Graham, A310 helical turn is essential for the proliferation-inhibiting properties of macrophage inflammatory protein-1 alpha (CCL3). *Blood* **107**, 1284-1291 (2006).
55. M. Metzemaekers, J. Van Damme, A. Mortier, P. Proost, Regulation of Chemokine Activity - A Focus on the Role of Dipeptidyl Peptidase IV/CD26. *Front Immunol* **7**, 483 (2016).
56. P. Proost *et al.*, Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor. *J Immunol* **160**, 4034-4041 (1998).
57. A. E. Starr, A. Dufour, J. Maier, C. M. Overall, Biochemical analysis of matrix metalloproteinase activation of chemokines CCL15 and CCL23 and increased glycosaminoglycan binding of CCL16. *J Biol Chem* **287**, 5848-5860 (2012).
58. U. Forssmann, H. J. Magert, K. Adermann, S. E. Escher, W. G. Forssmann, Hemofiltrate CC chemokines with unique biochemical properties: HCC-1/CCL14a and HCC-2/CCL15. *J Leukoc Biol* **70**, 357-366 (2001).
59. B. Lee *et al.*, Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J Biol Chem* **274**, 9617-9626 (1999).
60. S. Thiele *et al.*, Allosteric and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach. *J Biol Chem* **286**, 37543-37554 (2011).
61. P. C. Jensen *et al.*, Reversed binding of a small molecule ligand in homologous chemokine receptors - differential role of extracellular loop 2. *Br J Pharmacol* **166**, 258-275 (2012).

62. A. Ridiandries, J. T. Tan, C. A. Bursill, The Role of CC-Chemokines in the Regulation of Angiogenesis. *Int J Mol Sci* **17** (2016).
63. A. Viola, A. D. Luster, Chemokines and their receptors: drug targets in immunity and inflammation. *Annu Rev Pharmacol Toxicol* **48**, 171-197 (2008).
64. G. Marelli, A. Sica, L. Vannucci, P. Allavena, Inflammation as target in cancer therapy. *Curr Opin Pharmacol* **35**, 57-65 (2017).
65. T. J. Schall, A. E. Proudfoot, Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat Rev Immunol* **11**, 355-363 (2011).
66. A. Mantovani, Redundancy and robustness versus division of labour and specialization in innate immunity. *Semin Immunol* **36**, 28-30 (2018).
67. A. S. Hauser, M. M. Attwood, M. Rask-Andersen, H. B. Schioth, D. E. Gloriam, Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* **16**, 829-842 (2017).
68. Z. Rankovic, T. F. Brust, L. M. Bohn, Biased agonism: An emerging paradigm in GPCR drug discovery. *Bioorg Med Chem Lett* **26**, 241-250 (2016).
69. P. Donthamsetti, J. R. Quejada, J. A. Javitch, V. V. Gurevich, N. A. Lambert, Using Bioluminescence Resonance Energy Transfer (BRET) to Characterize Agonist-Induced Arrestin Recruitment to Modified and Unmodified G Protein-Coupled Receptors. *Curr Protoc Pharmacol* **70**, 2 14 11-12 14 14 (2015).
70. T. Loos, A. Mortier, P. Proost, Chapter 1. Isolation, identification, and production of posttranslationally modified chemokines. *Methods Enzymol* **461**, 3-29 (2009).
71. P. E. Brandish, L. A. Hill, W. Zheng, E. M. Scolnick, Scintillation proximity assay of inositol phosphates in cell extracts: high-throughput measurement of G-protein-coupled receptor activation. *Anal Biochem* **313**, 311-318 (2003).
72. S. Thiele *et al.*, Modulation in selectivity and allosteric properties of small-molecule ligands for CC-chemokine receptors. *J Med Chem* **55**, 8164-8177 (2012).
73. Schrodinger, LLC (2015) The PyMOL Molecular Graphics System, Version 1.8.
74. H. Nomiyama *et al.*, Human CC chemokine liver-expressed chemokine/CCL16 is a functional ligand for CCR1, CCR2 and CCR5, and constitutively expressed by hepatocytes. *Int Immunol* **13**, 1021-1029 (2001).

Manuscript 1 commentary

In the [5P7]CCL5-CCR5 structure solved by Zheng et al., a tilting of the core domain of [5P7]CCL5 was observed that leads to numerous interactions with the receptor extracellular loops from above[41]. This was not observed in other solved chemokine:receptor structures, establishing that large discreet interaction interfaces are employed by different groups within the chemokine family. As ECL2 is widely connected to ligand interactions within the GPCR family, the extensive contact surface between [5P7]CCL5 and ECL2 of CCR5 is a candidate for a chemokine:receptor interface engaged differently by various chemokine subtypes[126]. However, we did not regard the section of ECL2 closest to the 7TM-conserved disulfide bridge as the prime location for chemokine-selective interactions, as it has been consistently and closely involved in diverse chemokine interactions. For example, the vMIP2-CXCR4 X-ray structure employed an engineered disulfide bridge at position Cys+1 to tether vMIP2 to the receptor, and we have observed that the original aromatic residue there is indispensable to CXCL12-mediated activation (by Ala-mutation, not shown). The 7TM disulfide bridge itself, which borders the minor binding pocket, is also indispensable in other chemokine receptors[127-129]. This is in proximity to what is dubbed CRS1.5 – the region between CRS1 and CRS2 that is employed by all chemokine:receptor complexes yet solved – which marks the entry point that chemokine N-termini must pass to reach the receptor binding pocket.

Differential use of the minor and major receptor pocket

Across the receptor from CRS1.5 and the minor binding pocket, the beginning and end of ECL2 originates from the top of TM4 and TM5. It covers the major receptor binding pocket, and there are only reports that a few CC-chemokine receptors rely on this region, hence it would make sense that only a select subset of chemokines include it in their interaction interfaces[130]. The location of the aromatic cluster at the top of the transmembrane helices has not been defined clearly as a part of CRS1 or CRS2[29], which meant that we could not predict whether the role of the cluster would relate to chemokine binding or only to signaling. We found that this definition would have to be made for each receptor, as we observed for CCR1 and CCR5 (manuscript 1 figure S4).

While it was found that the aromatic Cys+4 residue was indispensable in only a subset of chemokine receptors, we did not exclude that beyond CCR1, -2 and -5, the region may also be involved in chemokine-selective interfaces of other receptors. Furthermore, it remains possible that some residues of the aromatic cluster could also be important to distinguish MIP chemokines from the eotaxins (CCL11, -24 and -26), which are primarily CCR3 chemokines, but display overlapping interactions with MIP and MCP chemokines on CCR2, CCR3 and CCR5 (see figure 5). This could indeed be the case, as it was found long ago that CCL3 and CCL11 must use distinct activation mechanisms on CCR1 and CCR3, respectively[28].

We previously investigated the role of aromatic residues at other positions along ECL2 in other chemokine receptors, part of these data were produced already during my master's project. In that process, we found that the CXC-chemokine receptors – where an aromatic Cys+4 residue was never needed – would sometimes be sensitive to alanine mutation of aromatic residues on ECL2 over the minor binding pocket. Specifically, an aromatic Cys+1 residue was required for chemokine signaling

and binding to CXCR1 and CXCR2, and an aromatic Cys+2 residue was required for chemokine signaling through CXCR3 and CXCR5. Aromatic residues at the Cys+1/2 positions of ECL2 would not be able to interact in an aromatic cluster with TM4 and TM5, but it is possible that their significance could also stem from a different binding mode of cognate chemokines. In both the vMIP2-CXCR4 and CXCL8-CXCR2 complexes, the core domain does not associate closely with the section of ECL2 over the major binding pocket, nor does the N-terminus move into the major binding pocket. Instead, the chemokine N-terminus and N-loop make many contact points within and near the minor binding pocket and ECL2 Cys+1/2[30, 40]. In a different example, the cryo-EM structure of the CCL20-CCR6 complex represents an interaction where the chemokine core domain moves close to the top of the major binding pocket, and the 30s-loop is positioned very close to the ECL2 Cys+4 residue[39]. However, in contrast to MIP chemokines, the CCL20 N-terminus consists of only five amino acids that bind in the receptor minor binding pocket, and the first three residues are fully dispensable[131]. As for the role of the receptor aromatic cluster, we found that only the Cys+4 residue is important, whereas the underlying TM4.63 and TM5.34 aromatic residues were dispensable (figure 7). This is in line with our findings for the MIP interactions (manuscript 1, figure 6), where we noted that chemokine N-terminal truncations could change the relative importance of the residues of the aromatic cluster.

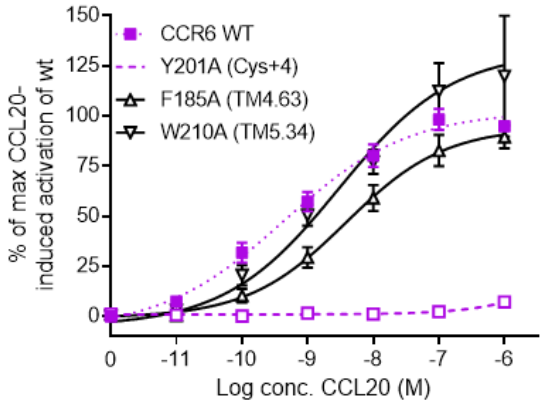


Figure 7 Mutational analysis of the aromatic cluster in CCR6, showing that only the Cys+4 residue is important to CCL20 signaling (IP accumulation).

A study on MCP chemokines with chimeric N-termini interacting with CCR2 concluded that the N-terminus of MCP chemokines likely moves closer to TM6, TM7 and ECL3, which could in part explain how the aromatic cluster of ECL2 has less significance in these interactions[132]. Comparing MIP and MCP N-terminal characteristics also makes sense in the context of receptor desensitization, for example for CCL5 3-68, which is functionally similar to CCL5 4-68[80, 81]. This CCL5 truncation variant does not induce THP-1 chemotaxis, but can still desensitize the cells to intact CCL3, CCL4 and CCL5[36]. CCL7, which is the MCP outlier that is sensitive to the Cys+4 mutation on CCR1 (in arrestin recruitment) is also affected by CCL5 3-68 desensitization, but this is not the case for CCL8.

The 30s-loop could help define MIP/MCP binding modes

In the [5P7]CCL5:CCR5 structure, the 30s-loop enters the major binding pocket to the point just above the N-terminus, with the backbones of the TSS motif being particularly exposed in the major binding pocket. CCL2, CCL7 and CCL8, with an RRI motif preceding the 30s-loop, are consistently less sensitive to mutations in the aromatic cluster, regardless of the paired receptor, relative to MIP chemokines with the shorter FE motif. If the N-termini of MCP chemokines truly moves to the opposite face of the major binding pocket, as predicted by Stone and colleagues[132], space may be available for a slightly longer MCP 30s-loop to contact the deeper binding pocket. This would not be entirely unprecedented, as the V3 loop of the viral envelope protein GP120 enters the CCR5 binding pocket in an angle consistent with the [5P7]CCL5 30s-loop, and overlaps with the N-terminal position of this chemokine variant[109]. Interestingly, this notion would imply that the cognate receptors can accommodate distinct MIP or MCP interactions, leaving it to the chemokines to specify a receptor interaction mode.

The MCP CCL13 does not match the RRI motif, and exhibits MIP-like ECL2-dependency on CCR1 and CCR5, and MCP-like dependency on CCR2. On one hand, this underscores the influence of the 30s-loop among other MCP members, but at the same time, it demonstrates that the receptors are, after all, also directing the chemokine:receptor interaction interface toward a preferred interaction mode, if the chemokine is sufficiently versatile. It is certainly clear that CCR1 and CCR5, which predominantly recognize MIP chemokines, share a number of traits around the aromatic cluster that are not found in CCR2. For example, at position Cys+1 and Cys+3 of CCR1 and CCR5, Ser and His side chains point toward the chemokine core domain, and the segment between Cys+4 and W5.34 is a 7-residue loop, compared to 3 residues in CCR2.

Manuscript 2:

Selective allosteric modulation of N-terminally cleaved, but not full length CCL3 in CCR1

Authors:

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Published in ACS Pharmacology & Translational Science November 2019

Introducing remarks:

The focus of manuscript 2 is on the role of the MIP chemokine extreme N-terminus on signaling and binding, and how this could be used to our advantage therapeutically, which in manuscript 1 was only introduced as a contributing aspect of overall chemokine-receptor interactions. Among MIP chemokines, the nature of the connection to the aromatic ECL2 cluster was not definitively established, though we did suggest that the cluster could play a role in the steric hindrance or access to the major receptor binding pocket (manuscript 1 figure 4). In manuscript 2, we find that many interactions exist between residues 1-4 of CCL3 and the deeper receptor binding pocket of CCR1. Interestingly, removing these residues increased the potency of CCL3, demonstrating that these amino acids provide a natural mechanism with a negative effect on signaling.

Perhaps most importantly, we show here that it is possible to develop small molecules interfering within the major binding pocket in a chemokine-specific manner to disturb the relative receptor affinity of individual MIP chemokines. The small molecule zinc chelators bipyridine and phenanthroline bind in the same binding pocket region as residues 1-4 of CCL3, meaning that they can only bind allosterically when the chemokine N-terminus has been truncated. We know that this truncation variant has been found in certain human tissues, but many more MIP and MCP chemokine truncation variants can be found in various other tissues[36, 133-135]. In fact, we could not find any published evidence of full length CCL3 detected in human tissue.

Interaction studies of chemokines and their receptors are increasingly recognizing and emphasizing how specific, or biased, chemokine:receptor interactions and their signaling patterns can affect distinct biological outcomes. It is also appreciated that chemokine specificity must be characterized on a molecular level in order to influence the many pathologies where chemokines are implicated[105, 136]. Here, by showing that it is possible to design small molecule drugs that display allosteric modulation to only a subtype of very similar chemokines on CCR1, giving this chemokine subtype a strong binding advantage relative to other cognate chemokines, we also present a strategy that could be used to control some aspects of biased signaling.

ACS Pharmacology & Translational Science

Article

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Selective Allosteric Modulation of N-Terminally Cleaved, but Not Full Length CCL3 in CCR1

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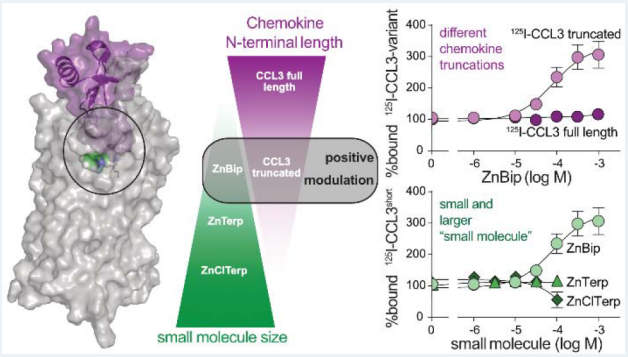
ABSTRACT: Chemokines undergo post-translational modification such as N-terminal truncations. Here, we describe how N-terminal truncation of full length CCL3$^{(1-70)}$ affects its activity at CCR1. Truncated CCL3$^{(5-70)}$ has 10-fold higher potency and enhanced efficacy in β-arrestin recruitment, but less than 2-fold increased potencies in G protein signaling determined by calcium release, cAMP and IP_3 formation. Small positive ago-allosteric ligands modulate the two CCL3 variants differently as the metal ion chelator bipyridine in complex with zinc (ZnBip) enhances the binding of truncated, but not full length CCL3, while a size-increase of the chelator to a chloro-substituted terpyridine (ZnClTerp), eliminates its allosteric, but not agonistic action. By employing a series of receptor mutants and *in silico* modeling we describe residues of importance for chemokine and small molecule binding. Notably, the chemokine receptor-conserved Glu287$^{7.39}$ interacts with the N-terminal amine of truncated CCL3$^{(5-70)}$ and with Zn^{2+} of ZnBip, thereby bridging their binding sites and enabling the positive allosteric effect. Our study emphasizes that small allosteric molecules may act differently toward chemokine variants and thus selectively modulate interactions of specific chemokine subsets with their cognate receptors.



KEYWORDS: *chemokine system, chemokine truncation, allosteric modulation, 7-transmembrane helix receptor, G protein-coupled receptor (GPCR), C−C chemokine receptor 1 (CCR1), metal ion chelator complexes*

INTRODUCTION

Chemokine receptors belong to the class A G protein-coupled receptors (GPCRs) and their ligands, the chemokines, are peptides of 8−12 kDa. Most chemokines contain four conserved cysteines, which form disulfide bridges that are name-giving for their subgrouping (CC-, CXC-, CX_3C-, and XC-group).[1] The CC/CXC-motif moreover separates the rigid chemokine core structure from its more flexible N-terminus.[2] In broad terms, these two regions have different roles in receptor interaction as the core domain provides the major share of receptor specificity and -affinity to extracellular receptor domains, while the N-terminus contributes more to receptor activation by interacting with transmembrane receptor residues. The effect of N-terminal modifications on activity further underpins the role of the chemokine N-terminus in receptor activation.[3,4] Most prominently, AOP-CCL5 acts as a potent CCR5 antagonist in Ca^{2+}-signaling and chemotaxis in monocytes, but is also more potent than wild type CCL5 at internalizing and desensitizing CCR5, thereby explaining its strong anti-HIV activity.[5,6] Many other N-terminal modifications, such as single amino acid additions,[7] chemical modifications,[8−10] or sequence alterations of the entire chemokine N-terminus[11−15] have been investigated, and emphasized the role of the N-terminus in the receptor-activating step.

Most endogenous nonpeptide ligands for class A GPCRs bind solely in the transmembrane receptor pocket.[16,17] Likewise, most synthetic small molecules for chemokine receptors possess a positive charge in their structure that interacts with a chemokine receptor-conserved glutamate (Glu) in transmembrane (TM) domain 7 (position 7.39

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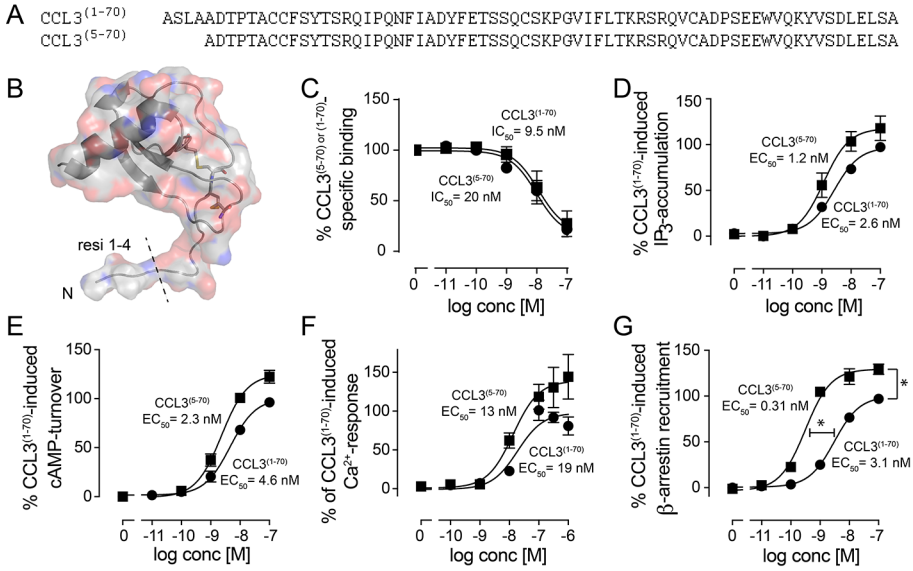

Figure 1. N-terminal truncation $CCL3^{(5-70)}$ of $CCL3^{(1-70)}$ binds with unaltered affinity, but higher potency and efficacy in arrestin recruitment. (A) Amino acid sequence of $CCL3^{(1-70)}$ and the truncated $CCL3^{(5-70)}$. (B) Structure of $CCL3^{(1-70)}$ (PDB: 2X69, manually added residues 1−3) and indication of the first four amino acids that will be cleaved off in $CCL3^{(5-70)}$. (C) Homologous binding curves with ^{125}I-$CCL3^{(1-70)}$ (squares) or ^{125}I-$CCL3^{(5-70)}$ (circles) against respective cold ligand, normalized to the specific binding of each tracer as 100% and binding in mock-transfected cells as 0% (nonspecific binding). (D−G) Dose−response curves of $CCL3^{(1-70)}$ (circles) and $CCL3^{(5-70)}$ (squares) in different *in vitro* assays: IP_3-accumulation (D), cAMP-generation (E), Ca^{2+}-release (F), and β-arrestin recruitment (G). All dose−response curves are normalized to the response obtained for $CCL3^{(1-70)}$ (100%) and background signaling (0%). The homologous binding curves are normalized to the specific binding of tracer (100%) and nonspecific binding on mock-transfected cells (0%). The values of all IC_{50} and EC_{50}-values are given. * $p \leq 0.0001$ (unpaired *t* test), $n \geq 3$.

according to the Ballesteros/Weinstein nomenclature[18]). This Glu is located centrally in the transmembrane binding pocket where most chemokine N-termini are projected to interact.[19,20] Since the binding site of small molecules is most often located in the transmembrane pocket, the molecules' effect on the chemokine is considered allosteric. However, recent crystal structures of chemokine receptors in complex with chemokine ligands, such as US28 with CX_3CL1,[21] or CCR5 with an antagonistic CCL5-derivative,[22] showed that the N-terminus of the chemokines reaches deeply into the transmembrane receptor domain. This raises the question of whether small molecules in the transmembrane binding pocket are solely allosteric or partially overlapping with the chemokines.

The chemokine system mediates leukocyte migration, and is central to homeostatic and inflammatory functions of the immune system. It also plays a role in hematopoiesis, angiogenesis, lymphoid organogenesis, and HIV-infection, and aberrant regulation of the chemokine system can result in allergic, inflammatory, or autoimmune diseases, or may promote cancer metastasis and tumor angiogenesis.[1,23] It has, therefore, been intensively studied in terms of its druggability. Numerous ligands with good *in vitro* antagonistic effects have been identified, but only three compounds have reached the market: the CXCR4 antagonist Plerixafor, the CCR5 antagonist Maraviroc, and Mogamulizumab, targeting CCR4 (only approved in Japan).[1] To fully exploit the pharmaceutical potential of chemokine receptors as drug targets, a better understanding of the complexity of the chemokine system is needed.

The chemokine receptor CCR1 binds several chemokines with CCL3 and CCL5 being most potent in addition to eight other CC-chemokines (CCL4, 7, 8, 13, 14, 15, 16, and 23) with lower affinity.[1] CCR1 has been investigated as a target for the treatment of multiple sclerosis, rheumatoid arthritis, and chronic obstructive pulmonary disease. Compounds such as BX471 (Schering AG),[24] MLN 3897 (Millenium),[25] CP-481,715 (Pfizer),[26] AZD4818 (Astra Zeneca),[27] C-4462 and C-6448 (Merck)[28] have been subject to clinical trials, but failed due to lack of *in vivo* efficacy.[1] The reasons could be insufficient receptor coverage,[29] or the need for chemokine neutralization rather than receptor inactivation, as suggested by the observation that CCL3 neutralization is completely protective in a mouse model for multiple sclerosis.[30]

CCL3 is available in various forms: the commercially available long variant $CCL3^{(1-70)}$, in addition to $CCL3^{(2-70)}$, and N-terminally truncated $CCL3^{(5-70)}$, numbered 23−92, 24−92, and 27−92, respectively, if the signal peptide is counted (Figure 1A,B). A specific role for the truncated variant in physiology is supported by the finding of $CCL3^{(5-70)}$ in ascites of patients with ovarian cancer.[31] We have previously described the pharmacological properties of a class of small molecule positive allosteric modulators consisting of complexes between a metal ion (Zn^{2+} or Cu^{2+}) and the chelators 2,2′-bipyridine, 1,10-phenanthroline, or 2,2′:6′,2′′-terpyridine (and their derivatives) in chemokine receptors. ZnBip and ZnPhe are low-potent agonists at a subset of chemokine receptors, and act as allosteric enhancers of CCL3-binding to CCR1 and CCR5.[32−36] Here, we investigate the impact of N-terminal CCL3 truncation for binding and activation of CCR1 and for the positive allosteric modulation of CCL3 in CCR1.

RESULTS

Truncation of $CCL3^{(1-70)}$ to $CCL3^{(5-70)}$ Results in More Potent and Efficacious Activation of CCR1. First, we determined the activity of $CCL3^{(1-70)}$ and $CCL3^{(5-70)}$ (Figure 1A,B) in a range of intracellular signal transduction assays: IP_3-

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accumulation (Figure 1D), cAMP-generation (Figure 1E), Ca^{2+}-signaling (Figure 1F), and β-arrestin recruitment (Figure 1G). In all G protein-mediated pathways (Figure 1D−F), $CCL3^{(5-70)}$ appeared to have slightly improved activity compared to $CCL3^{(1-70)}$ although this difference did not reach significance. However, with regard to β-arrestin recruitment, $CCL3^{(5-70)}$ was significantly improved with 10-fold higher potency and 130% of maximal efficacy compared to $CCL3^{(1-70)}$. We moreover investigated the binding properties of both CCL3-variants in a homologous competition binding setting with iodination of both variants. Using this approach, we observed no significant difference in the binding affinities with log K_d −8.0 ± 0.22 (n = 8) and −7.7 ± 0.31 (n = 6), for $CCL3^{(1-70)}$ and $CCL3^{(5-70)}$, respectively (Figure 1C). Similar high affinities were obtained in the heterologous settings with log IC50 values of −8.2 ± 0.45 (n = 3) for $CCL3^{(5-70)}$ in competition with ^{125}I-$CCL3^{(1-70)}$ and −8.0 ± 0.04 (n = 37) for $CCL3^{(5-70)}$ in competition with ^{125}I-$CCL3^{(1-70)}$, confirming an overall similar binding site of the two CCL3 forms.

Allosteric Modulation of CCR1 Depends on the Length of the Chemokine N-Terminus. As most small molecule ligands for chemokine receptors bind to the transmembrane pocket,[19,20] we explored whether the length of the chemokine N-terminus, which interacts with this receptor area,[3,4] influenced the action of allosteric modulators on CCR1. We used the previously described allosteric modulators,[32,33] Zn^{2+} complexed with the metal ion chelators 2,2′-bipyridine (ZnBip) or 1,10-phenanthroline (ZnPhe) (Figure 2A). ZnBip and ZnPhe are ago-allosteric modulators as agonists and positive allosteric modulators of $CCL3^{(5-70)}$ binding to CCR1.[32] In contrast to their ability to enhance the binding of $CCL3^{(5-70)}$, ZnBip and ZnPhe did not enhance the specific binding of the longer variant ^{125}I-$CCL3^{(1-70)}$ (Figure 2B,C). This suggests that the truncation of CCL3 allows positive allosteric effects of the small molecules ZnBip and ZnPhe, whereas the extended variant, $CCL3^{(1-70)}$, prevents this action.

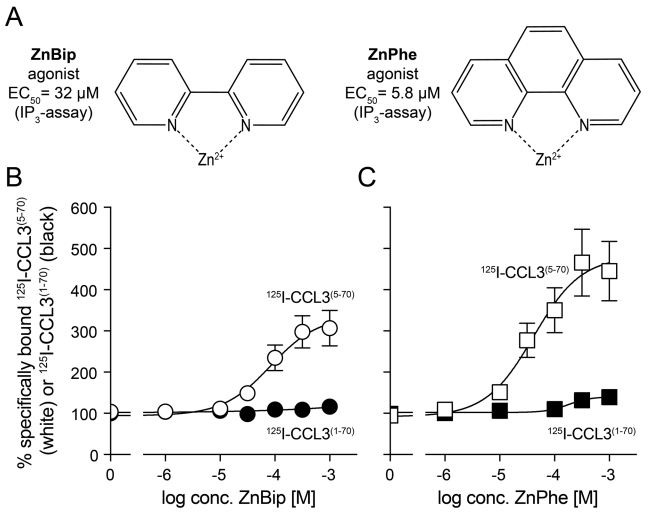

Figure 2. Truncation of the CCL3 N-terminus enables positive allosteric modulation by ZnBip and ZnPhe in CCR1. (A) The structure of ZnBip and ZnPhe with indication of their potency in IP_3-assays as determined previously.[34] (B,C) Heterologous competition binding assays with ^{125}I-$CCL3^{(5-70)}$ (white symbols) or ^{125}I-$CCL3^{(1-70)}$ (black symbols) in competition with ZnBip (circles, B) or ZnPhe (squares, C). Data are normalized to the specific binding of each tracer as 100% and nonspecific binding in mock-transfected cells as 0%. All experiments were repeated at least 3 times ($n \geq 3$).

The Allosteric Enhancement of $CCL3^{(5-70)}$ is Lost upon Increased Size of the Metal Ion Chelators. Considering that the long N-terminus in $CCL3^{1-70}$ excludes the positive allosteric modulation by ZnBip and ZnPhe (Figure 2), we speculated that an increase in the size of small molecule ligands could prevent the allosteric effect. We therefore included two additional metal ion chelator complexes, ZnTerp and ZnClTerp with a third pyridine ring (Figure 3A),

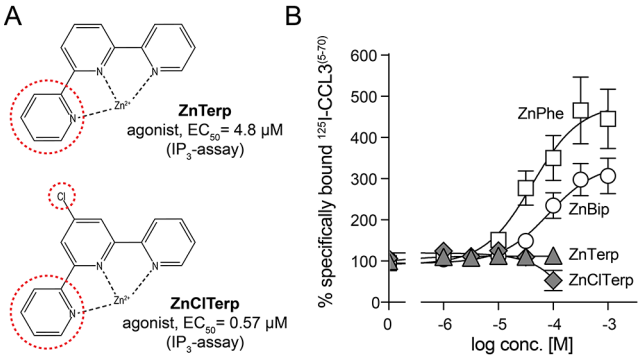

Figure 3. Larger chelator structures abrogates the positive allosteric modulation of $CCL3^{(5-70)}$. (A) The structure of ZnTerp with the change to the ZnBip structure circled in red and ZnClTerp with the added chlorine indicated, and indication of their agonistic potency in IP_3-assays, as determined previously.[34] (B) Heterologous competition binding assays with ^{125}I-$CCL3^{(5-70)}$ in competition with ZnTerp (gray triangles), ZnClTerp (gray diamonds), ZnBip (white circles), or ZnPhe (white squares). Notably, the positive allosteric enhancement that was found for ZnBip and ZnPhe is not observed for ZnTerp or ZnClTerp. Graphs are normalized to the specific binding of the tracer as 100% and its nonspecific binding in mock-transfected cells as 0%. $n \geq 3$.

compared to the smaller ZnBip with two pyridine rings (Figure 2A). These compounds were previously described as 6.7-fold and 56-fold more potent than ZnBip on CCR1.[34] Neither ZnTerp nor ZnClTerp were able to enhance the binding of ^{125}I-$CCL3^{(5-70)}$. In fact, ZnClTerp competed with ^{125}I-$CCL3^{(5-70)}$ with a 50%-displacement at 10 mM (Figure 3B). This suggests that the positive allosteric modulation between the small molecule ligands and CCL3 in CCR1 requires a short chemokine N-terminus and a small chelator size (Figure 2).

Computational Model of the Binding Site of ZnBip and ZnClTerp in CCR1. To understand the differences in agonism and allosteric action of ZnBip and ZnClTerp, we docked both into a model of CCR1 (Figure 4A,B), which was based on the X-ray crystal structure of CCR5 in complex with [5P7]CCL5 (PDB: 5UIW). As previously described, the chemokine receptor-conserved Glu287$^{7.39}$ was involved in the coordination of Zn^{2+} in both metal ion chelator complexes.[32] Zn^{2+} is preferentially coordinated in a tetrahedral manner[37] and, for ZnBip, the other three coordinating partners are the two nitrogens of the bipyridine (Bip), and the hydroxyl-group of Tyr113$^{3.32}$. In the chloro-substituted terpyridine ring (ZnClTerp), all three nitrogens contribute to the coordination of Zn^{2+}. Although this does not display a tetrahedral geometry, we observed that all three nitrogens are indeed required for receptor activation of this ligand. Removal or repositioning of the nitrogen in one outer ring of terpyridine abolishes the

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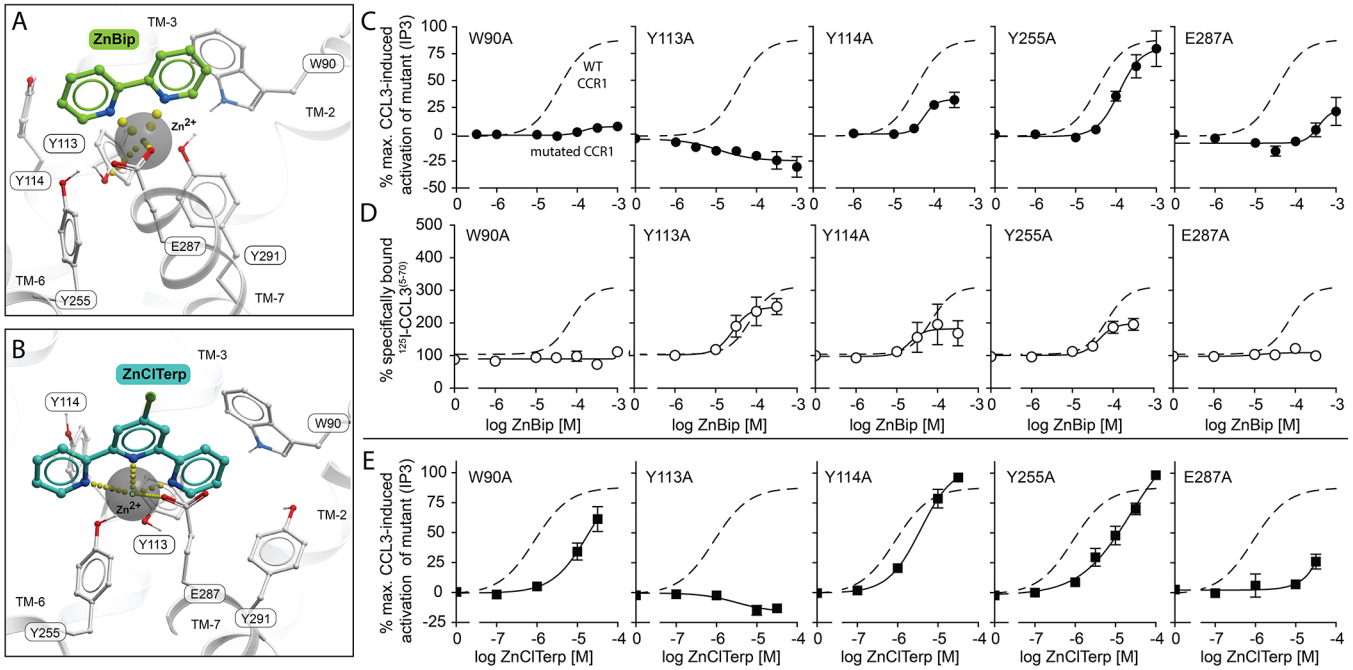

Figure 4. ZnBip and ZnPhe bind to Glu287$^{7.39}$ and aromatic residues in the transmembrane domain of CCR1. (A) Computational model of ZnBip (green) and (B) of ZnClTerp (cyan) bound to CCR1. Receptor residues near the ligand are represented as sticks, helices are indicated, and the ion Zn^{2+} is shown as a gray sphere. Projected interactions are indicated by yellow dotted lines. (C,D) IP_3 activity on key CCR1 mutants by ZnBip relative to wild type (dashed line, C), and positive allosteric binding modulation of CCL3$^{(5-70)}$ to the same residues (D). (E) Dissimilar IP_3 dose–response curves of the same mutations activated with ZnClTerp. IP_3 graphs are normalized to full CCL3$^{(1-70)}$ activation as 100% and basal CCR1 activity as 0%. Binding curves are normalized to the specific binding of the radioligand, $n \geq 3$.

activity of ZnClTerp (Figure 5), which is in support of a central role of all three nitrogens for ligand–receptor interaction. Furthermore, Trp90$^{2.60}$ makes edge-to-face aromatic interactions with the bipyridine of ZnBip. No other close interactions with the bipyridine were observed (Figure 4A). Like bipyridine, the chloro-terpyridine chelator interacts with Trp90$^{2.60}$ and additionally with Tyr114$^{3.33}$ and Tyr255$^{6.51}$ (Figure 4B).

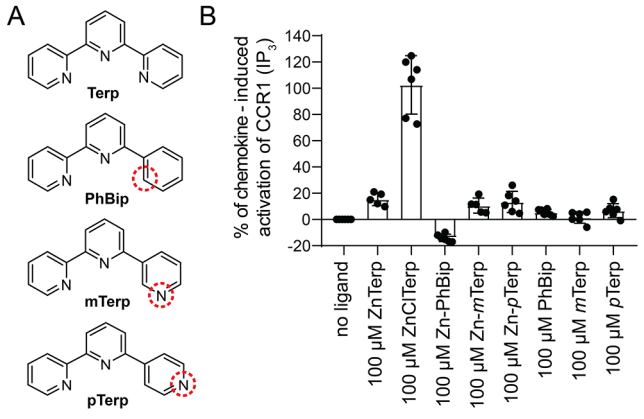

Figure 5. Role of the nitrogen in the third pyridyl-ring of ZnTerp. (A) Structure of Terp and Terp-analogues, which either lack the third nitrogen (PhBip), or in which this nitrogen is placed in a position that is unfavorable for zinc-complex formation (mTerp, pTerp). (B) Column diagram presenting the efficacy of these analogues alone or in complex with Zinc in comparison to the efficacy of ZnTerp, ZnClTerp, or no ligand. All compounds were given at 100 μM and the IP_3-accumulation of CCR1-expressing cells was tested. Data represent mean ± SEM of at least three repetitions and are normalized to no ligand as 0% and the efficacy of CCL3$^{(1-70)}$ as 100%.

The Effect of CCR1 Receptor Mutagenesis on the Activity of CCL3, ZnBip, and ZnClTerp. To verify the suggested binding poses of ZnBip and ZnClTerp in CCR1 (Figure 4), we prepared 27 mutants within the transmembrane pocket of CCR1, and tested whether they were activated by CCL3$^{(1-70)}$, ZnBip, and ZnClTerp in the IP_3-assay (Table 1). These residues were selected based on our modeling and previous work, where they were predicted to be available for chemokine binding in CCR1 (Leu87$^{2.57}$, Ser110$^{3.29}$, Gly207$^{5.46}$, and Glu287$^{7.39}$) or aligned well to similar small molecule binding in CCR1, CCR5, and CCR8 (Trp90$^{2.60}$, Lys94$^{2.64}$, Phe112$^{3.31}$, Tyr113$^{3.32}$, Tyr114$^{3.33}$, Trp195$^{5.34}$, Lys196$^{5.35}$, Phe206$^{5.45}$, Tyr255$^{6.51}$, and Tyr291$^{7.43}$).[32,33,38−42] In support of the surface expression and general functionality of the mutant library, we observed that CCL3$^{(1-70)}$ activated all mutants (Table 1). This also highlights that single point mutations within the transmembrane area of CCR1 do not significantly affect the activity of CCL3$^{(1-70)}$. The predicted interaction with Tyr113$^{3.32}$ was supported by the functional test of Y113A$^{3.32}$, at which ZnBip changed from agonist to inverse agonist (Figure 4C). Furthermore, Y113F$^{3.32}$ led to a 31-fold reduced (agonistic) potency, and E287A$^{7.39}$ abolished activity (Table 1). This supports the model in which Glu287$^{7.39}$ and the OH-group of Tyr113$^{3.32}$ coordinate zinc (Figure 4A). The W90A$^{2.60}$ mutation had a severe effect on the activity of ZnBip, despite the fact that virtual mutation of Trp90$^{2.60}$ to alanine only resulted in an increase of the ICM_VLS docking score for ZnBip from 8.8 to 10.3 (for comparison, mutating Glu287$^{7.39}$ to alanine increases the score from 8.8 to 21.2). Like ZnBip, ZnClTerp turned into an inverse agonist at Y113A$^{3.32}$ (Figure 4E), but had only 6.8-fold

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Table 1. Activity of CCL3$^{(1-70)}$, ZnBip and ZnClTerp at CCR1 and Mutant Receptors Measured in the IP$_3$-Assay

			CCL3$^{(1-70)}$					ZnBip					ZnClTerp				
			EC$_{50}$[a]					EC$_{50}$					EC$_{50}$				
		location	log ± SEM	(nM)	F_{mut}[b]	(n)[c]		log ± SEM	(μM)	F_{mut}	(n)		log ± SEM	(μM)	F_{mut}	(n)	
TM-2	WT		−8.42 ± 0.03	3.8	1.0	(118)		−4.49 ± 0.04	32	1.0	(52)		−6.11 ± 0.09	0.78	1.0	(12)	
	F83A	2.53/II:13[d]	−8.37 ± 0.17	4.2	1.1	(6)		−4.70 ± 0.11	20	0.62	(3)		−6.43 ± 0.11	0.37	0.48	(3)	
	L87F	2.57/II:17	−8.66 ± 0.14	2.2	0.57	(4)		−4.02 ± 0.09	95	2.9	(4)		n.d.				
	W90A	2.60/II:20	−8.67 ± 0.08	2.1	0.56	(9)		−3.93 ± 0.06	119	**3.7**	(5)		−4.68 ± 0.15	21	**27**	(3)	*[f]
	K94A	2.64/II:24	−8.75 ± 0.12	1.8	0.47	(8)		−5.01 ± 0.07	9.7	0.30	(6)		n.d.				
	K94L	2.64/II:24	−9.03 ± 0.03	0.9	0.25	(4)	*	−5.04 ± 0.03	9.1	0.28	(4)		n.d.				
TM-3	K107A	3.26/III:02	−7.86 ± 0.05	14	**3.6**	(5)	*	−4.18 ± 0.10	66	2.0	(5)		n.d.				
	L109A	3.28/III:04	−7.98 ± 0.07	10	2.7	(5)		−4.02 ± 0.08	97	3.0	(3)		−5.64 ± 0.09	2.3	2.9	(3)	
	S110A	3.29/III:05	−8.73 ± 0.10	1.8	0.49	(5)		−4.53 ± 0.08	29	0.91	(5)		n.d.				
	F112A	3.31/III:07	−8.47 ± 0.15	3.4	0.89	(7)		−4.96 ± 0.16	11	0.34	(4)		n.d.				
	Y113A[e]	3.32/III:08	−8.69 ± 0.09	2.1	0.54	(28)	*	-5.33 ± 0.19	4.7	0.15	(9)	*	-5.59 ± 0.26	2.6	3.3	(3)	*
	Y113F	3.32/III:08	−7.90 ± 0.06	13	**3.3**	(5)	*	>−3		**31**	(3)	*	−5.28 ± 0.18	5.3	**6.8**	(3)	*
	Y114A	3.33/III:09	−8.84 ± 0.16	1.4	0.38	(11)	*	−4.30 ± 0.10	50	1.5	(3)		−5.38 ± 0.15	4.1	**5.3**	(3)	*
	Y114F	3.33/III:09	−8.14 ± 0.09	7.2	1.9	(7)		−3.94 ± 0.08	114	**3.5**	(6)	*	−5.85 ± 0.07	1.4	1.8	(5)	
	L117A	3.36/III:12	−8.22 ± 0.07	6.0	1.6	(5)		−3.96 ± 0.10	109	**3.4**	(4)		−5.82 ± 0.10	1.5	2.0	(4)	
TM-5	W195A	5.31/V:-04	−7.56 ± 0.20	27	**7.2**	(4)	*	−3.72 ± 0.13	191	**5.9**	(3)	*	n.d.				
	K196A	5.32/V:-03	−7.98 ± 0.02	11	2.8	(6)		−4.85 ± 0.17	14	0.43	(4)		n.d.				
	F206A	5.42/V:08	−8.43 ± 0.16	3.7	0.97	(7)		−4.76 ± 0.22	17	0.54	(4)		n.d.				
	G207A	5.43/V:09	−8.29 ± 0.13	5.1	1.3	(8)		−4.62 ± 0.14	24	0.73	(5)		n.d.				
	G207F	5.43/V:09	−8.57 ± 0.18	2.7	0.71	(6)		−4.57 ± 0.24	27	0.84	(4)		n.d.				
TM-6	Y255A	6.51/VI:16	−8.46 ± 0.10	3.4	0.90	(20)		−3.91 ± 0.08	124	**3.8**	(10)	*	−5.05 ± 0.17	9.0	**12**	(5)	*
	Y255F	6.51/VI:16	−8.45 ± 0.20	3.5	0.92	(5)		−4.99 ± 0.12	10	0.31	(4)		−6.82 ± 0.04	0.15	0.20	(3)	*
TM-7	D280A	7.32/VII:-02	−8.30 ± 0.16	5.0	1.3	(3)		−4.15 ± 0.08	71	2.2	(4)		−5.93 ± 0.14	1.2	1.5	(3)	
	L281A	7.33/VII:-01	−8.05 ± 0.11	9.0	2.4	(3)		−4.76 ± 0.09	17	0.54	(3)		n.d.				
	E287A	7.39/VII:06	−8.29 ± 0.12	5.1	1.4	(12)		>−3		**31**	(9)	*	−3.85 ± 0.07	142	**183**	(5)	*
	A290S	7.42/VII:09	−8.45 ± 0.07	3.5	0.93	(5)		−4.34 ± 0.10	45	1.4	(4)		n.d.				
	Y291A	7.43/VII:10	−8.85 ± 0.14	1.4	0.37	(9)	*	−4.76 ± 0.08	17	0.53	(5)		n.d.				
	Y291F	7.43/VII:10	−8.54 ± 0.10	2.9	0.76	(3)		−4.61 ± 0.03	24	0.75	(3)		n.d.				

[a]EC$_{50}$ values are given in log and nM/μM. [b]F_{mut} is the factor presenting the fold-decrease of EC$_{50}$ for the mutant compared to WT CCR1. F_{mut} changes greater than a 3-fold decrease are highlighted in bold. [c](n) is the number of experiments. [d]Position of mutants according to the Ballesteros/Weinstein (left) and Baldwin/Schwartz (right) numbering system. [e]The ZnBip and ZnClTerp activity on this mutation refers to inverse agonism. [f]Statistics: one-way Anova of log ± SEM (n) of mutant compared to wt, $^{*}p \leq 0.05$.

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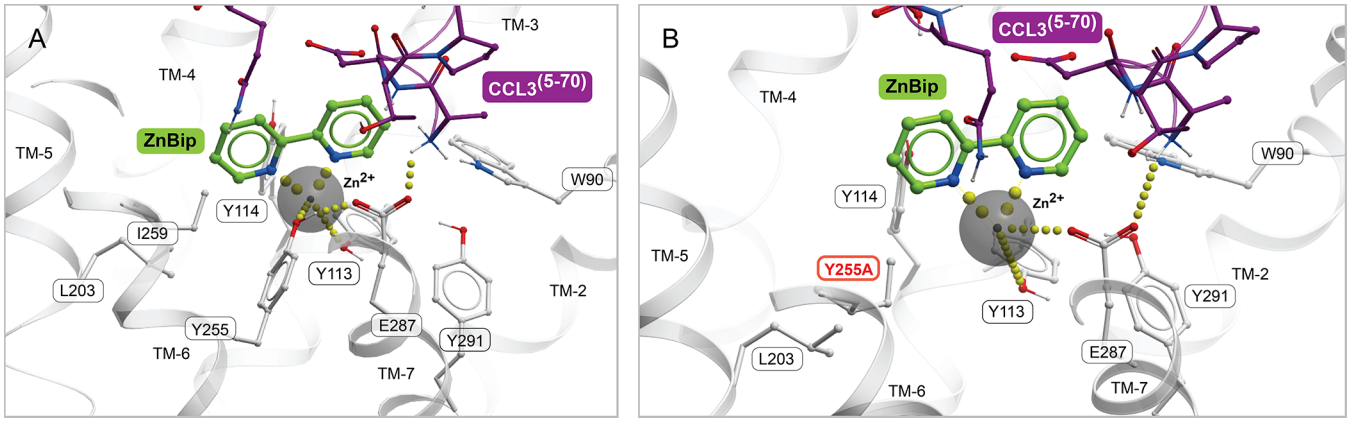

Figure 6. In the ternary complex model of ZnBip with CCL3$^{(5-70)}$ in CCR1, Glu287$^{7.39}$ interacts directly with the chemokine N-terminus and indirectly via Tyr255$^{6.51}$ with Zn^{2+} of ZnBip. (A) ZnBip bound to the CCR1-CCL3$^{(5-70)}$ complex; Zn^{2+} is coordinated by Tyr255$^{6.51}$, Tyr113$^{3.32}$, and Bip. Glu287$^{7.39}$ mediates interaction between ZnBip and CCL3$^{(5-70)}$. (B) ZnBip bound to the Y255A$^{6.51}$-CCR1-CCL3$^{(5-70)}$ complex. In the absence of Tyr255$^{6.51}$ Zn^{2+} is coordinated by Glu287$^{7.39}$, Tyr113$^{3.32}$, and Bip. Receptor residues near the ligand are represented as sticks, helices are indicated, and the ion Zn^{2+} is shown as a sphere. Bip is green; CCL3$^{(5-70)}$ is purple.

decreased potency at Y113F$^{3.32}$. In accordance with the model, this suggests that the OH-group of Tyr113$^{3.32}$ is not as central for ligand-interaction with ZnClTerp. Similarly to ZnBip, the activity of ZnClTerp was abolished by E287A$^{7.39}$, and in addition its potency decreased 27-fold by W90A$^{2.60}$, 12-fold by Y255A$^{6.51}$, and 5.3-fold by Y114A$^{3.33}$. These residues interacted with Zn^{2+} or the chelator in the model (Figure 4B). None of the remaining mutants impaired the activity of ZnClTerp. Altogether, this is in support of its suggested binding pose.

Computational Model of Concomitant Binding of ZnBip and CCL3$^{(5-70)}$ in CCR1. In order to understand the positive allosteric modulation of ZnBip for the binding of CCL3$^{(5-70)}$, we made a model of both ligands simultaneously bound to CCR1. For this purpose, we modeled CCL3$^{(5-70)}$ onto the chemokine coordinates from the crystal structure of 5P7-CCL5 with CCR5,[22] and proceeded with the receptor model and metal ion chelator complex docking as described above (Figure 6A). To our surprise, we found that in the presence of CCL3$^{(5-70)}$, there is no space for ZnBip to be coordinated by Glu287$^{7.39}$. Instead, Zn^{2+} is coordinated by Tyr255$^{6.51}$, Tyr113$^{3.32}$, and both nitrogens of bipyridine. However, the role of Glu287$^{7.39}$ is still central, as it coordinates the N-terminus of the chemokine on one side, and Tyr255$^{6.51}$ on the other. Thus, Glu287$^{7.39}$ bridges the chemokine to the metal ion chelator complex. In support of the lack of allosteric modulation of CCL3$^{(1-70)}$, it was not possible to create an energetically favorable model of CCL3$^{(1-70)}$ with the ago-allosteric modulator ZnBip (CCL3$^{(1-70)}$-ZnBip complex) or of the larger allosteric modulator with the shorter CCL3 (CCL3$^{(5-70)}$-ZnClTerp complex) in CCR1.

Receptor Residues Involved in the ZnBip-Mediated Positive Allosteric (PAM) Effect on CCL3$^{(5-70)}$. To validate the suggested binding mode of ZnBip and CCL3$^{(5-70)}$ in CCR1, we screened 11 selected mutants for their impact on the allosteric enhancement of ^{125}I- CCL3$^{(5-70)}$ binding by ZnBip (Table 2). As expected, Ala-substitution of Glu287$^{7.39}$ completely abrogated the ability of ZnBip to increase the binding of CCL3$^{(5-70)}$. The same was observed for W90A$^{2.60}$, possibly by creating space for the chemokine to move away from Glu287$^{7.39}$, thereby breaking the link to ZnBip. None of the other tested residues had any effect, including Y113A$^{3.32}$ and Y255A$^{6.51}$. To investigate why we did not observe an effect of Y255A$^{6.51}$ although it was shown to coordinate Zn^{2+}, we repeated the modeling in CCR1 Y255A$^{6.51}$ (Figure 6B), and found that in the absence of Tyr255$^{6.51}$, Glu287$^{7.39}$ directly coordinates Zn^{2+} and the N-terminus of CCL3$^{(5-70)}$ explaining why its mutation is tolerated. A similar redundancy mechanism is conceivable for the role of Tyr113$^{3.32}$, whose mutation to alanine likewise is tolerated in the allosteric binding assay. In Y113A$^{3.32}$, it is possible that the neighboring Tyr114$^{3.33}$ overtakes the coordination of Zn^{2+}.

DISCUSSION AND CONCLUSIONS

Effect of Chemokine Truncation on CCL3 Activity. The truncation of chemokines is often crucial for attenuation, neutralization, and termination of inflammatory responses and thus influences how immunological challenges are resolved. Chemokine truncation may occur at the N-terminal or C-terminal end and may have a multitude of consequences such as increasing or decreasing biological function, creation of antagonistic properties, complete inactivation, or change in receptor preference. Truncating proteases include CD13 and CD26, which exist in membrane-associated or soluble forms, for example, in plasma. CD13 cleaves N-terminal residues from, for example, CXCL5 and CXCL8 leading to an increased activity with moderate truncation, but loss of function after substantial truncation (reviewed in Vanheule et al.[43]). CD26 is a serine-protease that cleaves dipeptides from substrates with a (hydroxy)Pro or Ala in the penultimate position. Chemokine substrates for CD26 are, for example, CCL3L1, CCL4, and CCL5. Furthermore, matrix-metalloproteases (MMPs), stored in intracellular vesicles and released upon appropriate stimulus, cleave CCL2, 7, and 8, which results in retained receptor binding, but reduced Ca^{2+}-signaling and potentially antagonistic activities.[44−46] Here, we find that N-terminal truncation of CCL3 results in similar affinities and a tendency toward higher activity through G proteins. A similar activating effect was described for CD26-mediated truncation of CCL4, where increased Ca^{2+}-signaling properties via CCR1 and CCR2 were obtained.[47,48] In contrast CD26-mediated truncation of CCL5$^{(1-68)}$ to CCL5$^{(3-68)}$ impaired Ca^{2+}-signaling through CCR1 and CCR3, while improving it through CCR5.[49−51] Of note, we find that the truncation of CCL3 induces selectivity toward β-arrestin signaling with a 10-fold higher potency for

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Table 2. Competition Binding Assays of ^{125}I-CCL3$^{(5-70)}$ to CCR1 WT and Mutants Shows That Only Small Molecule PAM Activity Relies on Transmembrane Receptor Residues

			^{125}I-CCL3$^{(5-70)}$ against CCL3$^{(1-70)}$						^{125}I-CCL3$^{(5-70)}$ against ZnBip					
			IC$_{50}$[a]			B_{max} (fmol/100 000 cells)[b]		(n)[c]	IC$_{50}$			% enhancement[d]		(n)
		location	log ± SEM	(nM)	F_{mut}[e]	Mean ± SEM			log ± SEM	(μM)	F_{mut}	Mean ± SEM		
	WT		−8.0 ± 0.03	10	1.0	1695 ± 214		(33)	−4.2 ± 0.10	60	1.0	235 ± 22		(17)
TM-2	W90A	2.60/II:20	−7.8 ± 0.24	15	1.5	6131 ± 2349	*[g]	(3)	no enhanced binding	*				(3)
TM-3	S110W	3.29/III:05	−7.8 ± 0.05	17	1.8	4869 ± 1048	*	(3)	−3 ± ####		**17**	*		(3)
	Y113A	3.32/III:08	−8.3 ± 0.06	5.0	0.52	758 ± 158		(11)	−4.5 ± 0.21	32	0.54	264 ± 29		(5)
	Y114A	3.33/III:09	−8.1 ± 0.11	7.8	0.82	1640 ± 701		(8)	−4.7 ± 0.28	19	0.31	169 ± 42		(4)
	L117F	3.36/III:12	−7.7 ± 0.26	19	2.0	3332 ± 666		(2)	−3.9 ± 0.41	131	**2.2**	174 ± 27		(2)
TM-6	F248A	6.44/VI:09	−7.7 ± 0.10	18	1.9	3313 ± 347		(3)	−3.8 ± 0.12	168	**2.8**	223 ± 42		(3)
	W252A	6.48/VI:13	−8.6 ± 0.07	2.4	0.25	135 ± 27	*	(6)	−4.6 ± 0.01	26	0.44	363 ± 28	*	(4)
	Y255A	6.51/VI:16	−8.9 ± 0.22	1.4	0.15	149 ± 79	*	(5)	−4.3 ± 0.04	48	0.80	187 ± 17		(4)
TM-7	E287A	7.39/VII:06	−9.0 ± 0.15	0.9	0.09	68 ± 19	*	(6)	no enhanced binding	*				(5)
	Y291A	7.43/VII:10	−8.3 ± 0.10	4.9	0.52	1134 ± 503		(5)	−4.2 ± 0.17	63	1.1	344 ± 51		(5)

[a]IC$_{50}$ is concentration at which 50% of ^{125}I-CCL3$^{(5-70)}$ is displaced, IC$_{50}$ values are given in log and nM or μM. [b]B_{max} is total number of binding sites calculated as fmol/100.000 cells. [c](n) is the number of experiments. [d]%-Enhancement is the amount of ZnBip-induced enhanced binding relative to the amount of specific binding of CCL3$^{(5-70)}$ to each mutant (set to 100%). [e]F_{mut} is the fold-decrease of IC$_{50}$ of mutant compared to WT. F_{mut} changes greater than a 2-fold decrease are highlighted in bold. [f]Position of mutants according to the Ballesteros/Weinstein (left) and Baldwin/Schwartz (right) numbering system. [g]Statistics: one-way Anova of log ± SEM (n) of mutant compared to wt. *$p \leq 0.05$.

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truncated compared to full-length CCL3, which could be important for retention of receptors at the cell surface.

CCL3 exists in several N-terminal variants, of which $CCL3^{(5-70)}$ has been confirmed.[52] However, it does not carry the typical cleavage site for CD13, CD26, and MMPs,[53,54] and $CCL3^{(5-70)}$ found in ascites might therefore arise from CCL3L1 and resemble $CCL3^{(5-70)}$ in its exact mass and amino acid composition, with only two amino acid differences (S61 to G62 and G69 to S70 in CCL3 and CCL3L1, respectively).[31] These chemokines cannot be distinguished using mass spectrometry and the small alterations are unlikely to affect the allosteric modulation by small molecule drugs presented in this study. Consistent with our findings, increased affinity and potency of short compared to full length CCL3 at CCR1 have been reported previously for CCL3 and CCL3L1 truncations.[54,55] In addition, in a previous study, CCL3L1 displayed potent interactions with CCR1 and CCR5, with a 10-fold increase after N-terminal truncation, which is mirrored in our study of a CCL3 truncation.[36]

Chemokine-Dependent Allosteric Action and the Role of Glu7.39$^{7.39}$. The chemokine system is well suited for the development of allosteric modulators, as the main chemokine binding domain is formed by extracellular receptor regions.[2,56] Therefore, small molecule ligands that bind to the transmembrane receptor domain have often been classified as allosteric, that is, not overlapping with the chemokine. However, it is well established that the flexible chemokine N-termini interact with specific receptor residues in the transmembrane area to induce receptor activation,[2,56] as confirmed by crystal structures of chemokines in complex with their receptors.[21,22,57] One residue that seems to be important for both chemokines and small molecule ligands is the chemokine receptor-conserved glutamate in the top of TM7 (position 7.39) that is found in 74% of endogenous chemokine receptors, but in less than 1% of nonchemokine class A GPCRs.[19] This glutamate is crucial for activation by chemokines in a number of chemokine-receptor pairs such as CCL2 and −7 with CCR2, CCL11, with CCR3, CCL17, and −22 with CCR4 and CCL5 with CCR1 and CCR5.[3] In contrast, as shown here, and previously by others, CCL3 does *not* depend on Glu287$^{7.39}$ for CCR1 or CCR5 activation,[32,33] whereas it coordinates Zn^{2+} in the ZnBip-CCR1 and ZnClTerp-CCR1 models, and contributes to the binding of $CCL3^{(5-70)}$ in the ZnBip-CCR1 complex. Furthermore, we have previously shown that ZnBip and ZnPhe do not enhance ^{125}I-CCL5 binding, but rather compete with it, consistent with the fact that CCL5, like $CCL3^{(1-70)}$, carries a nine amino acid long N-terminus, which competes with the allosteric modulators for the binding to Glu287$^{7.39}$, as corroborated by mutagenesis studies proving this residue be necessary for CCL5 activity.[32]

To summarize, the transmembrane binding pocket of chemokine receptors, with Glu287$^{7.39}$ at its center, is occupied by the chemokine N-termini to varying degrees depending on the chemokine and truncation variants. Small allosteric ligands for chemokine receptors often use this Glu as anchor,[19] and it is therefore around this residue that a competitive situation may occur between chemokines and small molecule ligands.

From Agonist to Inverse Agonist and the Role of the Tyrosine Network for Efficacy. Mutation Y113A$^{3.32}$ results in a shift from agonist to inverse agonist for ZnBip and ZnClTerp (Table 1), while having no effect on the allosteric action of ZnBip on $CCL3^{(5-70)}$. The efficacy switch might result from an altered, conceivably deeper binding, of the metal ion chelator complexes in the transmembrane pocket that lock the receptor in an inactive conformation. Furthermore, one could argue that ZnBip is no longer able to establish a connection between helix 3 and helix 6 in Y113A$^{3.32}$, resulting in an inactivation of CCR1. This is in accordance with the earlier proposed activation mechanisms for GPCRs, suggesting a movement of the extracellular-facing part of TM-6 inward toward TM-3 for activation.[58] Interestingly, Tyr113$^{3.32}$ is one of four tyrosine residues at the bottom of the transmembrane binding pocket. The other three are the adjacent Tyr114$^{3.33}$, and Tyr255$^{6.51}$, and Tyr291$^{7.43}$. These positions have previously been shown to form an aromatic zipper of importance for a small molecule binding site in CXCR3, where a metal ion site was introduced between Asp$^{4.60}$ and His$^{3.29}$.[59] Upon binding of ZnBip via Zn^{2+} to this engineered metal ion site, an aromatic zipper consisting of the chelator 2,2′-bipyridine and Tyr$^{6.51}$, Phe$^{3.32}$, and Tyr$^{7.43}$ (in this order), pulls TM-6 toward TM-3, leading to the stabilization of the active conformation.[59] It is remarkable that these residues are also present in CCR1 and CCR5, which both are activated by metal ion chelator complexes.[32,34,35] However, in contrast to the site in CXCR3, the chelator in our CCR1 model binds centrally and above Glu287$^{7.39}$ and, instead of forming a zipper, the tyrosines are located below Zn^{2+}. This may allow the tyrosines to substitute for each other in their interaction with Zn^{2+}. Such redundancy would explain why mutation of either tyrosine alone has a relatively small effect. Furthermore, they might be of general importance for aromatic interactions of small molecules with the transmembrane receptor domain.

Clinical Relevance. In this study, we show that the action of allosteric modulators depends on the length of the chemokine N-terminus. To our knowledge, this is the first report of its kind, and it emphasizes that drug development programs for chemokine receptors and other peptide receptors, such as C3A and C5A receptors, or glucagon-like receptors (class B1 GPCRs) need to consider that differential post-translational ligand modification may influence the action of a drug candidate. To give an example; if chemokine receptor drug candidates were developed as antagonists for a long-variant chemokine, but, at the relevant sites *in vivo*, would meet a truncated chemokine variant, this could change their activity either in a biased manner, or even result in complete inactivity. This is further complicated by the difficulties involved in determining the exact amount and location of truncated chemokines *in vivo*. In the chemokine field in general, N-terminal truncation is often a ubiquitous and progressive set of events, and has been identified in more than half of all chemokines.[43] So far, it seems that the effect of chemokine truncations can only be determined on a case-by-case basis, and as a consequence, the same will hold true for the allosteric modulators in this GPCR group. Therefore, our study highlights that future drug development targeting chemokine (and other peptide) receptors should take this *in vivo* phenomenon into account on a pharmacological level to optimize the likelihood of finding compounds with promising clinical effects.

EXPERIMENTAL SECTION

Materials. Human $CCL3^{(5-70)}$ was purchased from R&D Biotechne (MN, U.S.), and human $CCL3^{(1-70)}$ was from Peprotech (NJ, U.S.). ^{125}I-$CCL3^{(5-70)}$ was produced in-house,

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and ^{125}I-CCL3$^{(1-70)}$ purchased from PerkinElmer (MA, U.S.). $ZnCl_2$, 2,2′-bipyridine, 4′-chloro-2,2′:6′,2″-terpyridine, and DMSO was purchased from Sigma-Aldrich and used without further purification. 6-Phenyl-2,2′-bipyridine (PhBip) was synthesized from 2,2′-bipyridine by reaction with phenyl lithium followed by oxidation with manganese oxide.[60] 2,2′:6′,3′′-Terpyridine (mTerp) and 2,2′:6′,4′′-terpyridine (pTerp) were synthesized by condensation of 3-(dimethylamino)-1-(pyridin-2-yl)prop-2-en-1-one[61] with 3-acetylpyridine or 4-acetylpyridine, respectively.[62] The highest concentrations of metal-ion chelator complexes were 20 mM for ZnBip, and 2 mM for ZnClTerp, and were made from 0.2 or 0.02 M $ZnCl_2$ in water, and 100 mM Bip or 10 mM ClTerp in DMSO, respectively, and were supplemented with water and 70% ethanol. The ratio of Zn^{2+}/chelator was 1:2. Dilutions were made in water. The human CCR1 cDNA was kindly provided by Tim Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland). The promiscuous G protein $G\alpha_{\Delta 6qi4myr}$ (abbreviated as G_{qi4myr}) was kindly provided by Evi Kostenis (University of Bonn, Germany) Myo[^{3}H]inositol (PT6−271) and 125-iodine were purchased from PerkinElmer (MA, U.S.). Polyethyleimine (PEI) for transfection was purchased from Polysciences (PA, U.S.). CHO-K1 cells stably expressing CCR1 were kindly provided by Hans Rudolf von Lüttichau (University of Copenhagen, Denmark).

Molecular Biology. Receptor mutations were introduced by the polymerase chain reaction overlap extension technique or the QuikChange technique (Agilent Technologies, CA, U.S.) using wild-type (WT) CCR5. All reactions were carried out using Pfu polymerase (Stratagene, CA, U.S.) under conditions recommended by the manufacturer. The mutations were cloned into pcDNA3.1+ for use in inositol-trisphosphate (IP_3) and binding assays (Invitrogen, CA, U.S.). All constructs were verified by restriction endonuclease digestion and DNA sequencing (GATC Biotech, Germany).

Transfection and Tissue Culture. COS-7 cells were grown in DMEM with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 180 U/mL penicillin and 45 μg/mL streptomycin at 37 °C in a 10% CO_2/90% air-humidified atmosphere. Transfection of 6×10^6 COS-7 cells in a T175 culture flask was carried out by the calcium phosphate precipitation method.[63,64] Briefly, plasmid DNA (20 μg of receptor cDNA and 30 μg G_{qi4myr} for IP_3-assays, or 40 μg receptor cDNA for ^{125}I-CCL3-binding assays) was mixed with TE buffer (10 mM Tris-HCl, 2 mM EDTA-Na_2, pH 7.5) and 30 μL calcium chloride (2 M) to a total volume of 480 μL, and was then added to the same amount of HEPES buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.2). The mixture was allowed to precipitate for 45 min at room temperature, after which the precipitate and 300 μL chloroquine (2 mg/mL) in 10 mL culture media were added to the 6×10^6 COS-7 cells seeded the day before. Transfection was stopped after 5 h by replacing to fresh medium, and cells were incubated overnight. HEK293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), containing 1% GlutaMAX, and supplemented with 10% FBS and 1% penicillin (180 U/mL)/streptomycin (45 μg/mL), and incubated at 37 °C, 10% CO_2 and 95% air humidity. CHO K1 cells stably transfected with CCR1 were grown in RPMI1640 supplemented with 10% FBS and 1% penicillin (180 U/mL)/streptomycin (45 μg/mL) and incubated in 37 °C, 5% CO_2 and 95% air humidity. For transfection of HEK293 or CHO K1 cells (1 000 000 cells/well for HEK293 and 500 000 cells/well for CHO-K1) were seeded in pretreated 6-well tissue culture plates before a polyethylenimine (PEI) DNA transfection was performed.[65] For the cAMP assays, CHO-K1 cells stably expressing CCR1 were transfected with 1 μg of CAMYEL (YFP-Epac-RLuc). For the ß-arrestin-2-assays, HEK293 cells were transfected with 0.33 μg of human CCR1 receptor, 0.042 μg of Rluc8-Arrestin3-SP1, 0.8 μg of mem-citrine-SH3 and 0.8 μg of GRK2. DNA constructs were mixed with PEI (ratio 1:2 or 1:1.5 (DNA/PEI) for cAMP- and ß-arrestin assays, respectively) and nonsupplemented DMEM, incubated at room temperature (RT) for 15 min, before the sample was added dropwise to the cells. The following day, transfection was terminated by replacing the cell transfection-medium with fresh supplemented DMEM medium.

Inositol-Trisphosphate (IP_3) Assay. One day after transfection, COS-7 cells (35 000 cells/well) were incubated with [^{3}H]myo-inositol (5 μL/mL, 2 μCi/mL) in 0.1 mL of media overnight in a 96-well plate. The following day, cells were washed twice in PBS and were incubated in 0.1 mL of Hank's balanced salt solution (Invitrogen) supplemented with 10 mM LiCl at 37 °C in the presence of various concentrations of ligands for 90 min. Assay medium was then removed, and cells were extracted by the addition of 50 μL of 10 mM formic acid to each well, followed by incubation on ice for 30−60 min. The [^{3}H]IPs in the formic acid cell lysates were thereafter quantified by adding YSi-poly-D-Lys-coated SPA beads. Briefly, 35 μL of cell extract was mixed with 80 μL of SPA bead suspension in H_2O (12.5 μg/μL) in a white 96-well plate. Plates were sealed, agitated for at least 30 min, and centrifuged for 5 min at 402 rcf. SPA beads were allowed to settle and react with the extract for at least 8 h before radioactivity was determined using a Packard Top Count NXT scintillation counter (PerkinElmer). All determinations were made in duplicate. These overall readouts have earlier been used effectively for CCR5, CXCR4, and other chemokine receptors.[33,34,66,67]

cAMP-Assay. Two days after transfection, cells were resuspended in PBS with 1% glucose (5 mM), before they were aliquoted at 80 μL into a white 96-well culture plate (PerkinElmer). Subsequently, the cells were stimulated to produce cAMP by adding 10 μM forskolin. Five minutes after incubation with forskolin, Coelenterazine h (Nanolight Technologies, Pinetop, AZ) was added in a final concentration of 5 μM. The reaction was started 10 min after forskolin stimulation by the addition of ligands (concentrations were ranging from 1 pM to 0.1 μM). After a 30 min incubation at room temperature (RT), the inhibition of forskolin-stimulated cAMP by the activated $G\alpha_i$-coupled chemokine receptor was measured as luminescence (Rluc 485/40 nm and YFP 530/25 nm) on a LB 940 Mithras Multimode Microplate Reader (Berthold Technologies GmbH & CO. KG, Bad Wildbad, Germany).

β-Arrestin Assay. The ß-arrestin recruitment assay was performed as previously described.[68] In short, 2 days after transfection, cells were resuspended in PBS with 1% glucose (5 mM), before they were aliquoted at 85 μL into a 96-well black-white iso-plate (PerkinElmer). Subsequently, Coelenterazine h (Nanolight Technologies) was added in a final concentration of 5 μM and the reaction was started immediately after, by the addition of ligands (concentrations were ranging from 1 pM to 0.1 μM). After 30 min incubation at RT, the luminescence (Rluc 485/40 nm and YFP 530/25 nm) was measured on a LB

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940 Mithras Multimode Microplate Reader (Berthold Technologies GmbH & CO. KG).

Ca^{2+}-Assay. Two days prior to the assay, CHO-K1 cells stably expressing CCR1 were seeded in clear-bottom black 96-well plates (Thermo Fisher, Invitrogen) at 35 000 cells/well. One vial (77 mg) probenecid (Thermo Fisher, Invitrogen) was dissolved in 550 μL of 0.6 M NaOH; 1 vial of Fluo-4 AM (50 μg) (Thermo Fisher, Invitrogen) was dissolved in 23 μL of DMSO. Wash buffer was prepared consisting of 50 mL of HBSS + 20 mM Hepes, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, supplemented with 250 μL of probenecid. Loading buffer contains wash buffer +0.2% Fluo-4 (e.g., 5.5 mL of wash buffer +11 μL of Fluo-4 solution). Upon start of the assay, the cell culture medium from the cell-plate was removed and 50 μL/well loading buffer was added and incubated in the dark at 37 °C for 1 h. During that time, a ligand plate (96-well v-bottom, Greiner bio-one, Austria) was prepared containing 5-fold the desired concentration of ligand diluted in wash buffer. Thereafter, cells were washed twice with 100 μL/well wash buffer. Finally, 100 μL/well wash buffer was added, and cell- and ligand-plates were placed into a Flexstation 3 (Molecular Devices, CA, U.S.), which adds ligands and measures fluorescence over 90 s.

Iodination of CCL3. Seventeen micrograms (ca. 2 nmol) of carrier-free CCL3 (RnD systems, Bio-Techne, MN, U.S.) was dissolved in 10 μL of iodination buffer (300 mM phosphate buffer pH 7.4). A 4 μL aliquot of 125-iodine (PerkinElmer, NEX033A, MA, U.S.) was added. Five microliters of a 3 μg/mL chloramine T solution in 300 mM phosphate buffer pH 7.4 was added six times at 1 min intervals while occasionally stirring. After 6 min, the reaction was stopped by the addition of 400 μL of water containing 0.1% trifluoroacetic acid. The reaction mixture was then purified on C18 column with an acetonitrile gradient from 20 to 80% over ca. 45 min.

Competition Binding. On the day after transfection, cells were transferred to 24- or 12-well culture plates. The number of cells seeded per well was determined by the apparent expression efficiency of the receptors, aiming for 5−10% specific binding of ^{125}I-CCL3. The number of cells thus ranged from 10 000 to 300 000. Two days after transfection, cells were assayed by competition binding for 3 h at 4 °C using 10−15 pM ^{125}I-CCL3 plus unlabeled ligand in 0.2 mL (24-well-plates) or 0.3 mL (12-well-plates) of 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% (w/v) bovine serum albumin. After incubation, cells were washed quickly two times with 4 °C binding buffer supplemented with 500 mM NaCl. Nonspecific binding was determined in the presence of 0.1 μM unlabeled CCL3. Determinations were made in duplicate.

Computational Modeling. A homology model of CCR1 (UniProt ID P32246) in complex with CCL3 was generated using the X-ray crystal structure of CCR5 in complex with 5P7-CCL5 (PDB 5UIW).[22] The N- and C-termini of CCR1 not covered by the template were not considered during model generation, and the structural waters of CCR5 were omitted. The models were built using the Full Model Builder of ICM 3.8-7b (Molsoft L.L.C.) and subsequently refined through 200 steps of all-atom Monte Carlo-minimization. The best model was selected by having the lowest overall energy strain based on the "Protein Health" tool in ICM, which assesses the relative energy of each amino acid residue. The chelator metal ion receptor complexes were optimized through 5000 Monte Carlo global optimization steps, each involving a random move of one of four types (pseudo-Brownian move, change of internal variable, group of angles or loop conformation), followed by a local energy minimization (100 calls, gradient RMSD < 0.05, tolFunc = 6 consecutive steps without energy improvement), a calculation of the complete energy including surface and advanced electrostatic terms and, finally, acceptance or rejection of the move based on the energy and the temperature. The chelator, metal ion and side chains were left free to move, which resulted in chelator and metal ion receptor-mediated interactions in agreement with metal ion interaction geometries observed in experimental structures.

Data Analysis. Nonlinear regression concentration−response curves, EC_{50} values, and IC_{50} values were determined using GraphPad Prism 8 for binding and all signaling assays. If included concentrations of small molecule or chemokine ligands did not reach a plateau, the EC_{50}/IC_{50} of the interaction was qualified as nondeterminable (no sign of sigmoidal plateau) or nearing saturation, in which case the Hill coefficient was set to 1 (or −1 for competitive binding) to determine the plateau. The values of efficacy, B_0, IC_{50}, and EC_{50} of the CCL3 variants were compared within each assay format using the unpaired two-tailed *t* test.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CCR1, C−C-chemokine receptor 1; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; HBSS, Hanks balanced salt solution; RT, room temperature; TE, Tris-EDTA; ZnBip, Zn^{2+} in complex with 2,2′-bipyridine; ZnPhe, Zn^{2+} in complex with 1,10-phenantrholine; ZnTerp, Zn^{2+} in complex 2,2′:6′,2′′-terpyridine; ZnClTerp, Zn^{2+} in complex with 4′-chloro-2,2′:6′,2′′-terpyridine.

REFERENCES

(1) Bachelerie, F., Ben-Baruch, A., Burkhardt, A. M., Combadiere, C., Farber, J. M., Graham, G. J., Horuk, R., Sparre-Ulrich, A. H., Locati, M., Luster, A. D., Mantovani, A., Matsushima, K., Murphy, P. M., Nibbs, R., Nomiyama, H., Power, C. A., Proudfoot, A. E., Rosenkilde, M. M., Rot, A., Sozzani, S., Thelen, M., Yoshie, O., and Zlotnik, A. (2014) International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family

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ACS Pharmacol. Transl. Sci. 2019, 2, 429−441

of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol. Rev. 66* (1), 1−79.
(2) Allen, S. J., Crown, S. E., and Handel, T. M. (2007) Chemokine: receptor structure, interactions, and antagonism. *Annu. Rev. Immunol. 25*, 787−820.
(3) Thiele, S., and Rosenkilde, M. M. (2014) Interaction of chemokines with their receptors–from initial chemokine binding to receptor activating steps. *Curr. Med. Chem. 21* (31), 3594−614.
(4) Kleist, A. B., Getschman, A. E., Ziarek, J. J., Nevins, A. M., Gauthier, P. A., Chevigne, A., Szpakowska, M., and Volkman, B. F. (2016) New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. *Biochem. Pharmacol. 114*, 53−68.
(5) Simmons, G. (1997) Potent Inhibition of HIV-1 Infectivity in Macrophages and Lymphocytes by a Novel CCR5 Antagonist. *Science 276* (5310), 276−279.
(6) Proudfoot, A. E., Buser, R., Borlat, F., Alouani, S., Soler, D., Offord, R. E., Schroder, J. M., Power, C. A., and Wells, T. N. (1999) Amino-terminally modified RANTES analogues demonstrate differential effects on RANTES receptors. *J. Biol. Chem. 274* (45), 32478−85.
(7) Proudfoot, A. E., Power, C. A., Hoogewerf, A. J., Montjovent, M. O., Borlat, F., Offord, R. E., and Wells, T. N. (1996) Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J. Biol. Chem. 271* (5), 2599−603.
(8) Townson, J. R., Graham, G. J., Landau, N. R., Rasala, B., and Nibbs, R. J. (2000) Aminooxypentane addition to the chemokine macrophage inflammatory protein-1alpha P increases receptor affinities and HIV inhibition. *J. Biol. Chem. 275* (50), 39254−61.
(9) Hartley, O., Gaertner, H., Wilken, J., Thompson, D., Fish, R., Ramos, A., Pastore, C., Dufour, B., Cerini, F., Melotti, A., Heveker, N., Picard, L., Alizon, M., Mosier, D., Kent, S., and Offord, R. (2004) Medicinal chemistry applied to a synthetic protein: development of highly potent HIV entry inhibitors. *Proc. Natl. Acad. Sci. U. S. A. 101* (47), 16460−5.
(10) Kawamura, T., Bruse, S. E., Abraha, A., Sugaya, M., Hartley, O., Offord, R. E., Arts, E. J., Zimmerman, P. A., and Blauvelt, A. (2004) PSC-RANTES blocks R5 human immunodeficiency virus infection of Langerhans cells isolated from individuals with a variety of CCR5 diplotypes. *J. Virol 78* (14), 7602−9.
(11) Kumar, S., Choi, W. T., Dong, C. Z., Madani, N., Tian, S., Liu, D., Wang, Y., Pesavento, J., Wang, J., Fan, X., Yuan, J., Fritzsche, W. R., An, J., Sodroski, J. G., Richman, D. D., and Huang, Z. (2006) SMM-chemokines: a class of unnatural synthetic molecules as chemical probes of chemokine receptor biology and leads for therapeutic development. *Chem. Biol. 13* (1), 69−79.
(12) Gaertner, H., Cerini, F., Escola, J. M., Kuenzi, G., Melotti, A., Offord, R., Rossitto-Borlat, I., Nedellec, R., Salkowitz, J., Gorochov, G., Mosier, D., and Hartley, O. (2008) Highly potent, fully recombinant anti-HIV chemokines: reengineering a low-cost microbicide. *Proc. Natl. Acad. Sci. U. S. A. 105* (46), 17706−11.
(13) Detheux, M., Standker, L., Vakili, J., Munch, J., Forssmann, U., Adermann, K., Pohlmann, S., Vassart, G., Kirchhoff, F., Parmentier, M., and Forssmann, W. G. (2000) Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor (CCR)1 and CCR5 agonist with anti-HIV properties. *J. Exp. Med. 192* (10), 1501−8.
(14) Richter, R., Schulz-Knappe, P., John, H., and Forssmann, W. G. (2000) Posttranslationally processed forms of the human chemokine HCC-1. *Biochemistry 39* (35), 10799−805.
(15) Sanchez, J., Lane, J. R., Canals, M., and Stone, M. J. (2019) Influence of Chemokine N-Terminal Modification on Biased Agonism at the Chemokine Receptor CCR1. *Int. J. Mol. Sci. 20* (10), 2417.
(16) Katritch, V., Cherezov, V., and Stevens, R. C. (2013) Structure-function of the G protein-coupled receptor superfamily. *Annu. Rev. Pharmacol. Toxicol. 53*, 531−56.
(17) Thal, D. M., Glukhova, A., Sexton, P. M., and Christopoulos, A. (2018) Structural insights into G-protein-coupled receptor allostery. *Nature 559* (7712), 45−53.
(18) Ballesteros, J. A., and Weinstein, H. (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors in *Methods in Neurosciences* (Stuart, C. S., Ed.) Vol. *25*, pp 366−428 Academic Press.
(19) Rosenkilde, M. M., and Schwartz, T. W. (2006) GluVII:06–a highly conserved and selective anchor point for non-peptide ligands in chemokine receptors. *Curr. Top. Med. Chem. 6* (13), 1319−33.
(20) Scholten, D. J., Canals, M., Maussang, D., Roumen, L., Smit, M. J., Wijtmans, M., de Graaf, C., Vischer, H. F., and Leurs, R. (2012) Pharmacological modulation of chemokine receptor function. *Br. J. Pharmacol. 165* (6), 1617−1643.
(21) Burg, J. S., Ingram, J. R., Venkatakrishnan, A. J., Jude, K. M., Dukkipati, A., Feinberg, E. N., Angelini, A., Waghray, D., Dror, R. O., Ploegh, H. L., and Garcia, K. C. (2015) Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. *Science 347* (6226), 1113−7.
(22) Zheng, Y., Han, G. W., Abagyan, R., Wu, B., Stevens, R. C., Cherezov, V., Kufareva, I., and Handel, T. M. (2017) Structure of CC Chemokine Receptor 5 with a Potent Chemokine Antagonist Reveals Mechanisms of Chemokine Recognition and Molecular Mimicry by HIV. *Immunity 46* (6), 1005−1017.
(23) Viola, A., and Luster, A. D. (2008) Chemokines and their receptors: drug targets in immunity and inflammation. *Annu. Rev. Pharmacol. Toxicol. 48*, 171−97.
(24) Liang, M., Mallari, C., Rosser, M., Ng, H. P., May, K., Monahan, S., Bauman, J. G., Islam, I., Ghannam, A., Buckman, B., Shaw, K., Wei, G. P., Xu, W., Zhao, Z., Ho, E., Shen, J., Oanh, H., Subramanyam, B., Vergona, R., Taub, D., Dunning, L., Harvey, S., Snider, R. M., Hesselgesser, J., Morrissey, M. M., Perez, H. D., and Horuk, R. (2000) Identification and characterization of a potent, selective, and orally active antagonist of the CC chemokine receptor-1. *J. Biol. Chem. 275* (25), 19000−8.
(25) Vallet, S., Raje, N., Ishitsuka, K., Hideshima, T., Podar, K., Chhetri, S., Pozzi, S., Breitkreutz, I., Kiziltepe, T., Yasui, H., Ocio, E. M., Shiraishi, N., Jin, J., Okawa, Y., Ikeda, H., Mukherjee, S., Vaghela, N., Cirstea, D., Ladetto, M., Boccadoro, M., and Anderson, K. C. (2007) MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. *Blood 110* (10), 3744−52.
(26) Gladue, R. P., Tylaska, L. A., Brissette, W. H., Lira, P. D., Kath, J. C., Poss, C. S., Brown, M. F., Paradis, T. J., Conklyn, M. J., Ogborne, K. T., McGlynn, M. A., Lillie, B. M., DiRico, A. P., Mairs, E. N., McElroy, E. B., Martin, W. H., Stock, I. A., Shepard, R. M., Showell, H. J., and Neote, K. (2003) CP-481,715, a potent and selective CCR1 antagonist with potential therapeutic implications for inflammatory diseases. *J. Biol. Chem. 278* (42), 40473−80.
(27) Kerstjens, H. A., Bjermer, L., Eriksson, L., Dahlstrom, K., and Vestbo, J. (2010) Tolerability and efficacy of inhaled AZD4818, a CCR1 antagonist, in moderate to severe COPD patients. *Respir Med. 104* (9), 1297−303.
(28) Naya, A., Ishikawa, M., Matsuda, K., Ohwaki, K., Saeki, T., Noguchi, K., and Ohtake, N. (2003) Structure-activity relationships of xanthene carboxamides, novel CCR1 receptor antagonists. *Bioorg. Med. Chem. 11* (6), 875−84.
(29) Schall, T. J., and Proudfoot, A. E. I. (2011) Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat. Rev. Immunol. 11* (5), 355−363.
(30) Karpus, W. J., Lukacs, N. W., McRae, B. L., Strieter, R. M., Kunkel, S. L., and Miller, S. D. (1995) An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. *J. Immunol 155* (10), 5003−10.
(31) Schutyser, E., Struyf, S., Proost, P., Opdenakker, G., Laureys, G., Verhasselt, B., Peperstraete, L., Van de Putte, I., Saccani, A., Allavena, P., Mantovani, A., and Van Damme, J. (2002) Identification

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ACS Pharmacol. Transl. Sci. 2019, 2, 429−441

of Biologically Active Chemokine Isoforms from Ascitic Fluid and Elevated Levels of CCL18/Pulmonary and Activation-regulated Chemokine in Ovarian Carcinoma. *J. Biol. Chem. 277* (27), 24584−24593.
(32) Jensen, P. C., Thiele, S., Ulven, T., Schwartz, T. W., and Rosenkilde, M. M. (2008) Positive versus negative modulation of different endogenous chemokines for CC-chemokine receptor 1 by small molecule agonists through allosteric versus orthosteric binding. *J. Biol. Chem. 283* (34), 23121−8.
(33) Thiele, S., Steen, A., Jensen, P. C., Mokrosinski, J., Frimurer, T. M., and Rosenkilde, M. M. (2011) Allosteric and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach. *J. Biol. Chem. 286* (43), 37543−54.
(34) Thiele, S., Malmgaard-Clausen, M., Engel-Andreasen, J., Steen, A., Rummel, P. C., Nielsen, M. C., Gloriam, D. E., Frimurer, T. M., Ulven, T., and Rosenkilde, M. M. (2012) Modulation in selectivity and allosteric properties of small-molecule ligands for CC-chemokine receptors. *J. Med. Chem. 55* (18), 8164−77.
(35) Karlshoj, S., Amarandi, R. M., Larsen, O., Daugvilaite, V., Steen, A., Brvar, M., Pui, A., Frimurer, T. M., Ulven, T., and Rosenkilde, M. M. (2016) Molecular Mechanism of Action for Allosteric Modulators and Agonists in CC-chemokine Receptor 5 (CCR5). *J. Biol. Chem. 291* (52), 26860−26874.
(36) Struyf, S., Menten, P., Lenaerts, J. P., Put, W., D'Haese, A., De Clercq, E., Schols, D., Proost, P., and Van Damme, J. (2001) Diverging binding capacities of natural LD78beta isoforms of macrophage inflammatory protein-1alpha to the CC chemokine receptors 1, 3 and 5 affect their anti-HIV-1 activity and chemotactic potencies for neutrophils and eosinophils. *Eur. J. Immunol. 31* (7), 2170−8.
(37) Laitaoja, M., Valjakka, J., and Janis, J. (2013) Zinc coordination spheres in protein structures. *Inorg. Chem. 52* (19), 10983−91.
(38) Steen, A., Thiele, S., Guo, D., Hansen, L. S., Frimurer, T. M., and Rosenkilde, M. M. (2013) Biased and constitutive signaling in the CC-chemokine receptor CCR5 by manipulating the interface between transmembrane helices 6 and 7. *J. Biol. Chem. 288* (18), 12511−21.
(39) Rummel, P. C., Thiele, S., Hansen, L. S., Petersen, T. P., Sparre-Ulrich, A. H., Ulven, T., and Rosenkilde, M. M. (2013) Extracellular disulfide bridges serve different purposes in two homologous chemokine receptors, CCR1 and CCR5. *Mol. Pharmacol. 84* (3), 335−45.
(40) Steen, A., Sparre-Ulrich, A. H., Thiele, S., Guo, D., Frimurer, T. M., and Rosenkilde, M. M. (2014) Gating function of isoleucine-116 in TM-3 (position III:16/3.40) for the activity state of the CC-chemokine receptor 5 (CCR5). *Br. J. Pharmacol. 171* (6), 1566−79.
(41) Rummel, P. C., Arfelt, K. N., Baumann, L., Jenkins, T. J., Thiele, S., Luttichau, H. R., Johnsen, A., Pease, J., Ghosh, S., Kolbeck, R., and Rosenkilde, M. M. (2012) Molecular requirements for inhibition of the chemokine receptor CCR8–probe-dependent allosteric interactions. *Br. J. Pharmacol. 167* (6), 1206−17.
(42) Jensen, P. C., Nygaard, R., Thiele, S., Elder, A., Zhu, G., Kolbeck, R., Ghosh, S., Schwartz, T. W., and Rosenkilde, M. M. (2007) Molecular interaction of a potent nonpeptide agonist with the chemokine receptor CCR8. *Mol. Pharmacol. 72* (2), 327−40.
(43) Vanheule, V., Metzemaekers, M., Janssens, R., Struyf, S., and Proost, P. (2018) How post-translational modifications influence the biological activity of chemokines. *Cytokine+ 109*, 29−51.
(44) Decock, B., Conings, R., Lenaerts, J. P., Billiau, A., and Van Damme, J. (1990) Identification of the monocyte chemotactic protein from human osteosarcoma cells and monocytes: detection of a novel N-terminally processed form. *Biochem. Biophys. Res. Commun. 167* (3), 904−9.
(45) Proost, P., Struyf, S., Couvreur, M., Lenaerts, J. P., Conings, R., Menten, P., Verhaert, P., Wuyts, A., and Van Damme, J. (1998) Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6−76) as a natural chemokine inhibitor. *J. Immunol 160* (8), 4034−41.
(46) McQuibban, G. A., Gong, J. H., Wong, J. P., Wallace, J. L., Clark-Lewis, I., and Overall, C. M. (2002) Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood 100* (4), 1160−7.
(47) Guan, E., Wang, J., Roderiquez, G., and Norcross, M. A. (2002) Natural truncation of the chemokine MIP-1 beta /CCL4 affects receptor specificity but not anti-HIV-1 activity. *J. Biol. Chem. 277* (35), 32348−52.
(48) Guan, E., Wang, J., and Norcross, M. A. (2004) Amino-terminal processing of MIP-1beta/CCL4 by CD26/dipeptidyl-peptidase IV. *J. Cell. Biochem. 92* (1), 53−64.
(49) Oravecz, T., Pall, M., Roderiquez, G., Gorrell, M. D., Ditto, M., Nguyen, N. Y., Boykins, R., Unsworth, E., and Norcross, M. A. (1997) Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J. Exp. Med. 186* (11), 1865−72.
(50) Struyf, S., De Meester, I., Scharpe, S., Lenaerts, J. P., Menten, P., Wang, J. M., Proost, P., and Van Damme, J. (1998) Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor. *Eur. J. Immunol. 28* (4), 1262−71.
(51) Proost, P., De Meester, I., Schols, D., Struyf, S., Lambeir, A. M., Wuyts, A., Opdenakker, G., De Clercq, E., Scharpe, S., and Van Damme, J. (1998) Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase IV. Conversion of RANTES into a potent inhibitor of monocyte chemotaxis and HIV-1-infection. *J. Biol. Chem. 273* (13), 7222−7.
(52) Schutyser, E., Struyf, S., Proost, P., Opdenakker, G., Laureys, G., Verhasselt, B., Peperstraete, L., Van de Putte, I., Saccani, A., Allavena, P., Mantovani, A., and Van Damme, J. (2002) Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *J. Biol. Chem. 277* (27), 24584−93.
(53) Townson, J. R., Barcellos, L. F., and Nibbs, R. J. (2002) Gene copy number regulates the production of the human chemokine CCL3-L1. *Eur. J. Immunol. 32* (10), 3016−26.
(54) Proost, P., Menten, P., Struyf, S., Schutyser, E., De Meester, I., and Van Damme, J. (2000) Cleavage by CD26/dipeptidyl peptidase IV converts the chemokine LD78beta into a most efficient monocyte attractant and CCR1 agonist. *Blood 96* (5), 1674−80.
(55) Nibbs, R. J., Yang, J., Landau, N. R., Mao, J. H., and Graham, G. J. (1999) LD78beta, a non-allelic variant of human MIP-1alpha (LD78alpha), has enhanced receptor interactions and potent HIV suppressive activity. *J. Biol. Chem. 274* (25), 17478−83.
(56) Kufareva, I., Salanga, C. L., and Handel, T. M. (2015) Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. *Immunol. Cell Biol. 93* (4), 372−83.
(57) Qin, L., Kufareva, I., Holden, L. G., Wang, C., Zheng, Y., Zhao, C., Fenalti, G., Wu, H., Han, G. W., Cherezov, V., Abagyan, R., Stevens, R. C., and Handel, T. M. (2015) Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. *Science 347* (6226), 1117−22.
(58) Schwartz, T. W., Frimurer, T. M., Holst, B., Rosenkilde, M. M., and Elling, C. E. (2006) Molecular Mechanism of 7tm Receptor Activation—a Global Toggle Switch Model. *Annu. Rev. Pharmacol. Toxicol. 46* (1), 481−519.
(59) Rosenkilde, M. M., Andersen, M. B., Nygaard, R., Frimurer, T. M., and Schwartz, T. W. (2007) Activation of the CXCR3 chemokine receptor through anchoring of a small molecule chelator ligand between TM-III, -IV, and -VI. *Mol. Pharmacol. 71* (3), 930−41.
(60) Dietrich-Buchecker, C. O., Nierengarten, J. F., Sauvage, J. P., Armaroli, N., Balzani, V., and De Cola, L. (1993) Dicopper(I) trefoil knots and related unknotted molecular systems: influence of ring size and structural factors on their synthesis and electrochemical and excited-state properties. *J. Am. Chem. Soc. 115* (24), 11237−11244.

DOI: 10.1021/acsptsci.9b00059
ACS Pharmacol. Transl. Sci. 2019, 2, 429−441

(61) Zeng, X. X., Zheng, R. L., Zhou, T., He, H. Y., Liu, J. Y., Zheng, Y., Tong, A. P., Xiang, M. L., Song, X. R., Yang, S. Y., Yu, L. T., Wei, Y. Q., Zhao, Y. L., and Yang, L. (2010) Novel thienopyridine derivatives as specific anti-hepatocellular carcinoma (HCC) agents: synthesis, preliminary structure-activity relationships, and in vitro biological evaluation. *Bioorg. Med. Chem. Lett. 20* (21), 6282−5.
(62) Constable, E. C., Housecroft, C. E., Kopecky, P., Martin, C. J., Wright, I. A., Zampese, J. A., Bolink, H. J., and Pertegas, A. (2013) Solution, structural and photophysical aspects of substituent effects in the N̂N ligand in [Ir(Ĉ N)2(N̂N)]+ complexes. *Dalton Trans 42* (22), 8086−103.
(63) Rosenkilde, M. M., Cahir, M., Gether, U., Hjorth, S. A., and Schwartz, T. W. (1994) Mutations along transmembrane segment II of the NK-1 receptor affect substance P competition with non-peptide antagonists but not substance P binding. *J. Biol. Chem. 269* (45), 28160−4.
(64) Kissow, H., Hartmann, B., Holst, J. J., Viby, N. E., Hansen, L. S., Rosenkilde, M. M., Hare, K. J., and Poulsen, S. S. (2012) Glucagon-like peptide-1 (GLP-1) receptor agonism or DPP-4 inhibition does not accelerate neoplasia in carcinogen treated mice. *Regul. Pept. 179* (1−3), 91−100.
(65) Donthamsetti, P., Quejada, J. R., Javitch, J. A., Gurevich, V. V., and Lambert, N. A. (2015) Using Bioluminescence Resonance Energy Transfer (BRET) to Characterize Agonist-Induced Arrestin Recruitment to Modified and Unmodified G Protein-Coupled Receptors. *Curr. Protoc Pharmacol 70* (2), 1−14.
(66) Brandish, P. E., Hill, L. A., Zheng, W., and Scolnick, E. M. (2003) Scintillation proximity assay of inositol phosphates in cell extracts: high-throughput measurement of G-protein-coupled receptor activation. *Anal. Biochem. 313* (2), 311−8.
(67) Thiele, S., Mungalpara, J., Steen, A., Rosenkilde, M. M., and Vabeno, J. (2014) Determination of the binding mode for the cyclopentapeptide CXCR4 antagonist FC131 using a dual approach of ligand modifications and receptor mutagenesis. *Br. J. Pharmacol. 171* (23), 5313−29.
(68) Gabe, M. B. N., van der Velden, W. J. C., Gadgaard, S., Smit, F. X., Hartmann, B., Brauner-Osborne, H., and Rosenkilde, M. M. (2019) Enhanced agonist residence time, internalization rate and signalling of the GIP receptor variant [E354Q] facilitate receptor desensitization and long-term impairment of the GIP system. *Basic Clin. Pharmacol. Toxicol.*, No. 13289, DOI: 10.1111/bcpt.13289.

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4. Extended results

The minimalized contribution by chemokine N-termini to overall chemokine-receptor interactions is dictated by the receptor

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Preliminary data

Foreword

The extended results section is a group of experimental results aimed at understanding some observations from our previous work that did not appear to match with any of the currently established models for chemokine interaction mechanisms. Part of the practical work was performed together with Sara Schuermans during her masters thesis, during collaboration with Paul Proost, who also supervised synthesis of the peptides used in the study.

There have been several updates, expansions and alternative suggestions to replace the previous models for chemokine activation, as I will describe in the introduction. Generally, the original two-site, two-step models that divides chemokine-receptor interactions into binding at site one and activation at site two is now regarded as being less distinct, with some degree of binding and activation attributed to the same interaction sites. This is highly relevant from our perspective, since in manuscript 2 we found that binding can be strongly influenced by modulation from the receptor pocket, otherwise more commonly associated with signaling. However, we also deduced from manuscript 1 that different receptors work differently when it comes to the role of individual chemokine domains, even in the case of CCR1 and CCR5 responding to the same chemokines.

We therefore set out to determine if it can be clearly decided whether chemokine receptor activation is achieved through different mechanisms depending on the ligand-receptor pair, or if the mechanism is governed by variations of a single theme. We were interested in focusing on the role of the chemokine N-terminus, where there is already evidence, both from us and others, that it may undertake various roles, and that it can often be manipulated extensively.

A study of chemokine-derived peptides has been reported before[137], but never of such a small molecular size, and may reveal some fundamental lessons: We synthesized the N-termini of a few MIP and MCP chemokines as free peptides, and tested them for their ability to activate chemokine receptors, or modulate the activity of other chemokines. Before we begin the introduction, it will be helpful to clarify the possible outcomes: The N-termini, defined by the segment preceding the CC/CXC motif, typically constitute 10-15% of the native chemokine, so if they do participate in recognition and binding to receptors, we should assume that it will be with a fraction of the affinity and potency of intact chemokines. We also know that the core domain, especially of MIP chemokines, could be an important contributor to the receptor activation mechanism, so that the N-terminus would be responsible for part of the total chemokine-induced signal. Finally, we wanted

to know if the extreme chemokine N-termini could act as allosteric modulators, akin to the metal ion chelators, since this was essentially the region we hypothesized was replaced and repurposed in manuscript 2.

Introduction

One of the most straightforward ways to influence the immune system would, in theory, be to direct immune cell trafficking and behavior by controlling chemotactic signaling. The chemokine system is responsible for this task, and under ideal conditions it attracts only the needed immune cell subset, only to the correct tissue, and eliciting only the appropriate behavior. The many disease conditions that follow when chemokine regulation goes awry, and the continuing efforts to successfully drug the chemotactic signaling of the system, underscore that it is still necessary to improve our understanding of how chemokine-receptor activation works in detail[53, 106, 138]. A major obstacle in that regard is that the scientific field has continuously attempted to revise a model of chemokine-receptor activation, without identifying potential future drug target sites more accurately.

Early data describing contributions to binding properties of chemokine-receptor complexes indicated that these properties would derive from distinct sub-regions of the ligand-receptor interaction interface. It had been shown that the receptor N-terminus of CCR1, CCR2 and CCR3 dictated the specific patterns of chemokine recognition displayed by the receptors, and that the receptor N-terminus was sufficient for strong chemokine binding on CCR2[28, 31, 139, 140]. Additionally, others had found that disturbing the N-terminus of the CXCL12 chemokine resulted in loss of signaling, but not binding of the cognate receptor[141]. The CXCL12 N-loop, belonging to the chemokine core domain and thus important for receptor binding, could be changed to allow recognition of CXCR1[142-144]. Variations of CCL5 with modifications to the N-terminus (Met- and AOP-RANTES) also led to loss of receptor signaling with retained binding, making them effective antagonists on CCR1 and CCR3, though CCR5 signaling was peculiarly unaffected[145-148]. All this led to a proposal that chemokine receptor activation followed a generalized model divided into two sites and two events; chemokine binding and subsequent receptor activation[149]. This view would also line up with data showing that the biased signaling properties, i.e. pathway-selective receptor activation, can be strongly influenced by modifications to the chemokine N-terminus[48].

Since then, much more information has emerged, including observations that chemokine interactions should more appropriately be viewed as a continuous interaction site, and that some interacting regions appear to contribute to both binding and activation mechanisms[30, 124, 132, 137]. The two-site two-step model also fails to explain some pharmacological observations. In the case of the CCL7-CCR1 interaction, it was presented that the full native binding affinity could not be achieved from the interaction between the receptor N-terminus (CRS1, the step one site one region)[150]. Furthermore, for the interaction of CXCL12 with ACKR3, it was shown that the receptor N-terminus is important for receptor dissociation, but not association of CXCL12, illustrating that another receptor region, i.e. the ECLs or receptor pocket, must be responsible for initial chemokine-receptor binding, and that the receptor N-terminus stabilizes the interaction to allow signaling[151]. In other

cases, it has been reported that the chemokine core domain plays a role in signaling or site 2 interactions[39, 41, 51, 152], that the chemokine N-terminus affects binding affinity or receptor selectivity[87, 135, 153], or that the N-terminus is mostly dispensable to chemokine interactions[131, 154], reinforcing the same conclusion.

The data therefore no longer back up viewing chemokine-receptor interactions as separated into two distinct regions with two distinct roles, and instead it is now necessary to formulate a new interaction mechanism that can encompass available data. So far, it has sensibly been suggested that the interaction be viewed as a continuous interface where any point can contribute to binding and activation, and that multiple steps must be involved in receptor activation, at a minimum including recognition, initial conformational activation, and stabilization of the active chemokine-receptor complex[37, 150, 155].

Here, we focus on a mechanistic perspective that has not received much attention – whether it can be confirmed that the chemokine N-terminus can display independent specific binding, which would mean that it is capable of initial binding to the receptor. It would also mean that the chemokine N-terminus participates in recognition, or specificity, of chemokine-receptor complexes. By applying synthetic chemokine N-termini from CCL3 and CCL5 to receptor signaling assays, we find that these peptides can indeed act independently. We therefore definitively confirm the suggestions by others that the chemokine N-terminus can participate in initial receptor recognition[151].

Surprisingly, we found that a very short segment of CCL3, residues 1-4, could allosterically enhance signaling by the CCL5 full length chemokine at high concentrations, yielding a 50% signal boost. This small CCL3 peptide represents the residues removed to create the naturally occurring CCL3 (5-70) variant[133]. The potency of the N-terminal peptides is inadequate for them to be functional drugs, but their activity both as agonists and positive allosteric modulators highlights some exciting points: 1) Chemokine N-terminal sequences can be used as a starting point for developing higher-affinity drugs that are as selective as chemokines, but fully soluble. 2) The natural N-terminal truncation mechanisms that regulate the activity of many chemokines could perhaps be utilized as the foundation for highly specific allosteric modulators, which we now know can be used both to direct binding and signaling patterns of native chemokines.

Results

To investigate if chemokine N-termini can bind to and activate chemokine receptors with specificity without their globular core domain, we produced N-terminal peptides of representative macrophage inflammatory protein (MIP) and monocyte chemoattractant protein (MCP) chemokines. The interaction network involving these chemokines and their receptors is characterized by highly similar, but still specific, recognition, and thus it is possible to compare the interaction mechanisms of related chemokine-receptor pairs. Based on our experiences derived from manuscript 1, we deemed it likely that major aspects of the recognition of CCL3 and CCL5 could be similar, but only truncation of the CCL5 N-terminus had a negative signaling impact on CCR1 and CCR5, and therefore this peptide appeared more likely to contribute to signaling alone. Additionally,

two past studies had shown that CCL5-derived N-terminal peptides from 5 to 10 residues display anti-HIV activity on CCR5-expressing cells[156, 157]. We also considered that the small segments of peptides that are naturally truncated from chemokine N-termini might be able to affect chemokine activity in some cases. Therefore, we focused on producing and testing the following peptides: CCL5 (1-9) (main MIP peptide representative), CCL2 (1-10) (an MCP non-promiscuous representative), CCL8 (1-10) (an MCP promiscuous representative), and the two short peptides CCL3 (1-4) and CCL5 (1-3) (representatives of the natural truncation process of MIP chemokines).

Since we are essentially testing less than 15% of the original chemokine, we can assume that even if the peptides bind specifically to their receptors, a majority of the interaction affinity could still be contributed from the now absent core domain. Therefore, we needed to obtain large amounts of peptide, which was possible using automated Fmoc solid phase peptide synthesis (SPPS), followed by cleavage from the solid resin and removal of amino acid side chain protecting groups using concentrated acid (TFA) and 1,2-ethanedithiol. We then purified the peptides using reversed-phase (RP) HPLC coupled to mass spectrometry to detect the pure peptide fractions in real time. This yielded milligram quantities of key peptides at high purity (example in figure 8)

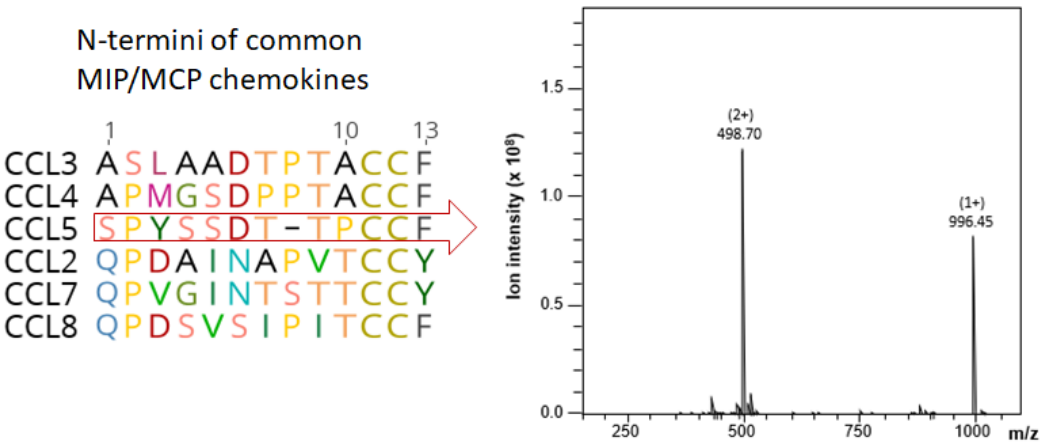


Figure 8 Alignment of select common MIP/MCP chemokine N-termini illustrating conserved and variable sequence features (left). Mass spectrometric analysis of the peptide acetyl (Ac) CCL5 (1-9), showing a single-charged ion-peak at the full peptide mass and a double-charged ion-peak at half the peptide mass (right).

The N-termini of natural chemokines are targets of degradation at varying degrees of specificity under physiological conditions[35, 158], and we therefore tested the most important peptide, CCL5 (1-9), with and without a protective acetyl (AC) group at the N-terminus, and with and without the presence of protease inhibitors (PI). We also considered that the half-life or active receptor residence time could be less than that of an intact chemokine, and addressed this by testing the peptides signaling capacity in two setups: First, a real-time measurement of Gαi-activity (forskolin inhibition) by co-transfecting CHO cells with CCR1 and the cAMP BRET-sensor CAMYEL, which can

measure changing cAMP concentrations in live cells (theoretically peaking at 20-30 minutes after forskolin stimulation, figure 9A). Second, measurement of accumulated Gαi-activity by co-transfection of CCR1 and the chimeric G protein GαΔ6qi4myr, which recognizes Gαi-coupled receptors but initiates the Gαq pathway. In this pathway, turnover of the second messenger inositol phosphates (IPs) can be blocked, enabling measurement of a signal accumulated over time (90 minutes ligand stimulation, figure 9B).

With the BRET system, we found that Ac-CCL5 (1-9) was able to significantly stimulate activity of CCR1, up to approximately 50% of full chemokine activation at 1 mM, with no effect on CCR1-deficient cells (figure 9C). The peptide did however seem more at risk of degradation, since the signal was enhanced by the N-terminal acetylation, and by the addition of broad spectrum protease inhibitors (against aspartic-, cysteine-, serine-, and metalloproteases and aminopeptidases, example result in figure 9D). Furthermore, over an extended time period, the peptide signal could not match 50% of native chemokine activation (example result in figure 9E), although this could also be related to a less stable peptide-receptor complex, if predictions by Stone and colleagues are correct[150]. This loss of activity could partially be recovered in the presence of protease inhibitors (example result in figure 9F).

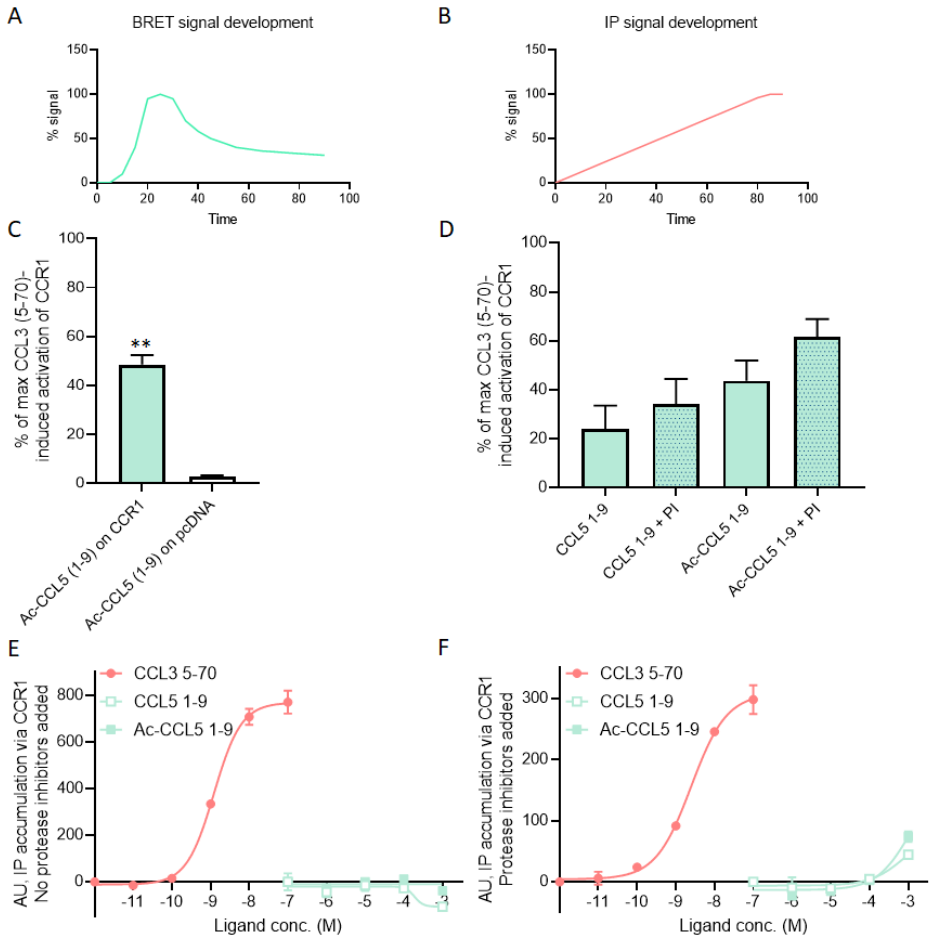


*Figure 9 (A-B) Idealized measurement of G protein signal over time when employing the (A) BRET system (B) or the IP system. (C) Signaling of Ac-CCL5 (1-9) on CCR1, compared to CCL3 (5-70). (D) BRET determination of CCR1 activity by CCL5 (1-9) and Ac-CCL5 (1-9) peptides ± protease inhibitors. (E and F) Accumulation of inositol phosphates over 90min after CCR1 stimulation with low CCL3 (5-70) concentrations or high CCL5 (1-9) concentrations, in the absence (E) or presence (F) of protease inhibitors. **: P ≤ 0.01*

Having determined that the chemokine receptor CCR1 can be partially stimulated with the N-terminus of CCL5 alone, we inferred that the natural role of the N-terminus does not have to follow binding of the chemokine core domain as "step two". This would also mean that the N-terminus may participate in initial receptor recognition. We next set out to determine if the same observations could be made with related chemokine N-termini of the MIP and MCP subgroups, and if the N-termini alone maintained the specificity observed for native chemokines. We found that CCR1 was particularly sensitive to stimulation by N-terminal peptides alone, as CCL8 (1-10) could stimulate the receptor to 42% of full chemokine activation, and even the C-terminally amidated CCL2 (1-10)-NH_2 displayed some agonistic activity, although less than the natural agonists (figure 10A). Shorter peptides representing N-terminal segments reported to be naturally proteolytically removed from CCL3 and CCL5 did not act as receptor agonists (figure 10D, E).

CCR5, which recognizes chemokines in a pattern very similar to CCR1, was surprisingly not activated significantly by any of the chemokine N-terminus-derived peptides (figure 10B). On CCR2, we only

obtained significant activation by the C-terminally amidated CCL2 (1-10)-NH_2 peptide, although the efficacy was only minor (figure 10C).

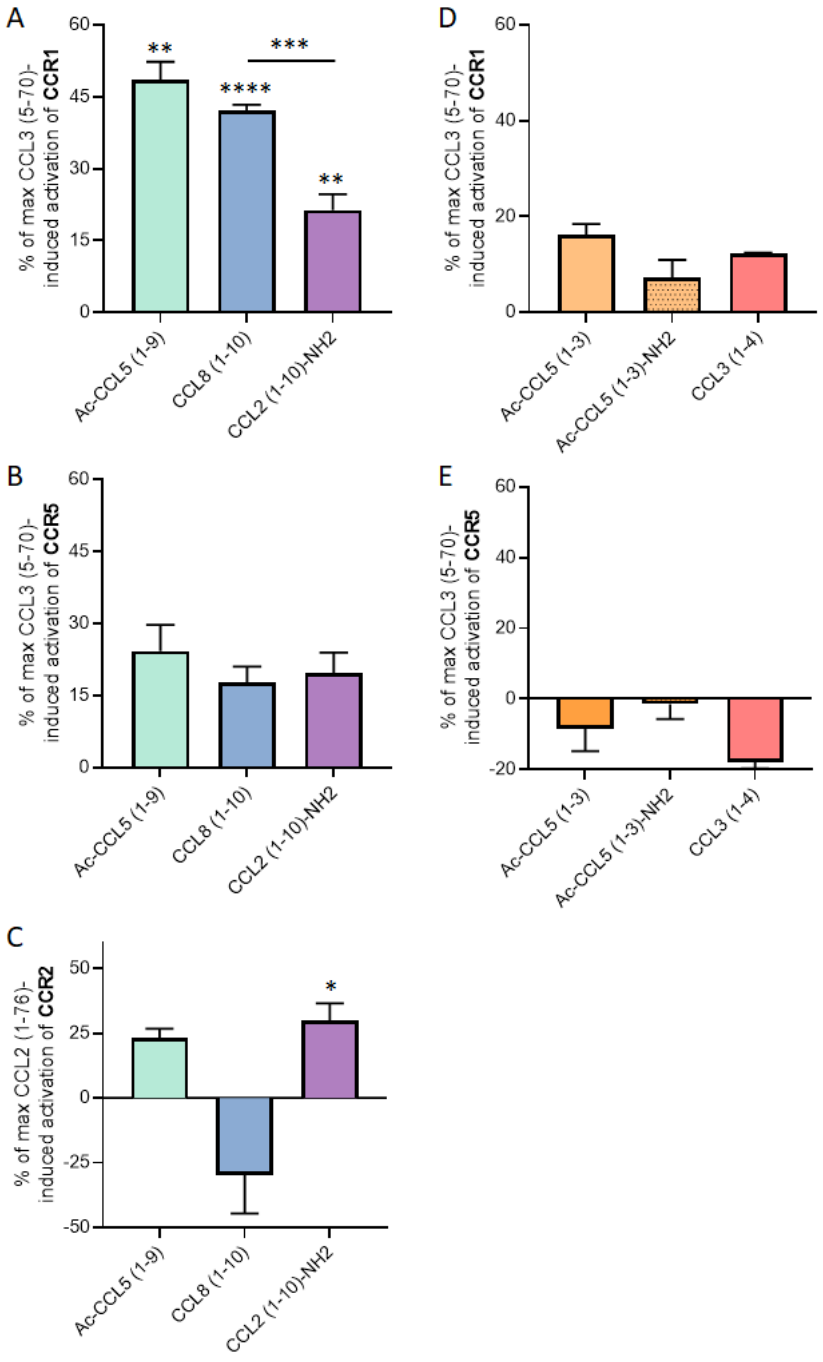


*Figure 10 (A-E) BRET cAMP assays of activity on (A, D) CCR1, (B, E) CCR5 and (C) CCR2, induced by various chemokine-derived N-terminal peptides at 1 mM. Significant activation based on statistical comparison to stimulation of pcDNA-transfected cells, using unpaired two-sided Students t test. *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****: P≤0.0001.*

Since only the longer N-termini-derived peptides could stimulate a chemokine receptor response, we tested if the shorter peptides were instead able to act as allosteric modulators of chemokine-induced receptor activity. This is because the small size of peptides of 3-4 residues could potentially fit in the small receptor pocket space even when a chemokine is bound. CCL3 (1-4) in particular would be interesting because it represents a natural truncation with opposite characteristics compared to the corresponding truncation of CCL5. However, on CCR1, no effect was observed on CCL5 signaling in the presence of CCL3 (1-4) (figure 11A). To our surprise however, applying the same combination as on CCR1 resulted in significant and substantial positive modulation of signal efficacy on CCR5 (by 51%, figure 11B). In previous experiences, we found that truncation of CCL3 was required for allosteric modulation by zinc ion chelators, seemingly because CCL3 residues 1-4 otherwise create steric hindrance of the chelator[87]. However, the modulatory effect of the CCL3 (1-4) peptide seemed to depend on the presence of the extreme N-terminus of the intact CCL5 chemokine, as the positive effect could not be detected for the truncation variant CCL5 (4-68) (figure 11C).

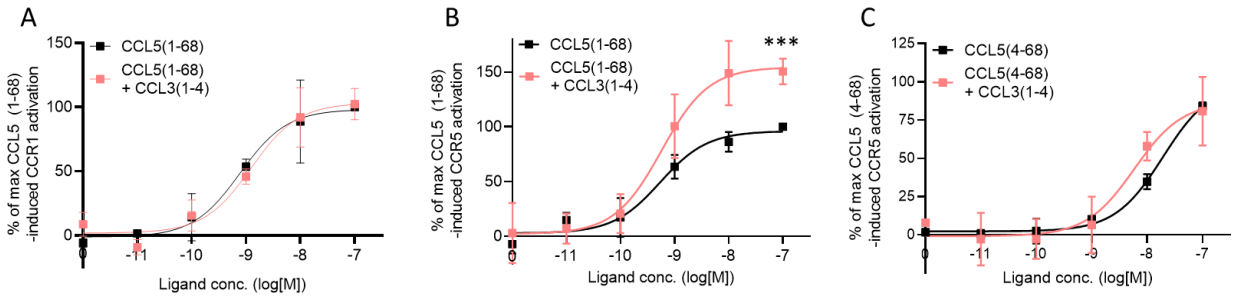


*Figure 11 (A) BRET cAMP assays of stimulation of CCR1 by CCL5 (1-68) ± the presence of CCL3 (1-4) at 1 mM. (B) Stimulation of CCR5 by CCL5 (1-68) ± the presence of CCL3 (1-4). (C) Stimulation of CCR5 (4-68) ± the presence of CCL3 (1-4). ***: P≤0.001.*

The CCL5 N-terminus is one residue shorter than many related MIP and MCP chemokines, so it is possible that the CCL3 (1-4) peptide acts as an allosteric modulator in the receptor pocket. Alternatively, it remains possible that the CCL3 (1-4) peptide could act by interfering with chemokine dimer/multimer formation by binding in the chemokine-chemokine interface, increasing the amount of chemokine available for signaling. Larger peptides, especially the Ac-CCL5 (1-9) peptide, may be more likely to disturb the interface of the natural homodimer that can be formed by CCL5 in solution compared to other peptide sequences, as this dimer involves interactions between the N-terminus and N-loop of the two CCL5 moieties[159-161]. We therefore tested if the peptides Ac-CCL5 (1-9) and CCL8 (1-10) would also be able to produce positive modulation of CCL5 (1-68) on CCR5, but found that this was not the case (figure 12).

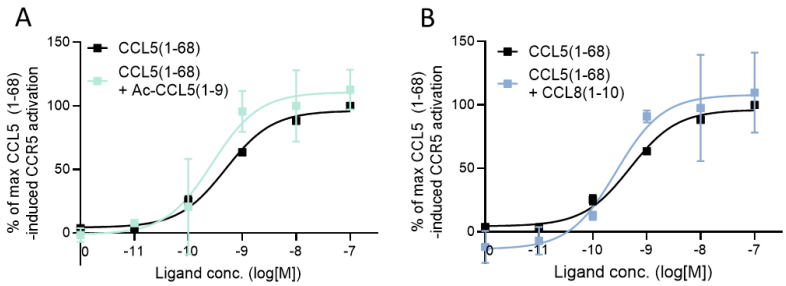


Figure 12 BRET cAMP assays to detect stimulation of CCR5 by CCL5 (1-68) with or without (A) Ac-CCL5 (1-9) or (B) CCL8 (1-10) at 1 mM.

To probe the degree of specificity of the modulation observed for CCL3 (1-4), we also tested whether CCL8 (1-10), derived from a more promiscuous MCP chemokine, could instead modulate an MCP chemokine on the various receptors. Signaling by CCL8 (1-76) on CCR1 and by CCL2 (1-76) on CCR2 was not affected by the presence of CCL8 (1-10) (figure 13A, B). However, we could detect a statistically significant but moderate increase of efficacy (by 24%) on CCR5 (figure 13C).

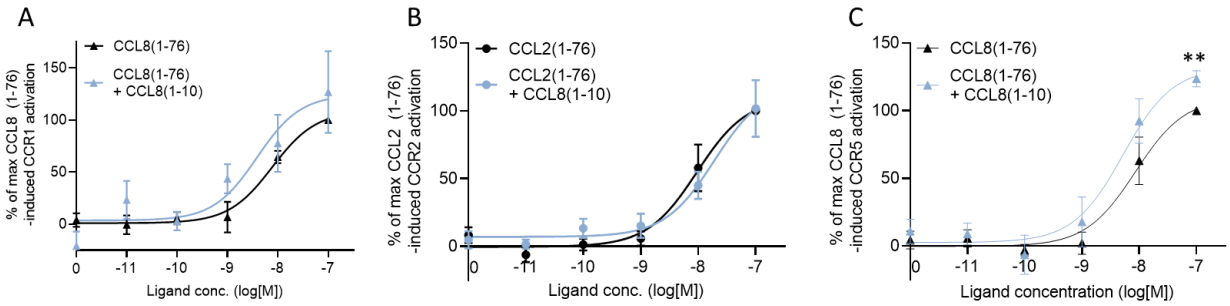


*Figure 13 BRET cAMP assays of stimulation of (A) CCR1 by CCL8 (1-76) ± the presence of CCL8 (1-10), (B) CCR2 by CCL2 (1-76) ± the presence of CCL8 (1-10) and (C) CCR5 by CCL8 (1-76) ± the presence of CCL8 (1-10). **: P≤0.01.*

Discussion

We uncovered two pharmacological properties that CC-chemokine N-termini can confer alone, receptor activation (agonism) and positive signal modulation. This has implications for our view on the role of interactions with the receptor pocket in the future, both mechanistically and for therapeutic targeting. Indeed, the success of drug development projects depends heavily on making correct assumptions about receptor activation mechanisms. In this study, we have learned that the chemokine interaction sites responsible for activation are tailored to each chemokine receptor, which should caution us against extrapolating activation mechanisms across receptors. It also reassures us that the receptor pocket is appropriate for highly specific drug targeting in this most redundant chemokine interaction network.

From a mechanistic perspective, the agonistic activity of short peptides shows that the chemokine N-terminus of intact chemokines cannot credibly be regarded as only “step two” of the interaction mechanism, at least for some ligand-receptor interaction pairs. This is because a step-wise interaction would imply that the receptor cannot accept the N-terminus unless it is in a chemokine core-bound state. A similar finding has been reported for CXCL12, where the free N-terminus was able to bind to CXCR4 with low affinity and stimulate signaling[137, 162]. Furthermore, combinations of the N-termini of CXCL11 and CXCL12 with CXCR3, CXCR4 and ACKR3 showed a specificity that matched the intact chemokines. It was reported that chemokine N-termini may interact differently with subdomains of the binding pocket of CC-, CXC- and atypical chemokine receptors[30, 41, 132, 154]. It is therefore important to affirm that the activity of free N-termini can be observed in different types of chemokine receptors.

We found that the activity of the N-terminal chemokine-derived peptides correlated with the specificity of the intact chemokines, with the notable exception of interactions with CCR5. Based on previous literature, CCR5 should indeed be the exception because it is less sensitive to changes of chemokine N-termini that would terminate signaling on other receptors, including truncations, chimeric N-termini and the AOP- and Met-CCL5 modifications[36, 121, 146, 148]. Other N-terminally truncated MIP chemokines CCL3, CCL4 and CCL14 are also capable of signaling via CCR5[135, 163-165]. On CCR1, chemokine truncations produce a much more variable effect on signaling[166]. However, while the N-terminal peptides in this study did not activate CCR5, they should be able to bind the receptor because several CCL5-based variants have been reported to display anti-CCR5-tropic HIV activity on PBMCs[156, 157].

The different peptide activities on CCR1 and CCR5 could also not be because the N-terminus binds to distinct regions in the two receptors, and therefore must be due to different general receptor activation mechanisms [130, 167]. Overall, this is very difficult to reconcile with a two-step and a two-site interaction, and instead agrees with the idea that there is a continuous interaction interface where separate roles are not easily designated to separate interaction sites, as suggested by others[37, 168]. We can however add that it may be improbable to build an activation model that unifies the broader chemokine receptor family.

The other main finding in this study was the positive modulation of CCL5-CCR5 signaling by CCL3 (1-4). However, it remains to be determined if the peptide accomplishes this by interacting with the receptor or the chemokine. Other small molecule agonists/modulators have been reported to interact with the binding pocket of CCR5 in a manner that both mimics the presumed binding site of the extreme chemokine N-terminus, here represented by the CCL3 (1-4) peptide, and enhances both CCL3 and CCL4 receptor binding[87, 169]. However, it is not obvious from a structural perspective that the small molecule modulators or CCL3 (1-4) would be able to increase the activity of full-length chemokines, especially if they do not enhance the truncated variant, as we saw for CCL3 (1-4). One explanation for this could be if chemokines generally activate CCR5 using the core domain, and that the main requirement of the chemokine N-terminus is rather to regulate that activation. In the [5P7]CCL5-CCR5 structure, it is clear that the chemokine core domain has tilted downwards for

extensive interactions with ECL2 and the major receptor pocket. We know from Manuscript 1 that this pocket is important for receptor activation, but not binding, unless CCL5 is truncated at the N-terminus. In addition, CCL2 and CCL7, which are not CCR5 ligands, will signal through CCR5 if the extracellular recognition domains are replaced by those of CCR2[170, 171], which means that the transmembrane interactions between the chemokine N-terminus and the CCR5 receptor pocket are likely not very specific. CCL3 (1-4) could in that setting improve the activity of CCL5 by taking up space where the CCL5 N-terminus could otherwise limit receptor activation.

Alternatively, CCL3 (1-4) could have an effect by interfering with extracellular oligomerization or glycosaminoglycan (GAG) binding of CCL5. These properties are essential *in vivo* for chemokine-induced inflammation, but are not required for direct receptor interactions[160, 172, 173]. In CC-chemokines, part of the N-terminus and most of the N-loop is often involved with dimerization[161, 174, 175], and dimerization in turn influences extracellular GAG interactions[176-178]. However, monomeric CC-chemokines are still regarded as the state that interacts with cognate receptors[24], and therefore agents that can separate CC-chemokines into monomers could have a positive influence on receptor signaling[179]. Interfering with the GAG-binding properties of chemokines as a strategy to change receptor activity has been demonstrated for other chemokines, where it displayed a decisive effect on immune cell behavior[180, 181].

It remains uncertain if CCL3 (1-4) can positively modulate CCL5-CCR5 signaling by interacting with the receptor or the chemokine, as more data are needed to make that assessment, but it is nonetheless a novel phenomenon. The CCL3 (1-4) modulation we describe is highly specific to the CCL5-CCR5 interaction and depends on the presence of the full CCL5 N-terminus. Furthermore, as a small peptide at 360 Dalton, CCL3 (1-4) could in the future be used as a molecular scaffold to develop higher-potency modulators with a similar specificity, representing an alternative class of chemokine-derived small molecule drug.

In summary, we show that chemokine activation mechanisms vary based on the receptor, and that at least in some receptors, the chemokine N-terminus may participate in the initial receptor recognition and binding. Our data agrees with previous studies showing that the chemokine N-terminus and receptor pocket are of major importance to the stability of the chemokine-receptor complex[150, 151]. Our data also connect to many earlier studies by highlighting a limited role for the chemokine N-terminus in CCR5 signaling. This implies that the core domain may undertake this task almost independently, and contrasts a long-standing mechanistic dogma in the field. Finally, our results show that there is basis for targeting the MIP-chemokine receptors by developing these chemokine-derived sequences, which can lead to compounds with an as of yet unparalleled chemokine-receptor specificity.

5. Overall conclusions

Chemokine signaling is a prerequisite for the participation of immune cells in many disease settings, both where that participation worsens or alleviates a disease. Chemokine receptors, being GPCRs, are expected to be straightforward pharmacological targets, as GPCRs remain the most common type of target for marketed drugs today[182]. However, this has been successful only in situations where the drug targets an exclusive one-to-one ligand-receptor interaction. Many compounds have been developed that target receptors of promiscuous chemokines effectively *in vitro*, but this has not yet led to approved therapeutics[29, 53].

The most promiscuous chemokine network involving CCR1, CCR2 and CCR5 is being investigated for its involvement in multiple disease settings, and plays a particularly important role in peripheral tissue immune responses[68, 101, 104]. It is believed that past efforts to therapeutically target this network of receptors failed mainly because of a lack of drug efficacy or a lack of specificity[79, 105, 136, 183]. However, as we describe, certain characteristics likely must be accounted for first: In this interaction network, chemokines can often use the redundancy feature to "rescue" the effects of a single disabled interaction, at least to maintain major immune cell recruitment[74, 79, 184]. For more specific biological activities, it could be necessary to target unique ligand-receptor interactions, or interaction characteristics, from among a redundant interaction group[136].

In my thesis I approached this challenge by combining available structural data on chemokine-receptor interactions with pharmacological characterization of the interactions that occur in the interaction network. In **manuscript 1**, we show that there is merit in classifying interaction points of overlapping chemokine-receptor complexes into at least three tiers: Common, subgroup-specific and singleton, so that we can distinguish the probable outcomes of targeting receptor regions pharmacologically. We guided our attention to the extracellular segments of TM4 and TM5, and the major receptor pocket, where we did not observe a tight fit for the vMIP-II-CXCR4 and CX_3CL1-US28 complexes compared to the [5P7]CCL5-CCR5 complex. This is because our classification of interaction points does not account for the *steric* hindrances that could occur by pharmacologically targeting regions that are very commonly tightly involved in chemokine-receptor interactions (regardless of *chemical* specificity).

Commonly conserved residues would likely be better to avoid targeting pharmacologically if the aim is to affect specific members and functions of the chemokine system. On chemokines, we found a subgroup-specific sequence conservation that distinguished the MIP chemokines from MCP chemokines, which matched a distinct pharmacological role of a conserved receptor region. Since an alanine mutation of the Cys+4 residue had an effect on other receptors than CCR1 and CCR5, they too might recognize their chemokine ligands in a way similar to MIP recognition. However, only CCR3 recognizes multiple CC chemokine subgroups. Thus, it would therefore be relevant to investigate if CCR3 also distinguishes ligands via the aromatic cluster. The singleton, or non-conserved, residues at the beginning of the MIP chemokine N-termini displayed a highly variable pharmacological role, which appeared to change according to the exact chemokine-receptor pair. This aligned with our expectations as we know the biological role varies to some extent among MIP subgroup members[185-189], which should be reflected in some level of sequence variation. That the

major receptor pocket recognizing the extreme chemokine N-terminus is therefore of great interest. Pharmacological targeting of this area has different characteristics than if the subgroup-specific 30s-loop-ECL2 interface was targeted, because it presumably results in greater selectivity at the cost of decreased robustness in the face of redundancy.

One unexpected but interesting detail when ordering interactions into subgroup-specific and singleton areas was that the N-termini of CCL3 and CCL5 had a moderating role on the overall activity elicited by the chemokines, and appeared to control if the subgroup-conserved interaction with the aromatic receptor cluster could take place. This speaks to the power of the regulatory mechanism of N-terminal truncations, which can reduce the redundancy of MIP chemokines on a case by case basis[36, 135]. It also indicates that drugs designed for high specificity can be efficacious if appropriately applied.

It should likewise be feasible to develop compounds binding to the extracellular side of ECL2 of MIP-chemokine receptors that can modulate interactions with subgroups of chemokines, potentially interfering with some shared immunological effects such as inflammation of peripheral tissues by monocyte-derived cells. As we have discussed in **manuscript 1**, the promise of this approach is that it may defeat several difficulties connected to the druggability of the chemokine system, because **i)** it should overcome the potential redundancy that MIP chemokines could elicit on a single cell, **ii)** it appears to ignore ligand and receptor bias and **iii)** the target site is fully required for the pharmacological efficacy of many MIP chemokines.

Ligands with a redundant receptor recognition pattern can display aspects of specificity in multiple ways, and one that is relatively easy to understand and study is signaling bias. For chemokines, signaling bias is often attributed to interactions between the chemokine N-terminus and the receptor pocket, which indicates that key interaction variations can be found in this region[122, 137]. In **manuscript 2**, we investigated if small molecules targeting the major receptor pocket could be connected to the specific N-terminal variation of MIP chemokines. On wild type CCR1, smaller zinc chelators were able to enhance binding of N-terminally truncated CCL3 more than 3-fold relative to full length CCL3. However, it is important to notice that they must be present at high concentrations to do so. However, mutagenesis of the zinc chelator-binding region indicated the possibility that new variants could be developed with greater affinity or altered function. A number of exposed binding pocket residues were dispensable to both the chelator and chemokine, and they may therefore be available for chelator interactions for increased affinity. Mutating CCR1 Y113 resulted in an inverse agonistic activity of the chelators while they still induced enhanced chemokine binding. Thus chelators that do not interact with this residue would conceivably not display any intrinsic activity unless truncated CCL3 is present.

A lesson learned in these manuscripts should be that chemokine-receptor interactions in redundant networks can be targeted precisely and efficaciously, but that drug binding sites must be selected carefully. In the **extended results**, we test truly isolated interactions between chemokine N-termini and cognate receptors, and describe how the closely related chemokine receptors CCR1, CCR2 and CCR5 may interpret the chemokine N-terminus very differently. In line with results presented in our manuscripts, several chemokine N-terminal peptides can signal through CCR1, whereas some

peptides were able to enhance signaling of native chemokines in their full length form. This difference may make it difficult to design compounds that display a similar activity or antagonism on both CCR1 and CCR5, if they target the receptor pocket. However it may help explain why many such compounds are specific to a single receptor[29].

In similar fashion, it may be wise to be careful about extrapolating future models of receptor activation mechanisms beyond the receptors where experimental data were gathered. This is because a conventional model where the chemokine N-terminus interacts with the receptor pocket only after being guided to this interaction site by the core domain is incompatible with our results. Our data show that the CCL5 and CCL8 N-terminal peptides are independently recognized by CCR1. For CCR5, the same peptides may bind to the receptor without activity, or they may not bind at all. Either way the conclusion must be that the chemokine N-terminus is involved in decidedly different interaction mechanisms in the two receptors. This is certainly inconvenient information in terms of formulating a broadly applicable receptor activation model, but could prove essential in the quest to understand and manipulate the chemokine system in a truly specific and efficacious manner.

Fortunately, it should be feasible to apply the same approaches described in this thesis to other interaction networks. This includes the search for broad interaction determinants of redundant chemokine groups, where not only MIP chemokines are genetically clustered. There exist also MCP, eotaxin, GRO and IP10 regions where key sequences may be conserved. Many more individual chemokines could also be compared at the level of their N-terminal truncation variants to determine when small molecule allosteric modulators can be effective.

Of course, another significant challenge that remains important is to determine whether and when it is appropriate to target either specific or redundant interactions on a biological level[190]. While this is in many ways a separate task, it could actually be easier to first develop a variety of compounds that target specific or redundant interactions, as their effect could be observed in model organisms. It could therefore be of great value to follow up our findings in a drug compound development project to transition our results into biological settings.

6. References

1. Nygaard, R., Frimurer, T.M., Holst, B., Rosenkilde, M.M. & Schwartz, T.W. Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci* **30**, 249-259 (2009).
2. Ballesteros, J.A. & Weinstein, H. [19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors, in *Methods in Neurosciences*, Vol. Volume 25. (ed. C.S. Stuart) 366-428 (Academic Press, 1995).
3. Katritch, V., Cherezov, V. & Stevens, R.C. Structure-function of the G protein-coupled receptor superfamily. *Annu Rev Pharmacol Toxicol* **53**, 531-556 (2013).
4. Zhou, Q. *et al.* Common activation mechanism of class A GPCRs. *Elife* **8** (2019).
5. Venkatakrishnan, A.J. *et al.* Molecular signatures of G-protein-coupled receptors. *Nature* **494**, 185-194 (2013).
6. Wescott, M.P. *et al.* Signal transmission through the CXC chemokine receptor 4 (CXCR4) transmembrane helices. *Proc Natl Acad Sci U S A* **113**, 9928-9933 (2016).
7. Schwartz, T.W., Frimurer, T.M., Holst, B., Rosenkilde, M.M. & Elling, C.E. Molecular mechanism of 7TM receptor activation--a global toggle switch model. *Annu Rev Pharmacol Toxicol* **46**, 481-519 (2006).
8. Alexander, S.P., Mathie, A. & Peters, J.A. Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* **164 Suppl 1**, S1-324 (2011).
9. Zhao, S., Wu, B. & Stevens, R.C. Advancing Chemokine GPCR Structure Based Drug Discovery. *Structure* **27**, 405-408 (2019).
10. Fredriksson, R., Lagerstrom, M.C., Lundin, L.G. & Schioth, H.B. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**, 1256-1272 (2003).
11. Stevens, R.C. *et al.* The GPCR Network: a large-scale collaboration to determine human GPCR structure and function. *Nat Rev Drug Discov* **12**, 25-34 (2013).
12. Simon, M.I., Strathmann, M.P. & Gautam, N. Diversity of G proteins in signal transduction. *Science* **252**, 802-808 (1991).
13. Rasmussen, S.G. *et al.* Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**, 549-555 (2011).
14. Cabrera-Vera, T.M. *et al.* Insights into G protein structure, function, and regulation. *Endocr Rev* **24**, 765-781 (2003).
15. Dorsam, R.T. & Gutkind, J.S. G-protein-coupled receptors and cancer. *Nat Rev Cancer* **7**, 79-94 (2007).
16. Fimia, G.M. & Sassone-Corsi, P. Cyclic AMP signalling. *J Cell Sci* **114**, 1971-1972 (2001).
17. Pearson, G. *et al.* Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* **22**, 153-183 (2001).
18. Pende, M. *et al.* S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**, 3112-3124 (2004).
19. Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S. & Caron, M.G. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem* **274**, 32248-32257 (1999).
20. Vishnivetskiy, S.A. *et al.* How does arrestin respond to the phosphorylated state of rhodopsin? *J Biol Chem* **274**, 11451-11454 (1999).
21. Maxfield, F.R. Role of endosomes and lysosomes in human disease. *Cold Spring Harb Perspect Biol* **6**, a016931 (2014).
22. Baggiolini, M. Chemokines and leukocyte traffic. *Nature* **392**, 565-568 (1998).

23. Servant, G. *et al.* Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287**, 1037-1040 (2000).
24. Allen, S.J., Crown, S.E. & Handel, T.M. Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* **25**, 787-820 (2007).
25. Zlotnik, A. & Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121-127 (2000).
26. Murphy, P.M. *et al.* International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **52**, 145-176 (2000).
27. Thiele, S. & Rosenkilde, M.M. Interaction of chemokines with their receptors--from initial chemokine binding to receptor activating steps. *Curr Med Chem* **21**, 3594-3614 (2014).
28. Pease, J.E., Wang, J., Ponath, P.D. & Murphy, P.M. The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1alpha and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. *J Biol Chem* **273**, 19972-19976 (1998).
29. Scholten, D.J. *et al.* Pharmacological modulation of chemokine receptor function. *Br J Pharmacol* **165**, 1617-1643 (2012).
30. Qin, L. *et al.* Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. *Science* **347**, 1117-1122 (2015).
31. Monteclaro, F.S. & Charo, I.F. The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1alpha receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *J Biol Chem* **271**, 19084-19092 (1996).
32. Wells, T.N. *et al.* Selectivity and antagonism of chemokine receptors. *J Leukoc Biol* **59**, 53-60 (1996).
33. Metzemaekers, M., Van Damme, J., Mortier, A. & Proost, P. Regulation of Chemokine Activity - A Focus on the Role of Dipeptidyl Peptidase IV/CD26. *Front Immunol* **7**, 483 (2016).
34. Proost, P. *et al.* Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor. *J Immunol* **160**, 4034-4041 (1998).
35. Proost, P. *et al.* Chemokine isoforms and processing in inflammation and immunity. *J Autoimmun* **85**, 45-57 (2017).
36. Struyf, S. *et al.* Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor. *Eur J Immunol* **28**, 1262-1271 (1998).
37. Kleist, A.B. *et al.* New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. *Biochem Pharmacol* **114**, 53-68 (2016).
38. Burg, J.S. *et al.* Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. *Science* **347**, 1113-1117 (2015).
39. Wasilko, D.J. *et al.* Structural basis for chemokine receptor CCR6 activation by the endogenous protein ligand CCL20. *Nat Commun* **11**, 3031 (2020).
40. Liu, K. *et al.* Structural basis of CXC chemokine receptor 2 activation and signalling. *Nature* **585**, 135-140 (2020).
41. Zheng, Y. *et al.* Structure of CC Chemokine Receptor 5 with a Potent Chemokine Antagonist Reveals Mechanisms of Chemokine Recognition and Molecular Mimicry by HIV. *Immunity* **46**, 1005-1017 e1005 (2017).
42. Proudfoot, A.E.I., Johnson, Z., Bonvin, P. & Handel, T.M. Glycosaminoglycan Interactions with Chemokines Add Complexity to a Complex System. *Pharmaceuticals (Basel)* **10** (2017).
43. Smith, J.S., Lefkowitz, R.J. & Rajagopal, S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov* **17**, 243-260 (2018).
44. Steen, A., Larsen, O., Thiele, S. & Rosenkilde, M.M. Biased and g protein-independent signaling of chemokine receptors. *Front Immunol* **5**, 277 (2014).

45. Kenakin, T. Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**, 296-302 (2011).
46. Whalen, E.J., Rajagopal, S. & Lefkowitz, R.J. Therapeutic potential of beta-arrestin- and G protein-biased agonists. *Trends Mol Med* **17**, 126-139 (2011).
47. Rajagopal, S. *et al.* Biased agonism as a mechanism for differential signaling by chemokine receptors. *J Biol Chem* **288**, 35039-35048 (2013).
48. Sanchez, J., Lane, J.R., Canals, M. & Stone, M.J. Influence of Chemokine N-Terminal Modification on Biased Agonism at the Chemokine Receptor CCR1. *Int J Mol Sci* **20** (2019).
49. Corbisier, J., Gales, C., Huszagh, A., Parmentier, M. & Springael, J.Y. Biased signaling at chemokine receptors. *J Biol Chem* **290**, 9542-9554 (2015).
50. Forster, R., Davalos-Misslitz, A.C. & Rot, A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* **8**, 362-371 (2008).
51. Hjorto, G.M. *et al.* Differential CCR7 Targeting in Dendritic Cells by Three Naturally Occurring CC-Chemokines. *Front Immunol* **7**, 568 (2016).
52. Zidar, D.A., Violin, J.D., Whalen, E.J. & Lefkowitz, R.J. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc Natl Acad Sci U S A* **106**, 9649-9654 (2009).
53. Bachelerie, F. *et al.* International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* **66**, 1-79 (2014).
54. Nagasawa, T., Tachibana, K. & Kishimoto, T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin Immunol* **10**, 179-185 (1998).
55. Bonecchi, R. & Graham, G.J. Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response. *Front Immunol* **7**, 224 (2016).
56. Rot, A. Chemokine patterning by glycosaminoglycans and interceptors. *Front Biosci (Landmark Ed)* **15**, 645-660 (2010).
57. Fong, A.M. *et al.* Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med* **188**, 1413-1419 (1998).
58. Goda, S. *et al.* CX3C-chemokine, fractalkine-enhanced adhesion of THP-1 cells to endothelial cells through integrin-dependent and -independent mechanisms. *J Immunol* **164**, 4313-4320 (2000).
59. Busillo, J.M. & Benovic, J.L. Regulation of CXCR4 signaling. *Biochim Biophys Acta* **1768**, 952-963 (2007).
60. Campbell, J.J. *et al.* 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. *J Cell Biol* **141**, 1053-1059 (1998).
61. McQuibban, G.A. *et al.* Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* **100**, 1160-1167 (2002).
62. Sandblad, K.G. *et al.* Chemokine receptor expression on monocytes from healthy individuals. *Clin Immunol* **161**, 348-353 (2015).
63. Eltayeb, S. *et al.* Temporal expression and cellular origin of CC chemokine receptors CCR1, CCR2 and CCR5 in the central nervous system: insight into mechanisms of MOG-induced EAE. *J Neuroinflammation* **4**, 14 (2007).
64. Reichel, C.A. *et al.* Chemokine receptors Ccr1, Ccr2, and Ccr5 mediate neutrophil migration to postischemic tissue. *J Leukoc Biol* **79**, 114-122 (2006).
65. Ruster, M., Sperschneider, H., Funfstuck, R., Stein, G. & Grone, H.J. Differential expression of beta-chemokines MCP-1 and RANTES and their receptors CCR1, CCR2, CCR5 in acute rejection and chronic allograft nephropathy of human renal allografts. *Clin Nephrol* **61**, 30-39 (2004).

66. Zhang, L. *et al.* In vivo distribution of the human immunodeficiency virus/simian immunodeficiency virus coreceptors: CXCR4, CCR3, and CCR5. *J Virol* **72**, 5035-5045 (1998).
67. Weber, C. *et al.* Differential chemokine receptor expression and function in human monocyte subpopulations. *J Leukoc Biol* **67**, 699-704 (2000).
68. Dyer, D.P. *et al.* Chemokine Receptor Redundancy and Specificity Are Context Dependent. *Immunity* **50**, 378-389 e375 (2019).
69. Zlotnik, A., Yoshie, O. & Nomiyama, H. The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol* **7**, 243 (2006).
70. Menten, P. *et al.* The LD78beta isoform of MIP-1alpha is the most potent CCR5 agonist and HIV-1-inhibiting chemokine. *J Clin Invest* **104**, R1-5 (1999).
71. Nibbs, R.J., Yang, J., Landau, N.R., Mao, J.H. & Graham, G.J. LD78beta, a non-allelic variant of human MIP-1alpha (LD78alpha), has enhanced receptor interactions and potent HIV suppressive activity. *J Biol Chem* **274**, 17478-17483 (1999).
72. Heath, H. *et al.* Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J Clin Invest* **99**, 178-184 (1997).
73. Teixeira, M.M., Williams, T.J. & Hellewell, P.G. Description of an in vivo model for the assessment of eosinophil chemoattractants in the mouse. *Mem Inst Oswaldo Cruz* **92 Suppl 2**, 211-214 (1997).
74. Uguccioni, M., D'Apuzzo, M., Loetscher, M., Dewald, B. & Baggiolini, M. Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1 alpha and MIP-1 beta on human monocytes. *Eur J Immunol* **25**, 64-68 (1995).
75. Zamilpa, R. *et al.* CC chemokine receptor 5 deletion impairs macrophage activation and induces adverse remodeling following myocardial infarction. *Am J Physiol Heart Circ Physiol* **300**, H1418-1426 (2011).
76. Gu, L. *et al.* Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* **404**, 407-411 (2000).
77. Gschwandtner, M., Derler, R. & Midwood, K.S. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front Immunol* **10**, 2759 (2019).
78. Mantovani, A. The chemokine system: redundancy for robust outputs. *Immunol Today* **20**, 254-257 (1999).
79. Mantovani, A. Redundancy and robustness versus division of labour and specialization in innate immunity. *Semin Immunol* **36**, 28-30 (2018).
80. Lim, J.K., Burns, J.M., Lu, W. & DeVico, A.L. Multiple pathways of amino terminal processing produce two truncated variants of RANTES/CCL5. *J Leukoc Biol* **78**, 442-452 (2005).
81. Lim, J.K., Lu, W., Hartley, O. & DeVico, A.L. N-terminal proteolytic processing by cathepsin G converts RANTES/CCL5 and related analogs into a truncated 4-68 variant. *J Leukoc Biol* **80**, 1395-1404 (2006).
82. Schwartz, J.D. *et al.* Soluble factor(s) released from neutrophils activates endothelial cell matrix metalloproteinase-2. *J Surg Res* **76**, 79-85 (1998).
83. Starr, A.E., Dufour, A., Maier, J. & Overall, C.M. Biochemical analysis of matrix metalloproteinase activation of chemokines CCL15 and CCL23 and increased glycosaminoglycan binding of CCL16. *J Biol Chem* **287**, 5848-5860 (2012).
84. Mortier, A., Gouwy, M., Van Damme, J. & Proost, P. Effect of posttranslational processing on the in vitro and in vivo activity of chemokines. *Exp Cell Res* **317**, 642-654 (2011).
85. Giambernardi, T.A. *et al.* Overview of matrix metalloproteinase expression in cultured human cells. *Matrix Biol* **16**, 483-496 (1998).
86. Itoh, Y. & Seiki, M. MT1-MMP: a potent modifier of pericellular microenvironment. *J Cell Physiol* **206**, 1-8 (2006).
87. Larsen, O. *et al.* Selective Allosteric Modulation of N-Terminally Cleaved, but Not Full Length CCL3 in CCR1. *ACS Pharmacology & Translational Science* **2**, 429-441 (2019).

88. Cynis, H. *et al.* Isolation of an isoenzyme of human glutaminyl cyclase: retention in the Golgi complex suggests involvement in the protein maturation machinery. *J Mol Biol* **379**, 966-980 (2008).
89. Valenti, M.T. *et al.* Increased glutaminyl cyclase expression in peripheral blood of Alzheimer's disease patients. *J Alzheimers Dis* **34**, 263-271 (2013).
90. Cynis, H. *et al.* The isoenzyme of glutaminyl cyclase is an important regulator of monocyte infiltration under inflammatory conditions. *EMBO Mol Med* **3**, 545-558 (2011).
91. Serbina, N.V. & Pamer, E.G. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* **7**, 311-317 (2006).
92. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
93. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
94. de Visser, K.E., Eichten, A. & Coussens, L.M. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6**, 24-37 (2006).
95. Korbecki, J. *et al.* CC Chemokines in a Tumor: A Review of Pro-Cancer and Anti-Cancer Properties of the Ligands of Receptors CCR1, CCR2, CCR3, and CCR4. *Int J Mol Sci* **21** (2020).
96. Ridiandries, A., Tan, J.T. & Bursill, C.A. The Role of CC-Chemokines in the Regulation of Angiogenesis. *Int J Mol Sci* **17** (2016).
97. Lazennec, G. & Richmond, A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med* **16**, 133-144 (2010).
98. Getschman, A.E. *et al.* Protein engineering of the chemokine CCL20 prevents psoriasiform dermatitis in an IL-23-dependent murine model. *Proc Natl Acad Sci U S A* **114**, 12460-12465 (2017).
99. Harper, E.G. *et al.* Th17 cytokines stimulate CCL20 expression in keratinocytes in vitro and in vivo: implications for psoriasis pathogenesis. *J Invest Dermatol* **129**, 2175-2183 (2009).
100. Wu, Y. & Li, J. Expression of CC chemokine ligand 20 and CC chemokine receptor 6 mRNA in patients with psoriasis vulgaris. *J Huazhong Univ Sci Technolog Med Sci* **24**, 297-299 (2004).
101. Miyabe, Y., Lian, J., Miyabe, C. & Luster, A.D. Chemokines in rheumatic diseases: pathogenic role and therapeutic implications. *Nat Rev Rheumatol* **15**, 731-746 (2019).
102. Haringman, J.J., Smeets, T.J., Reinders-Blankert, P. & Tak, P.P. Chemokine and chemokine receptor expression in paired peripheral blood mononuclear cells and synovial tissue of patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis. *Ann Rheum Dis* **65**, 294-300 (2006).
103. Garcia-Vicuna, R. *et al.* CC and CXC chemokine receptors mediate migration, proliferation, and matrix metalloproteinase production by fibroblast-like synoviocytes from rheumatoid arthritis patients. *Arthritis Rheum* **50**, 3866-3877 (2004).
104. Mollica Poeta, V., Massara, M., Capucetti, A. & Bonecchi, R. Chemokines and Chemokine Receptors: New Targets for Cancer Immunotherapy. *Front Immunol* **10**, 379 (2019).
105. Schall, T.J. & Proudfoot, A.E. Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat Rev Immunol* **11**, 355-363 (2011).
106. Viola, A. & Luster, A.D. Chemokines and their receptors: drug targets in immunity and inflammation. *Annu Rev Pharmacol Toxicol* **48**, 171-197 (2008).
107. Hardy, W.D. *et al.* Two-year safety and virologic efficacy of maraviroc in treatment-experienced patients with CCR5-tropic HIV-1 infection: 96-week combined analysis of MOTIVATE 1 and 2. *J Acquir Immune Defic Syndr* **55**, 558-564 (2010).
108. Gulick, R.M. *et al.* Maraviroc for previously treated patients with R5 HIV-1 infection. *N Engl J Med* **359**, 1429-1441 (2008).
109. Shaik, M.M. *et al.* Structural basis of coreceptor recognition by HIV-1 envelope spike. *Nature* **565**, 318-323 (2019).
110. Lee, B. *et al.* Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J Biol Chem* **274**, 9617-9626 (1999).

111. Hendrix, C.W. *et al.* Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J Acquir Immune Defic Syndr* **37**, 1253-1262 (2004).
112. Liles, W.C. *et al.* Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. *Blood* **102**, 2728-2730 (2003).
113. DiPersio, J.F. *et al.* Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *J Clin Oncol* **27**, 4767-4773 (2009).
114. Mohle, R. *et al.* The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood* **91**, 4523-4530 (1998).
115. Nademanee, A.P. *et al.* Plerixafor plus granulocyte colony-stimulating factor versus placebo plus granulocyte colony-stimulating factor for mobilization of CD34(+) hematopoietic stem cells in patients with multiple myeloma and low peripheral blood CD34(+) cell count: results of a subset analysis of a randomized trial. *Biol Blood Marrow Transplant* **18**, 1564-1572 (2012).
116. Ishida, T. *et al.* Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin Cancer Res* **9**, 3625-3634 (2003).
117. Ishii, T. *et al.* Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. *Clin Cancer Res* **16**, 1520-1531 (2010).
118. Shinkawa, T. *et al.* The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* **278**, 3466-3473 (2003).
119. Vela, M., Aris, M., Llorente, M., Garcia-Sanz, J.A. & Kremer, L. Chemokine receptor-specific antibodies in cancer immunotherapy: achievements and challenges. *Front Immunol* **6**, 12 (2015).
120. Solari, R., Pease, J.E. & Begg, M. "Chemokine receptors as therapeutic targets: Why aren't there more drugs?". *Eur J Pharmacol* **746**, 363-367 (2015).
121. Blanpain, C. *et al.* A chimeric MIP-1alpha/RANTES protein demonstrates the use of different regions of the RANTES protein to bind and activate its receptors. *J Leukoc Biol* **69**, 977-985 (2001).
122. Eiger, D.S., Boldizsar, N., Honeycutt, C.C., Gardner, J. & Rajagopal, S. Biased agonism at chemokine receptors. *Cell Signal* **78**, 109862 (2021).
123. Hauser, A.S., Attwood, M.M., Rask-Andersen, M., Schioth, H.B. & Gloriam, D.E. Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* **16**, 829-842 (2017).
124. Kufareva, I., Gustavsson, M., Zheng, Y., Stephens, B.S. & Handel, T.M. What Do Structures Tell Us About Chemokine Receptor Function and Antagonism? *Annu Rev Biophys* **46**, 175-198 (2017).
125. Shindyalov, I.N. & Bourne, P.E. Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. *Protein Eng* **11**, 739-747 (1998).
126. Peeters, M.C., van Westen, G.J., Li, Q. & AP, I.J. Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends Pharmacol Sci* **32**, 35-42 (2011).
127. Barington, L. *et al.* Role of Conserved Disulfide Bridges and Aromatic Residues in Extracellular Loop 2 of Chemokine Receptor CCR8 for Chemokine and Small Molecule Binding. *J Biol Chem* **291**, 16208-16220 (2016).
128. Rummel, P.C. *et al.* Extracellular disulfide bridges serve different purposes in two homologous chemokine receptors, CCR1 and CCR5. *Mol Pharmacol* **84**, 335-345 (2013).
129. Ai, L.S. & Liao, F. Mutating the four extracellular cysteines in the chemokine receptor CCR6 reveals their differing roles in receptor trafficking, ligand binding, and signaling. *Biochemistry* **41**, 8332-8341 (2002).

130. Blanpain, C. *et al.* The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle. *J Biol Chem* **278**, 5179-5187 (2003).
131. Riutta, S.J. *et al.* Mutational analysis of CCL20 reveals flexibility of N-terminal amino acid composition and length. *J Leukoc Biol* **104**, 423-434 (2018).
132. Huma, Z.E. *et al.* Key determinants of selective binding and activation by the monocyte chemoattractant proteins at the chemokine receptor CCR2. *Sci Signal* **10** (2017).
133. Schutyser, E. *et al.* Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *J Biol Chem* **277**, 24584-24593 (2002).
134. Decock, B., Conings, R., Lenaerts, J.P., Billiau, A. & Van Damme, J. Identification of the monocyte chemotactic protein from human osteosarcoma cells and monocytes: detection of a novel N-terminally processed form. *Biochem Biophys Res Commun* **167**, 904-909 (1990).
135. Guan, E., Wang, J., Roderiquez, G. & Norcross, M.A. Natural truncation of the chemokine MIP-1 beta /CCL4 affects receptor specificity but not anti-HIV-1 activity. *J Biol Chem* **277**, 32348-32352 (2002).
136. Dyer, D.P. Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine mediated leukocyte recruitment. *Immunology* (2020).
137. Szpakowska, M. *et al.* Different contributions of chemokine N-terminal features attest to a different ligand binding mode and a bias towards activation of ACKR3/CXCR7 compared with CXCR4 and CXCR3. *Br J Pharmacol* **175**, 1419-1438 (2018).
138. Marelli, G., Sica, A., Vannucci, L. & Allavena, P. Inflammation as target in cancer therapy. *Curr Opin Pharmacol* **35**, 57-65 (2017).
139. Monteclaro, F.S. & Charo, I.F. The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1. Receptor activation by a pseudo-tethered ligand. *J Biol Chem* **272**, 23186-23190 (1997).
140. Duchesnes, C.E., Murphy, P.M., Williams, T.J. & Pease, J.E. Alanine scanning mutagenesis of the chemokine receptor CCR3 reveals distinct extracellular residues involved in recognition of the eotaxin family of chemokines. *Mol Immunol* **43**, 1221-1231 (2006).
141. Crump, M.P. *et al.* Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J* **16**, 6996-7007 (1997).
142. Hebert, C.A., Vitangcol, R.V. & Baker, J.B. Scanning mutagenesis of interleukin-8 identifies a cluster of residues required for receptor binding. *J Biol Chem* **266**, 18989-18994 (1991).
143. Lowman, H.B. *et al.* Exchanging interleukin-8 and melanoma growth-stimulating activity receptor binding specificities. *J Biol Chem* **271**, 14344-14352 (1996).
144. Fernando, H., Nagle, G.T. & Rajarathnam, K. Thermodynamic characterization of interleukin-8 monomer binding to CXCR1 receptor N-terminal domain. *FEBS J* **274**, 241-251 (2007).
145. Elsner, J. *et al.* The CC chemokine antagonist Met-RANTES inhibits eosinophil effector functions through the chemokine receptors CCR1 and CCR3. *Eur J Immunol* **27**, 2892-2898 (1997).
146. Kiss, D.L., Longden, J., Fechner, G.A. & Avery, V.M. The functional antagonist Met-RANTES: a modified agonist that induces differential CCR5 trafficking. *Cell Mol Biol Lett* **14**, 537-547 (2009).
147. Proudfoot, A.E. *et al.* Amino-terminally modified RANTES analogues demonstrate differential effects on RANTES receptors. *J Biol Chem* **274**, 32478-32485 (1999).
148. Elsner, J. *et al.* Differential activation of CC chemokine receptors by AOP-RANTES. *J Biol Chem* **275**, 7787-7794 (2000).
149. Szpakowska, M. *et al.* Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. *Biochem Pharmacol* **84**, 1366-1380 (2012).
150. Sanchez, J. *et al.* Evaluation and extension of the two-site, two-step model for binding and activation of the chemokine receptor CCR1. *J Biol Chem* **294**, 3464-3475 (2019).

151. Gustavsson, M., Dyer, D.P., Zhao, C. & Handel, T.M. Kinetics of CXCL12 binding to atypical chemokine receptor 3 reveal a role for the receptor N terminus in chemokine binding. *Sci Signal* **12** (2019).
152. Jorgensen, A.S. *et al.* Biased Signaling of CCL21 and CCL19 Does Not Rely on N-Terminal Differences, but Markedly on the Chemokine Core Domains and Extracellular Loop 2 of CCR7. *Front Immunol* **10**, 2156 (2019).
153. Fox, J.C. *et al.* Structure-function guided modeling of chemokine-GPCR specificity for the chemokine XCL1 and its receptor XCR1. *Sci Signal* **12** (2019).
154. Miles, T.F. *et al.* Viral GPCR US28 can signal in response to chemokine agonists of nearly unlimited structural degeneracy. *Elife* **7** (2018).
155. Ziarek, J.J. *et al.* Structural basis for chemokine recognition by a G protein-coupled receptor and implications for receptor activation. *Sci Signal* **10** (2017).
156. Nishiyama, Y., Murakami, T., Kurita, K. & Yamamoto, N. Low-molecular-weight anti-HIV-1 peptides from the amino-terminal sequence of RANTES: possible lead compounds for coreceptor-directed anti-HIV-1 agents. *Bioorg Med Chem Lett* **9**, 1357-1360 (1999).
157. Nishiyama, Y., Murakami, T., Shikama, S., Kurita, K. & Yamamoto, N. Anti-HIV-1 peptides derived from partial amino acid sequences of CC-chemokine RANTES. Regulated upon activation, normal T-cell expressed and secreted. *Bioorg Med Chem* **10**, 4113-4117 (2002).
158. Liang, W.G., Ren, M., Zhao, F. & Tang, W.J. Structures of human CCL18, CCL3, and CCL4 reveal molecular determinants for quaternary structures and sensitivity to insulin-degrading enzyme. *J Mol Biol* **427**, 1345-1358 (2015).
159. Czaplewski, L.G. *et al.* Identification of amino acid residues critical for aggregation of human CC chemokines macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, and RANTES. Characterization of active disaggregated chemokine variants. *J Biol Chem* **274**, 16077-16084 (1999).
160. Proudfoot, A.E. *et al.* Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* **100**, 1885-1890 (2003).
161. Liang, W.G. *et al.* Structural basis for oligomerization and glycosaminoglycan binding of CCL5 and CCL3. *Proc Natl Acad Sci U S A* **113**, 5000-5005 (2016).
162. Loetscher, P., Gong, J.H., Dewald, B., Baggiolini, M. & Clark-Lewis, I. N-terminal peptides of stromal cell-derived factor-1 with CXC chemokine receptor 4 agonist and antagonist activities. *J Biol Chem* **273**, 22279-22283 (1998).
163. Proost, P. *et al.* Cleavage by CD26/dipeptidyl peptidase IV converts the chemokine LD78beta into a most efficient monocyte attractant and CCR1 agonist. *Blood* **96**, 1674-1680 (2000).
164. Struyf, S. *et al.* Diverging binding capacities of natural LD78beta isoforms of macrophage inflammatory protein-1alpha to the CC chemokine receptors 1, 3 and 5 affect their anti-HIV-1 activity and chemotactic potencies for neutrophils and eosinophils. *Eur J Immunol* **31**, 2170-2178 (2001).
165. Detheux, M. *et al.* Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor (CCR)1 and CCR5 agonist with anti-HIV properties. *J Exp Med* **192**, 1501-1508 (2000).
166. Vanheule, V., Metzemaekers, M., Janssens, R., Struyf, S. & Proost, P. How post-translational modifications influence the biological activity of chemokines. *Cytokine* **109**, 29-51 (2018).
167. Zoffmann, S., Turcatti, G., Galzi, J., Dahl, M. & Chollet, A. Synthesis and characterization of fluorescent and photoactivatable MIP-1alpha ligands and interactions with chemokine receptors CCR1 and CCR5. *J Med Chem* **44**, 215-222 (2001).
168. Kufareva, I. Chemokines and their receptors: insights from molecular modeling and crystallography. *Curr Opin Pharmacol* **30**, 27-37 (2016).
169. Karlshoj, S. *et al.* Molecular Mechanism of Action for Allosteric Modulators and Agonists in CC-chemokine Receptor 5 (CCR5). *J Biol Chem* **291**, 26860-26874 (2016).
170. Thiele, S. *et al.* Allosteric and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach. *J Biol Chem* **286**, 37543-37554 (2011).

171. Samson, M. *et al.* The second extracellular loop of CCR5 is the major determinant of ligand specificity. *J Biol Chem* **272**, 24934-24941 (1997).
172. Crijns, H., Vanheule, V. & Proost, P. Targeting Chemokine-Glycosaminoglycan Interactions to Inhibit Inflammation. *Front Immunol* **11**, 483 (2020).
173. Appay, V., Brown, A., Cribbes, S., Randle, E. & Czaplewski, L.G. Aggregation of RANTES is responsible for its inflammatory properties. Characterization of nonaggregating, noninflammatory RANTES mutants. *J Biol Chem* **274**, 27505-27512 (1999).
174. Paavola, C.D. *et al.* Monomeric monocyte chemoattractant protein-1 (MCP-1) binds and activates the MCP-1 receptor CCR2B. *J Biol Chem* **273**, 33157-33165 (1998).
175. Li, J.Y., Chen, Y.C., Lee, Y.Z., Huang, C.H. & Sue, S.C. N-terminal Backbone Pairing Shifts in CCL5-(12)AAA(14) Dimer Interface: Structural Significance of the FAY Sequence. *Int J Mol Sci* **21** (2020).
176. McCornack, M.A., Boren, D.M. & LiWang, P.J. Glycosaminoglycan disaccharide alters the dimer dissociation constant of the chemokine MIP-1 beta. *Biochemistry* **43**, 10090-10101 (2004).
177. Zhao, B. & Liwang, P.J. Characterization of the interactions of vMIP-II, and a dimeric variant of vMIP-II, with glycosaminoglycans. *Biochemistry* **49**, 7012-7022 (2010).
178. Dyer, D.P., Salanga, C.L., Volkman, B.F., Kawamura, T. & Handel, T.M. The dependence of chemokine-glycosaminoglycan interactions on chemokine oligomerization. *Glycobiology* **26**, 312-326 (2016).
179. Tan, J.H. *et al.* Design and receptor interactions of obligate dimeric mutant of chemokine monocyte chemoattractant protein-1 (MCP-1). *J Biol Chem* **287**, 14692-14702 (2012).
180. Jorgensen, A.S. *et al.* CCL19 with CCL21-tail displays enhanced glycosaminoglycan binding with retained chemotactic potency in dendritic cells. *J Leukoc Biol* **104**, 401-411 (2018).
181. Vanheule, V. *et al.* The Positively Charged COOH-terminal Glycosaminoglycan-binding CXCL9(74-103) Peptide Inhibits CXCL8-induced Neutrophil Extravasation and Monosodium Urate Crystal-induced Gout in Mice. *J Biol Chem* **290**, 21292-21304 (2015).
182. Rask-Andersen, M., Masuram, S. & Schioth, H.B. The druggable genome: Evaluation of drug targets in clinical trials suggests major shifts in molecular class and indication. *Annu Rev Pharmacol Toxicol* **54**, 9-26 (2014).
183. Roy, I., Getschman, A.E., Volkman, B.F. & Dwinell, M.B. Exploiting agonist biased signaling of chemokines to target cancer. *Mol Carcinog* **56**, 804-813 (2017).
184. Repeke, C.E. *et al.* Evidences of the cooperative role of the chemokines CCL3, CCL4 and CCL5 and its receptors CCR1+ and CCR5+ in RANKL+ cell migration throughout experimental periodontitis in mice. *Bone* **46**, 1122-1130 (2010).
185. Bromley, S.K., Mempel, T.R. & Luster, A.D. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol* **9**, 970-980 (2008).
186. Aldinucci, D. & Colombatti, A. The inflammatory chemokine CCL5 and cancer progression. *Mediators Inflamm* **2014**, 292376 (2014).
187. Li, Y. *et al.* Chemokines CCL2, 3, 14 stimulate macrophage bone marrow homing, proliferation, and polarization in multiple myeloma. *Oncotarget* **6**, 24218-24229 (2015).
188. Liu, L. *et al.* Multiple myeloma hinders erythropoiesis and causes anaemia owing to high levels of CCL3 in the bone marrow microenvironment. *Sci Rep* **10**, 20508 (2020).
189. Castellino, F. *et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* **440**, 890-895 (2006).
190. Anderson, C.A., Solari, R. & Pease, J.E. Biased agonism at chemokine receptors: obstacles or opportunities for drug discovery? *J Leukoc Biol* **99**, 901-909 (2016).

Appendix I: Structural analysis

Chemokines presented in figure 6 were aligned in Pymol using combinatorial extension alignment (CEAlign). The [5P7]CCL5 chain of pdb: 5UIW was used as the core reference alignment structure after it was confirmed that it matched the core domain of CCL5 pdb: 5COY chain A. All non-MCP CC-chemokines and CX_3CL1 were aligned directly to [5P7]CCL5. When more than two chemokine structures were available, a representative structure for use in alignments could be estimated based on structural variations to the N-loop, 3_{10} helical turn, 30s-, 40s- and 50s-loops.

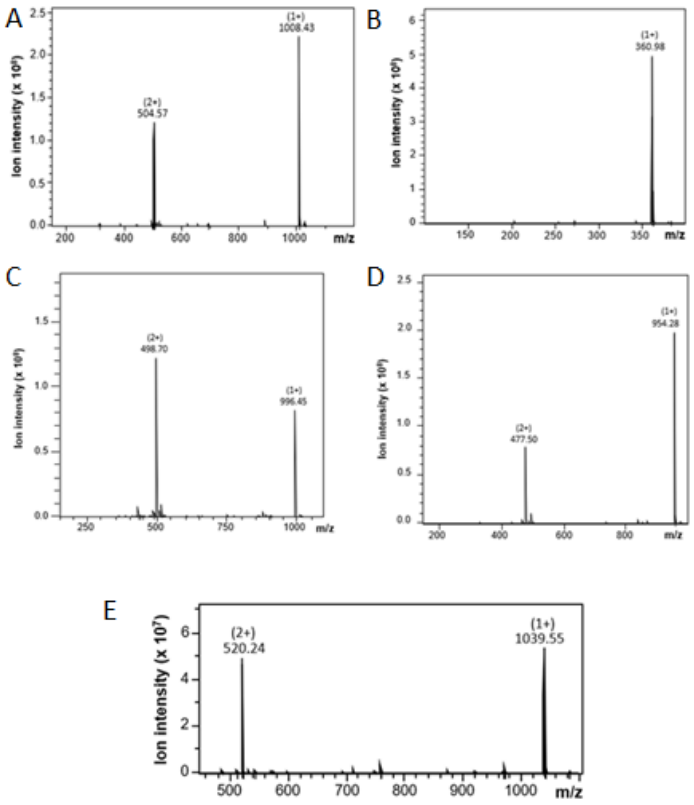
CCL2 and CXCL12 were also aligned directly to [5P7]CCL5, after which they were used as alignment reference for MCP chemokines (CCL1, -7, -8, -11 and -13) and CXC/XC chemokines, respectively.

Pdb references of chemokine structures included in the structural alignments are: CCL1:4OIJ chain A, CCL2: 1DOK chain A, CCL3: 2X69 chain A, CCL4: 1HUM chain A, CCL5: 5UIW chain B, CCL7: 1BO0, CCL8: 1ESR, CCL11: 1EOT, CCL13: 2RA4 chain A, CCL14: 2QR8 chain E, CCL15: 2HCC, CCL16: 5LTL chain A, CCL17: 1NR2 chain A, CCL18: 4MHE chain A, CCL19: 2MP1, CCL20: 1M8A chain A, CCL21: 2L4N state 1, CCL23: 1G91, CCL27: 2KUM state 1, CCL28: 6CWS state 1, CXCL2: 1QNK chain A state 1, CXCL4: 4R9W chain A, CXCL5: 2MGS state 1, CXCL7: 1NAP chain A, CXCL8: 5D14, CXCL10: 1LV9 state 1, CXCL11: 1RJT state 1, CXCL12: 2KEC state 1, CXCL14: 2HDL state 1, XCL1: 2N54 state 1, CX_3CL1: 4XT3 chain B.

Chemokine sequences were aligned with ClustalW in the Geneious software to determine conserved residues. The complete sequence alignment determined 4 residues with at least 93 % conservation (C11, C34, C50 and P53, numbered according to CCL5), and the CC-chemokine alignment determined another 6 residues with at least 80 % conservation (C10, L/I19, F41, T43, V58 and L65). In the MIP chemokine alignment, another 22 conserved residues with at least 80 % conservation were identified, 10 of which were uniquely conserved in the MIP subgroup (Y14, P20, D/E26, F/Y28, E29, S35, R/K44, R/K45, Q48 and S54). The additional conserved residues could also be identified in a separate MCP chemokine alignment, and are thus not included as unique MIP chemokines. An comparable procedure was applied to produce the MCP chemokine alignment.

Appendix II: Mass spectra (MS) of peptides used in this thesis

Peptides were synthesized by solid phase peptide synthesis and purified using HPLC-MS, allowing real-time verification of peptide quality. MS results are presented for CCL2 (1-10) (A), CCL3 (1-4) (B), Ac-CCL5 (1-9) (C), CCL5 (1-9) (D), CCL8 (1-10) (E).



Appendix III: Co-authorship declarations

UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Olav Larsen
E-mail	olavl@sund.ku.dk
Name of principal supervisor	Mette M. Rosenkilde
Title of the PhD thesis	Impact of N-terminal Chemokine Modifications on Cooperativity

2. The declaration applies to the following article	
Title of article	A conserved chemokine receptor motif confers selective engagement between MIP and MCP chemokines
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ■ Date: 02/02-2020	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	B
2. Development of the key methods	D
3. Planning of the experiments and methodology design and development	B
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	C

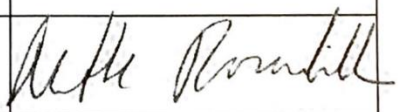
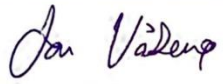
3. The PhD student's contribution to the article *(please use the scale A-F as benchmark)* Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	B

Provide a short description of the PhD student's specific contribution to the article.[i]

Performed pharmacological testing of receptor G protein signaling, binding studies with radioligands, generation of radioligands and produced receptor mutants by PCR. Performed analysis of data, analysis of available structural data, literature study for scientific reference/context, hypothesis formulation, writing of the manuscript and coordination with contributing co-authors.

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ■ No: ☐
If yes, please state name of the author and title of thesis / dissertation.	Olav Larsen Role of aromatic receptor residues of ECL2 in chemokine recognition
If the article is part of another author's academic degree, please describe the PhD student's and the author's contributions to the article so that the individual contributions are clearly distinguishable from one another.	The manuscript data presented in figure 2, figure S2 and some data from figure 4 was made during the master thesis of Olav Larsen.

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	19/10-2020	Mette M Rosenkilde	Prof Md PhD	
2.	21/10-2020	JON VÅBENØ	Dr. scient.	

5. Signatures of the co-authors[iii]				
3.	22/10/2020	PAUL PROOST	Prof. PhD	
4.	8/12/2020	Sara Schueremans	MSc	
5.				
6.				
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor
I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge. Date: Principal supervisor:

7. Signature of the PhD student
I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge. Date: PhD student:

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

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[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

5. Signatures of the co-authors[ii]				
3.	24/2-2021	Stefanie Kavlshoj	PhD	
4.	26-01-2021	Wijnand van der Velden	MSc	
5.	01/02-2021	Pia Cwarzko Rummel	PhD	
6.	05/02-2021	Martin Gustavsson	PhD	
7.	09/02-2021	MAŠA MAVRI	MSc	
8.	8/3-2021	Mette Rosenkilde	Professor	
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 8/3-2021

Principal supervisor:

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 9/3-2021

PhD student:

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

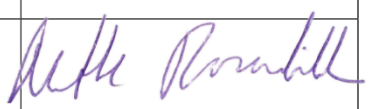
1. Declaration by	
Name of PhD student	Olav Larsen
E-mail	olavl@sund.ku.dk
Name of principal supervisor	Mette M. Rosenkilde
Title of the PhD thesis	Impact of N-terminal Chemokine Modifications on Cooperativity

2. The declaration applies to the following article	
Title of article	Selective Allosteric Modulation of N-terminally Cleaved, but Not Full Length CCL3 in CCR1
Article status	
Published ■ Date: 13/11-2019	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	ACS Pharmacology & Translational Science, 2019, vol 2, page 429-441, DOI 10.1021/acsptsci.9b00059

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	C
2. Development of the key methods	D
3. Planning of the experiments and methodology design and development	C
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	C

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
5. Conducting the analysis of data	C
6. Interpretation of the results	C
7. Writing of the first draft of the manuscript	C
8. Finalisation of the manuscript and submission	B
Provide a short description of the PhD student´s specific contribution to the article.[i] Performed pharmacological testing of receptor G protein signaling, binding studies with radioligands and generation of radioligands. Performed analysis of parts of the data, finalizing of the manuscript and coordination with contributing co-authors. Worked on responses for reviewer comments.	

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ■
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[iii]				
	Date	**Name**	**Title**	**Signature**
1.	19/10-2020	Mette M Rosenkilde	Prof Md PhD	
2.				

5. Signatures of the co-authors[4]				
3.	29.01.2021	Stefanie Karlstad	PhD	S. Karlstad
4.	26-01-2021	Wijnand van der Velden	MSc	W. van der Velden
5.	27-01-2021	Michael Lichtenstein	PhD	M. Lichtenstein
6.	28.01.21	Marta Oliva Santiago	MSc	
7.	08.02.21	Thomas Frimurer	PhD	Thomas Frimurer
8.	8/2-21	TROND ULVEN	PhD	Trond Ulven
9.	10.02.2021	Matjaž BRVAR	PhD	
10.	8/3-2021	Mette Rosenkilde	Professor	Mette Rosenkilde

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 8/3-2021

Principal supervisor: Mette Rosenkilde

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 9/3-2021

PhD student: Ole Larsen

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[4] This can be supplemented with an additional letter if needed.

[5] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

Appendix IV: Publication list

1) van der Velden WJC, Smit FX, Christiansen CB, Møller TC, Hjortø GM, **Larsen O**, Schiellerup SP, Bräuner-Osborne H, Hartmann B, Frimurer TM, Rosenkilde MM.
GLP-1 Val8: A biased GLP-1R agonist with altered binding kinetics and impaired release of pancreatic hormones in rats.
ACS Pharmacol. Transl. Sci. 2021, https://doi.org/10.1021/acsptsci.0c00193

2) **Larsen O**, Lückmann M, van der Velden WJC, Oliva-Santiago M, Brvar M, Ulven T, Frimurer TM, Karlshøj S, Rosenkilde MM.
Selective allosteric modulation of N-terminally cleaved, but not full length CCL3 in CCR1.
ACS Pharmacol. Transl. Sci. 2019, 2 (6), 429-441. https://doi.org/10.1021/acsptsci.9b00059

3) Jørgensen AS, **Larsen O**, Uetz-von Allmen E, Lückmann M, Legler DF, Frimurer TM, Veldkamp CT, Hjortø GM, Rosenkilde MM.
Biased signaling of CCL21 and CCL19 does not rely on N-terminal differences, but markedly on the chemokine core domains and extracellular loop 2 of CCR7.
Front Immunol. 2019;10:2156. Published 2019 Sep 13. doi:10.3389/fimmu.2019.02156

4) Fox JC, Thomas MA, Dishman AF, **Larsen O**, Nakayama T, Yoshie O, Rosenkilde MM, Volkman BF.
Structure-function guided modeling of chemokine-GPCR specificity for the chemokine XCL1 and its receptor XCR1.
Sci Signal. 2019;12(597):eaat4128. Published 2019 Sep 3. doi: 10.1126/scisignal.aat4128

5) Svendsen B, **Larsen O**, Gabe MBN, Christiansen CB, Rosenkilde MM, Drucker DJ, Holst JJ.
Insulin secretion depends on intra-islet Glucagon signaling.
Cell Rep. 2018;25(5):1127–1134.e2. doi:10.1016/j.celrep.2018.10.018

6) Janssens R, Boff D, Ruytinx P, Mortier A, Vanheule V, **Larsen O**, Daugvilaite V, Rosenkilde MM, Noppen S, Liekens S, Schols D, De Meester I, Opdenakker G, Struyf S, Teixeira MM, Amaral FA, Proost P.
Peroxynitrite exposure of CXCL12 impairs monocyte, lymphocyte and endothelial cell chemotaxis, lymphocyte extravasation in vivo and anti-HIV-1 activity.
Front Immunol. 2018;9:1933. Published 2018 Aug 28. doi:10.3389/fimmu.2018.01933

7) Riutta SJ, **Larsen O**, Getschman AE, Rosenkilde MM, Hwang ST, Volkman BF.
Mutational analysis of CCL20 reveals flexibility of N-terminal amino acid composition and length.
J Leukoc Biol. 2018 Aug;104(2):423-434. doi: 10.1002/JLB.1VMA0218-049R.

8) Miles TF, Spiess K, Jude KM, Tsutsumi N, Burg JS, Ingram JR, Waghray D, Hjorto GM, **Larsen O**, Ploegh HL, Rosenkilde MM, Garcia KC.
Viral GPCR US28 can signal in response to chemokine agonists of nearly unlimited structural degeneracy

eLife 2018;7:e35850. DOI: https://doi.org/10.7554/eLife.35850

9) Kuhre RE, Wewer Albrechtsen NJ, **Larsen O**, Jepsen SL, Balk-Møller E, Andersen DB, Deacon CF, Schoonjans K, Reimann F, Gribble FM, Albrechtsen R, Hartmann B, Rosenkilde MM, Holst JJ.
Bile acids are important direct and indirect regulators of the secretion of appetite- and metabolism-regulating hormones from the gut and pancreas.
Mol Metab. 2018 May;11:84-95. doi: 10.1016/j.molmet.2018.03.007. Epub 2018 Mar 17.

10) Getschman AE, Imai Y, **Larsen O**, Peterson FC, Wu X, Rosenkilde MM, Hwang ST, Volkman BF.
Protein engineering of the chemokine CCL20 prevents psoriasiform dermatitis in an IL-23-dependent murine model.
Proc Natl Acad Sci U S A. 2017 Nov 21;114(47):12460-12465. doi: 10.1073/pnas.1704958114. Epub 2017 Nov 6.

11) González-Motos V, Jürgens C, Ritter B, Kropp KA, Durán V, **Larsen O**, Binz A, Ouwendijk WJD, Lenac Rovis T, Jonjic S, Verjans GMGM, Sodeik B, Krey T, Bauerfeind R, Schulz TF, Kaufer BB, Kalinke U, Proudfoot AEI, Rosenkilde MM, Viejo-Borbolla A.
Varicella zoster virus glycoprotein C increases chemokine-mediated leukocyte migration.
PLoS Pathog. 2017 May 25;13(5):e1006346. doi: 10.1371/journal.ppat.1006346. eCollection 2017 May.

12) Janssens R, Mortier A, Boff D, Ruytinx P, Gouwy M, Vantilt B, **Larsen O**, Daugvilaite V, Rosenkilde MM, Parmentier M, Noppen S, Liekens S, Van Damme J, Struyf S, Teixeira MM, Amaral FA, Proost P.
Truncation of CXCL12 by CD26 reduces its CXC chemokine receptor 4- and atypical chemokine receptor 3-dependent activity on endothelial cells and lymphocytes.
Biochem Pharmacol. 2017 May 15;132:92-101. doi: 10.1016/j.bcp.2017.03.009. Epub 2017 Mar 18.

13) Hjortø GM*, **Larsen O***, Steen A*, Berg C, Fares S, Hansen M, Ali S, Rosenkilde MM
*Shared first authorship
Differential CCR7 targeting and dendritic cell activation by three naturally occurring CC-chemokines
Front Immunol. 2016 Dec 9;7:568. doi: 10.3389/fimmu.2016.00568.
-*Corrigendum added to include the Carlsberg Foundation as a funding body.*

14) Frank T, Reichel A, **Larsen O**, Stilp A-C, Rosenkilde MM, Stamminger T, Ozawa T, Tschammer N
Attenuation of chemokine receptor function and surface expression as an immunomodulatory strategy employed by HCMV is linked to vGPCR US28
Cell Commun Signal. 2016 Dec 12;14(1):31.

15) Karlshøj S, Amarandi RM, **Larsen O**, Daugvilaite V, Steen A, Brvar M, Pui A, Frimurer TM, Ulven T, Rosenkilde MM.
Molecular mechanism of action for allosteric modulators and agonists in CC-chemokine receptor 5 (CCR5).
J Biol Chem. 2016 Dec 23;291(52):26860-26874. doi: 10.1074/jbc.M116.740183.

16) Hassing HA, Fares S, **Larsen O**, Pad H, Hauge M, Jones RM, Schwartz TW, Hansen HS, Rosenkilde MM.
Biased signaling of lipids and allosteric actions of synthetic molecules for GPR119.
Biochem Pharmacol. 2016 Aug 25. pii: S0006-2952(16)30236-2. doi: 10.1016/j.bcp.2016.08.018.

17) Janssens R, Mortier A, Boff D, Vanheule V, Gouwy M, Franck C, **Larsen O**, Rosenkilde MM, Damme JV, Amaral FA, Teixeira MM, Struyf S, Proost P.
Natural nitration of CXCL12 reduces its signaling capacity and chemotactic activity in vitro and abrogates intra-articular lymphocyte recruitment in vivo.
Oncotarget. 2016 Sep 20;7(38):62439-62459. doi: 10.18632/oncotarget.11516.

18) Barington L, Rummel PC, Lückmann M, Pihl H, **Larsen O**, Daugvilaite V, Johnsen AH, Frimurer TM, Karlshøj S, Rosenkilde MM.
Role of conserved disulphide bridges and aromatic residues in extracellular loop 2 of chemokine receptor CCR8 for chemokine and small molecule binding
J Biol Chem. 2016 Jul 29;291(31):16208-20. doi: 10.1074/jbc.M115.706747. Epub 2016 May 19

19) Milanos L, Brox R, Frank T, Poklukar G, Palmisano R, Waibel R, Einsiedel J, Dürr M, Ivanović-Burmazović I, **Larsen O**, Hjortø GM, Rosenkilde MM, Tschammer N.
Discovery and characterization of biased allosteric agonists of the chemokine receptor CXCR3.
J Med Chem. 2016 Mar 10;59(5):2222-43. doi: 10.1021/acs.jmedchem.5b01965. Epub 2016 Feb 22.

20) Spiess K, Jeppesen MG, Malmgaard-Clausen M, Krzywkowski K, Dulal K, Cheng T, Hjortø GM, **Larsen O**, Burg JS, Jarvis MA, Garcia KC, Zhu H, Kledal TN, Rosenkilde MM.
Rationally designed chemokine-based toxin targeting the viral G protein-coupled receptor US28 potently inhibits cytomegalovirus in vivo
Proc Natl Acad Sci U S A. 2015 Jul 7;112(27):8427-32. doi: 10.1073/pnas.1509392112. Epub 2015 Jun 15.

21) Steen A, **Larsen O**, Thiele S and Rosenkilde MM.
Biased and G protein-independent signaling in chemokine receptors.
Front Immunol. 2014 Jun 23;5:277. doi: 10.3389/fimmu.2014.00277. Review.