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by

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SIGNALING, FUNCTION AND MODULATION OF CXCR3 VARIANTS

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Affidavit

I hereby confirm that the PhD thesis entitled ‘Signaling, function and modulation of CXCR3 variants’ has been written independently and without any other sources than cited.

Luxembourg, 24/05/2022 Name Nathan Reynders

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Table of content

List of Abbreviations

AA: Amino acid
AC: Adenylyl cyclase
ACKR: Atypical chemokine receptor
cAMP: Cyclic adenosine monophosphate
CCKR: Classical chemokine receptor
CCL: CC chemokine ligand
CCR: CC chemokine receptor
CD26: Dipeptidyl peptidase 4
CKR: Chemokine receptor
CRS: Chemokine recognition site
CTL: Cytotoxic T lymphocyte (CD8+)
CXCL: CXC chemokine ligand
CXCR: CXC chemokine receptor
DC: Dendritic cell
DMEM: Dulbecco's modified eagle medium
EC: Endothelial cell
ECL: Extracellular loops
ERK: Extracellular signal-regulated kinase
GAG: Glycosaminoglycans
GDP: Guanosine diphosphate
GPCR: G protein-coupled receptor(s)
GRK: G protein receptor kinases
GTP: Guanosine triphosphate
HEK: Human embryonic kidney
HuMVEC: Human microvascular endothelial cells
ICL: Intracellular loops
IFN: Interferon
KO: Knock-out
mAb: Monoclonal antibody
MMP: matrix metalloproteinases
MPAK: p44/42 MAP kinase
NanoBiT: Nanoluciferase complementation
NanoBRET: Bioluminescence resonance energy transfer
NK: Natural killer
PKA: Protein kinase A
PKC: Protein kinase C
PLC: Phospholipase C
RE: response element
SEM: standard error of the mean
Th: T helper cell
TIL: Tumor-infiltrating leukocytes
TM: Transmembrane
TME: Tumor microenvironment
TNF: Tumor necrosis factor
Tregs: Regulatory T cells
WT: Wild type

Summary

The chemokine-receptors field has been rapidly changing over the last three decades. First thought to only mediate leukocyte trafficking to ensure immune surveillance, their biological roles have been extended to cell proliferation, differentiation and angiogenesis. Their functions in several pathologies, including cancer development, autoimmune diseases, and inflammation have also been described. Furthermore, the identification and characterization of atypical chemokine receptors added another level of complexity to the already intricate chemokine receptor network. At present, around 50 chemokines and 20 chemokine receptors have been identified. Each chemokine-receptor induces specific cellular functions depending on their spatial and temporal expression that result in appropriate physiological responses.

CXCR3 is one of the 18 classical chemokine receptors. Together with CXCL9, CXCL10, and CXCL11, it mediates the trafficking of activated immune cells towards the site of inflammation. Interestingly, this chemokine receptor can be transcribed into three variants: CXCR3-A, CXCR3-B, and CXCR3-Alt. CXCR3-A and –B chemokine receptor variants differ in their amino sequences and display different characteristics: CXCR3-A induces cell proliferation and migration while CXCR3-B has an extended N terminus, shows opposing cellular effects and has been described to induce apoptosis. However, the underlying mechanism for CXCR3-B biology is still unclear. CXCR3-Alt lacks two transmembrane helices and displays an alternated C terminal region compared to CXCR3-A, recognizes all the CXCR3 ligands and its activation leads to its internalization. CXCR3-Alt's signaling properties are yet to be discovered. However, CXCR3-Alt has been recently shown as a positive marker for efficient chemotherapy treatment in bladder cancer.

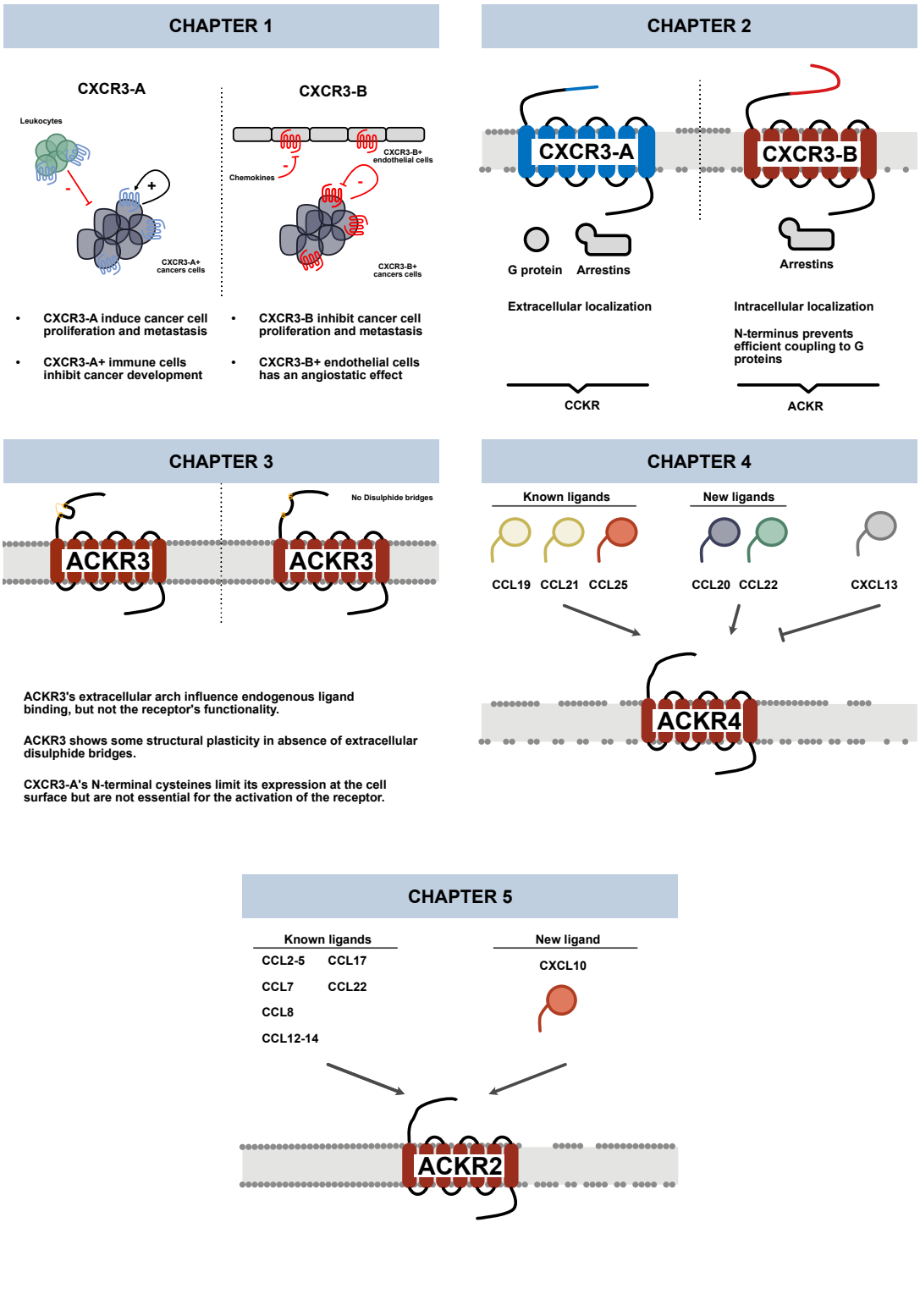
This PhD dissertation particularly focuses on the CXCR3 chemokine receptor variants CXCR3-A and CXCR3-B. In **chapter 1**, an in-depth literature review of their cellular effects in the tumor microenvironment was conducted. Here, we described that CXCR3-A attracts immune cells to the tumor bed to counter its development. However, cancer cells are able to hijack CXCR3-A's ability to promote immune cell proliferation and metastasis. CXCR3-B, on the other hand, attenuates cancer development, as overexpression in these cells reduced their proliferation, as well as angiogenesis.

In the next chapter, a pharmacological comparison of both CXCR3 chemokine receptor variants was performed. We revealed distinct signaling, receptor distribution, and trafficking characteristics for each CXCR3 variant. CXCR3-A induced a strong Gi/o protein activation, β-arrestin recruitment and was internalized following ligand binding. Contrasting to CXCR3-A, CXCR3-B possess many properties of the ACKR subfamily. For instance, CXCR3-B does not induce G protein-mediated signaling while maintaining β-arrestin recruitment capabilities. Furthermore, it is intracellularly localized in the absence of ligands, shows different receptor trafficking patterns following receptor activation, and is able to uptake extracellular CXCR3 chemokines (**chapter 2**). These atypical attributes of CXCR3-B could potentially explain their different biological effects in the tumor environment.

This PhD thesis also contains several studies that do not focus on CXCR3-A and CXCR3-B. In **chapter 3**, a study was conducted to investigate the roles of the two cysteine residues present in the N terminal extracellular domain of ACKR3 and CXCR3 compared to classical chemokine receptors on ligand binding and activation. Here, we showed that ACKR3's activation is less affected by structural changes than classical chemokine receptors and that ACKR3's N terminal arch influences the binding of its endogenous ligands but not its functionality. Similarly, removal of the adjacent cysteines in CXCR3-A's N terminus did not alter its functionally, however it limited its expression at the cell surface.

The last two chapters describes the discovery of new ligands for ACKR2 and ACKR4. By screening all the known chemokines on these two ACKRs, using our highly sensitive β-arrestin recruitment assay, we found that CCL20 and CCL22 are agonists for ACKR4 (**chapter 4**) and CXCL10 a ligand of ACKR2 (**chapter 5**).

Graphical summary



Introduction

1. G protein-coupled receptors

G Protein-Coupled Receptors (GPCRs) form the largest and the most diverse cell membrane receptor family found in humans. They are grouped into 5 subfamilies, according to the GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin) classification system, and can detect a wide variety of extracellular stimuli, including photons, ions, small molecules, and proteins [2, 3]. GPCRs are expressed on various cell types and can regulate many physiological processes, including vision, cell migration, and neurotransmission. However, changes in their structure, expression, or regulation can result in various pathologies, such as cancer, neurological disorders, or hypertension. Consequently, GPCRs are seen as high potential drug targets, which is illustrated by the number of drugs found on the market today that target this family of receptors [4-6].

1.1. GPCR's tertiary structure

GPCRs have highly diverse amino acid (AA) sequences. Yet, their overall tertiary structures are much alike [7, 8]. GPCRs fold into a seven helical structure that transverse the plasma membrane forming an extracellular, a transmembrane (TM), and an intracellular domain (**figure 1**). The extracellular domain comprises an N-terminus and three extracellular loops (ECLs). Together, they recognize and bind most extracellular signals. Moreover, the ECL2 loop differs in AA sequences and one conserved disulphide bridge between the ECL2 and the top of TM3 is found in many GPCRs. The TM domain is composed of 7 TM alpha helices and has a specific architecture that is held together by inter-TM contacts made by position-conserved equivalent AAs. Particularly, the third TM has an important structural role in maintaining the GPCR's fold in an inactive, intermediate, or active state. The intracellular domain consists of three intracellular loops (ICLs) and a flexible C-terminus. This domain forms the interface between the receptor and the different intracellular effectors, such as heterotrimeric G proteins, that transduce the extracellular stimuli into the cellular responses [2] and regulators, including G protein-coupled receptor kinases (GRKs) and arrestins involved in receptor desensitization.

1.2. GPCR activation

GPCRs are dynamic proteins: they can adopt diverse conformational states, for example active or inactive. In the absence of ligand, GPCRs can exhibit different levels of intrinsic or basal activity. However, GPCRs can also shift from an inactive to an active conformation after ligand binding by undergoing structural changes [2, 4]. Based on available structural data of GPCRs within the rhodopsin subfamily in an active and inactive conformation, a general trend in structural rearrangement is observed: activation of the receptor induces changes in the TM3-TM5 and TM5-6-7 interface that results in a narrower binding pocket and a large-scale rearrangement of the cytoplasmic side. The former strengthens the interactions between the ligand and the receptor, while the latter gives rise

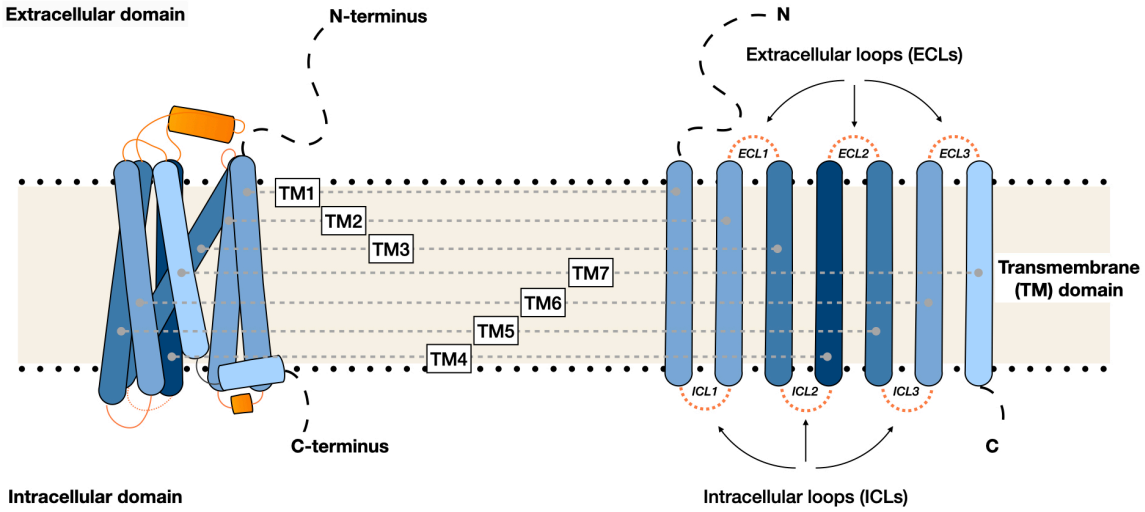


Figure 1 | Topology and structure of GPCRs. GPCRs are composed of 7 transmembrane alpha helices (TM) (blue) that are connected by three extracellular (ECLs) and intracellular loops (ICLs) (orange).

to an opening intracellular side used for the binding of intracellular effectors or regulators. However, not all ligands necessarily fully activate the receptors. Some receptors enter an intermediate conformational state after ligand stimulation and become only fully active once effector molecules or other interacting partners are recruited [2].

The binding of a ligand to a GPCR mostly triggers an intracellular response, which is concentration-dependent. The representation of the cellular response in function of the ligand concentration is called a dose-response curve. From this representation, various types of ligands are observed: full agonists, partial agonists, antagonists, and inverse agonists. A ligand that produces a maximal response at the highest concentration tested is called a full agonist. Ligands that induce only a fraction of the maximal response after binding to the receptor are referred to as partial agonists. Antagonists are unable to elicit a response and inverse agonists can lower a receptor's basal intrinsic activity [9].

1.3. GPCR's signaling and desensitization

GPCRs couple to heterotrimeric G proteins. These protein consist of a Gα subunit (Gαi/o, Gαs, Gαq or Gα12/13) and a heterodimeric complex Gβγ formed by a Gβ and Gγ subunit. Upon receptor activation, G proteins are recruited to the receptor and undergo conformational changes that allow the exchange of GDP for GTP, which leads to the dissociation of the Gα and Gβγ subunits. Once dissociated, each subunit activates effector enzymes, such as adenylyl cyclases (AC), phospholipase C (PLC-β) and ion channels, that induce changes in secondary messenger levels, including cAMP and calcium (**figure 2 and figure 3**). However, most GPCRs only activate one or a set of specific signaling pathways, which is dependent on the activated Gα subunit, due to specific receptor-G protein

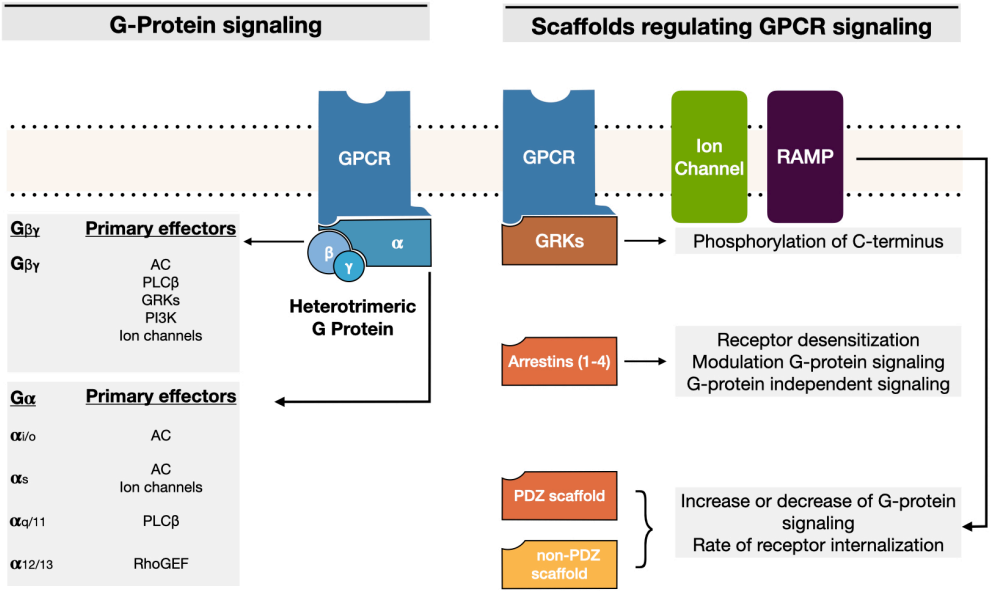


Figure 2 | Schematic representation of GPCR effectors signaling and their function. GPCRs couple to heterotrimeric G proteins (Gα, Gβ and Gγ). Upon activation, the heterotrimeric G protein dissociates into the Gα and Gβγ subunit. Each subunit initiates primary effectors, which leads to distinct signaling events. The G protein specific signaling pathways are mediated by various G protein-modulating proteins, including GRKs and arrestins, PDZ and non-PDZ scaffolds, ion channels and RAMPs. AC: adenylyl cyclase, PLC: phospholipase C, GRK: G protein-coupled receptor kinase, PI3K: phosphoinositide 3-kinases, GEF: guanine nucleotide exchange factor, RAMPs: receptor activity-modifying proteins.

interactions formed by unique residues in the N terminus and C-terminal helix of the Gα subunit and intracellular binding pocket of the receptor [10-12].

To dampen the G protein-mediated signaling, GPCRs need to be desensitized. The latter is initiated by G protein receptor kinases (GRKs) or other proteins, including PKA and PKC, and is mediated by arrestins. GRKs phosphorylate specific threonine and serine residues present in the receptor's C terminal tail and ICLs, generating arrestin-binding bar codes, which are used by arrestins to bind to the receptor, uncouple G proteins from the receptor by steric competition, and initiate receptor endocytosis in a clathrin-dependent or independent manner. After being internalized, the receptor is either targeted for degradation or will be dephosphorylated and recycled back to the cell surface (**figure 3**). Of note, these arrestin bar codes induce different arrestin conformations that influence the binding to the receptor and activation of arrestins. Dependent on the conformation, binding sites for certain effectors are exposed, which regulate the activated G-protein signaling and the receptor's desensitization [5, 13-15].

Besides their role in receptor desensitization, it was proposed that arrestins can initiate the MAPK pathway in a G protein-independent manner [16, 17]. This β-arrestin-dependent signaling was recently questioned using 'zero functional G protein' knockout HEK293 cells. These knock-out cells were unable to phosphorylate ERK proteins and

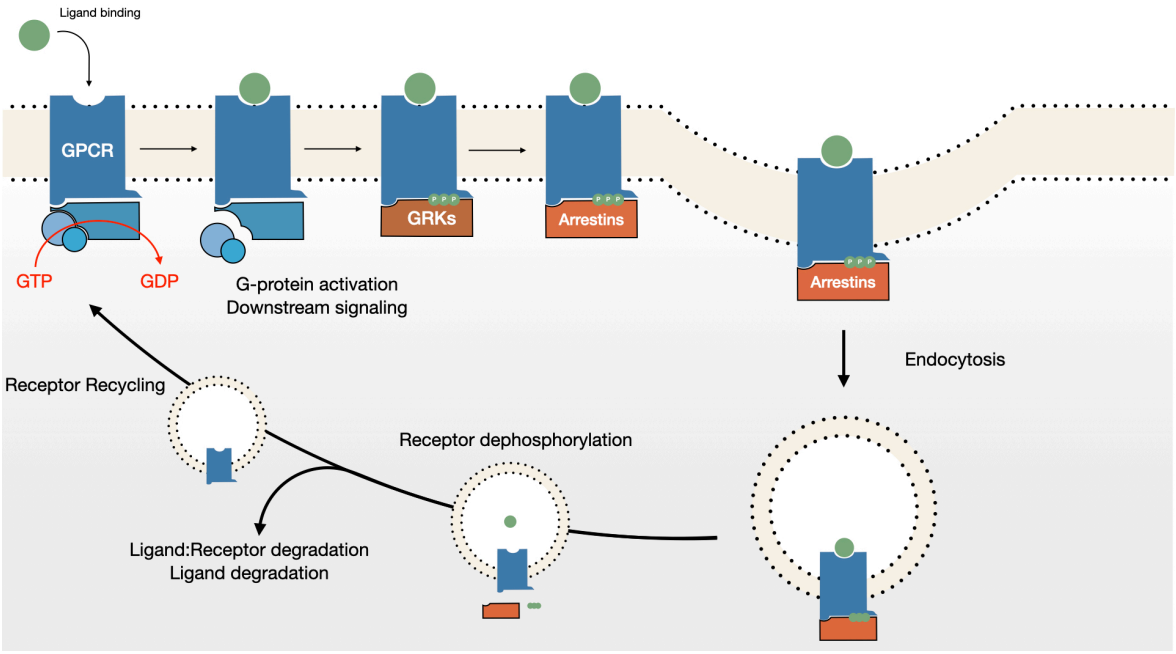


Figure 3 | GPCRs activation and desensitization. The binding of a ligand to its receptor induces structural rearrangements, which promotes the exchange of GDP for GTP that leads to the dissociation and activation of the coupled heterotrimeric G protein. The dissociated G protein subunits activate secondary messengers that initiate the corresponding downstream signaling pathways. The desensitization of the receptors involves the recruitment of G protein-coupled kinases (GRKs) to phosphorylate the receptor's C-terminus. The latter creates a scaffold for the binding of arrestins, which uncouples the receptor-bound G protein, due to steric hindrance, and initiates the internalization of the receptor. Once the ligand-receptor complex is internalized, dissociation of the ligand and arrestin together with the dephosphorylation of the C terminus occur. Subsequently, the receptor is either degraded, together with its ligand, or is recycled back to the plasma membrane.

induce whole-cell responses. However, receptor internalization through β-arrestins was still possible [18, 19]. Yet, another report described a decrease in ERK signaling after down-regulation of β-arrestin, using silencing RNA (siRNA), confirming the β-arrestin-dependent ERK signaling and the unpredictability of cellular effects of G protein and β-arrestin knock-out cells [20]. Together, both studies illustrate the complexity of G protein and β-arrestins functionality in regulating GPCRs and raise caution in interpreting data originated from knockout experiments as the overall complexity of cells cannot be reduced to simplistic models [21].

1.4. Biased signaling of GPCRs

Although GPCRs can trigger numerous downstream signaling events, certain ligands or receptors preferably activate or inhibit particular signaling pathways. This phenomenon is referred to as "biased agonism" or "functional selectivity" and can be linked to any of the components that contribute to GPCR signaling, including the ligand, the receptor, and the system (tissue or cell) that expresses the receptors and the effectors [2, 5].

Ligand bias refers to different ligands that bind to a common GPCR but trigger distinct or preferential downstream signaling pathways. These ligands induce and stabilize different GPCR conformations that result in a different coupling of intracellular effectors, for example, a preferred G protein instead of arrestin coupling. Despite G proteins and

arrestins sharing a common binding site, the selectivity for arrestin or G proteins binding is achieved by slight conformational changes or subtle fluctuations that favor one over the other by strengthening or weakening certain interactions [4, 5, 22]. **Receptor bias** describes the induction of distinct signaling events by two receptors after activation by a common ligand. These two receptors are phylogenetically different or can be generated by alternative splicing of the reference receptor gene resulting in mutations, C-terminal or N-terminal modifications. These modifications change the receptor's overall structure compared to the reference receptor, which alters the receptor's ability to bind certain intracellular effectors resulting in the activation of distinct signaling pathways. For example, a GPCR that lacks phosphorylation sites at its C-terminus will be unable to recruit arrestin and trigger different signaling events compared to a GPCR with phosphorylation sites, while binding a similar ligand. **Tissue bias** is observed when two different tissues, which express the same receptor, induce different biological effects following receptor activation by the same ligand. This bias occurs when the expression of specific effectors or regulators, such as GRKs, regulators of G protein signaling (RGS), arrestins, or the elements of the downstream signaling pathways, differs in a particular cell type or tissue compared to another. For example, a higher expression of a particular GRK or RGS in a cell type could influence a more pronounced arrestin-mediated pathway than in a cell type lacking GRKs, which will induce stronger G protein-mediated signaling (**figure 4**) [5].

1.5. Ligand selectivity of GPCRs

GPCRs can recognize one or multiple endogenous ligands and are able to discriminate them by adopting different architectures of their ligand binding pocket according to their distinct AA sequences. Their different tertiary structures result in orthosteric binding pockets that enable them to specifically accommodate their endogenous ligands and discriminate non-cognate molecules.

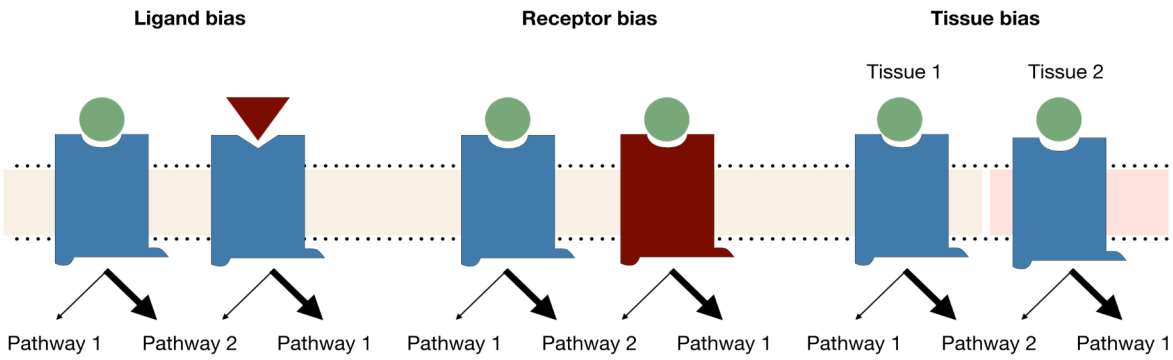


Figure 4 | Biased GPCR signaling. Ligand bias occurs when different ligands that bind to a common GPCR trigger distinct or preferential downstream signaling pathways. **Receptor bias** refers to the induction of distinct signaling events by two receptors after activation by a common ligand. **Tissue bias** is observed when two different tissues, which express the same receptor, induce different biological effects following receptor activation by the same ligand.

For GPCRs within the rhodopsin and secretin subfamily, the orthosteric site is formed by the TM domains and the ECLs and comprises of two binding pockets: the minor binding pockets, which form an interface with the TM1, 2, 3, and TM7 near the N-terminus, and the major binding pocket, which interacts with all TMs except with TM1 and TM2. The major binding pocket is the preferred binding pocket for the endogenous small ligands, while larger ligands, such as peptide and protein ligands, interact with both the minor and the major pocket [23]. The diverse AAs within the orthosteric binding pockets position the different ligands in a particular way that allows them to discriminate endogenous ligands [24, 25]. Additionally, the N-terminus and the ECL2 of the rhodopsin-like GPCRs, for instance, fold into varying, conserved structures, including β-hairpins and short α-helices, which participate in different binding modes depending on the ligand. In particular, they either shield the ligand-binding pocket from the environment, for example the binding of hydrophobic molecules, or leave the orthosteric site water-accessible after the binding of water-soluble ligands [2].

As mentioned above, the orthosteric site of the rhodopsin and secretin GPCR family is similar. However, based on recent cryo-EM structures of two secretin GPCRs, the calcitonin receptor and the GLP-1 receptor with their endogenous ligands, the binding site of these ligands appears to be larger and more extended than the orthosteric site of rhodopsin-like GRPCs [26, 27].

The glutamate subfamily distinguishes itself from the other GPCRs by its extracellular domain, which contains a Venus flytrap that functions as its primary binding site for endogenous ligands, and adhesion GPCRs differ from the other GPCRs by its large, multi-domain N termini and an autocatalytic GPCR proteolysis site. This autoproteolytic cleavage site can expose a tethered ligand, which can activate this receptor’s signaling [28]. Frizzled GPCRs, on the other hand, have a cysteine-rich extracellular region that binds glycoproteins from the Wingless/Int1 (WNT) family. Structural data of WNT-8-FDZ8-dimeric cysteine-rich domain (CRD) complex revealed that the lipid moiety of the ligand lies in a lipophilic groove on the CRD [29]. This binding site differentiates Frizzled GPCRs from other GPCRs subfamilies.

1.6. Alternative splicing of GPCRs

Many genes encoding for GPCRs contain one or multiple introns. Removing or adding one of these introns by alternative mRNA splicing, generates receptor isoforms or receptors with structural differences compared to the reference receptor. These structural changes can occur in many different receptor regions, however, each subfamily within the GPCR shows a ‘preferred’ splicing outcome. For example, isoforms within the secretin receptor family have truncated N terminal ends, which alter their interactions with extracellular matrix proteins and their functionality. In contrast, glutamate receptor isoforms mainly present shorter C terminus, which impairs their oligomerization potential. Alternative splicing of

rhodopsin-like receptors can result in isoform with either N or C terminal truncations [30, 31].

Within a specific tissue, the reference receptor and its isoforms can be expressed at the same time. This simultaneous expression of both receptors could lead to a non-desired tissue response than when the reference receptor or isoform would solely be expressed in the same tissue. This biological effect indicates that isoform-dependent biological effects are important factors to be considered when predicting the outcome of a particular drug [30]. For instance, the adenosine 2A receptor and its isoform, which lacks a phosphorylation site at its C terminus, form dimers with the dopaminergic D2 receptor. However, the dimer formed with the A2 receptors isoform exerts a negative allosteric modulation following the binding of selective A2AR antagonists. This negative modulation was found to be the root cause for the molecule's anti-Parkinsonian effect. Therefore, evaluating the overall expression of the receptor isoforms in particular tissues, as well as understanding the isoform-specific signaling events and structure to develop isoform-specific drugs, would allow to obtain a more precise biological effect that would lead to a desired outcome [31, 32].

2. The chemokine receptor family

2.1. Chemokine receptors and their ligands

Chemokines are small, secreted proteins that belong to the family of the cytokines and were named based on their ability to induce directional migration of leukocytes (chemotaxis). Chemokines vary in molecular weight (8-14 kDa) and share a conserved tertiary structure, despite having a low sequence identity. Their structure consists of an adaptable N-terminus, followed by a cysteine motif and an N-loop, three β-sheets, that are connected by a 30s and 40s loop, a C-terminal α-helix, and a flexible C-terminus (**figure 5**). The cysteine motif forms intramolecular disulfide bridges that are crucial to maintain their conserved fold and allows us to categorize the chemokines into four families (C, CC, CXC, and CX3C). The first three families are separated based on the number of AA in-between the two N-terminal cysteines within the cysteine motif: the CC-family has adjacent cysteines, and the CXC- and CX3C-families have one and three AAs that separates the cysteines, respectively. Also, sub-families can be distinguished based on additional features within the CC and CXC chemokines. CC chemokines can have additional cysteines at their C-terminus and CXC chemokines can be distinguished by the presence or absence of the ELR (glutamic acid-leucine-arginine) motif. Exceptions are the two chemokines CX3CL1 and CXCL16, which have an α-helical transmembrane C-terminal extension [33-35].

Chemokine receptors (CKRs) belong to the rhodopsin-like GPCR family and are divided into two groups: the classical (CCKRs) and atypical (ACKRs) chemokine receptors.

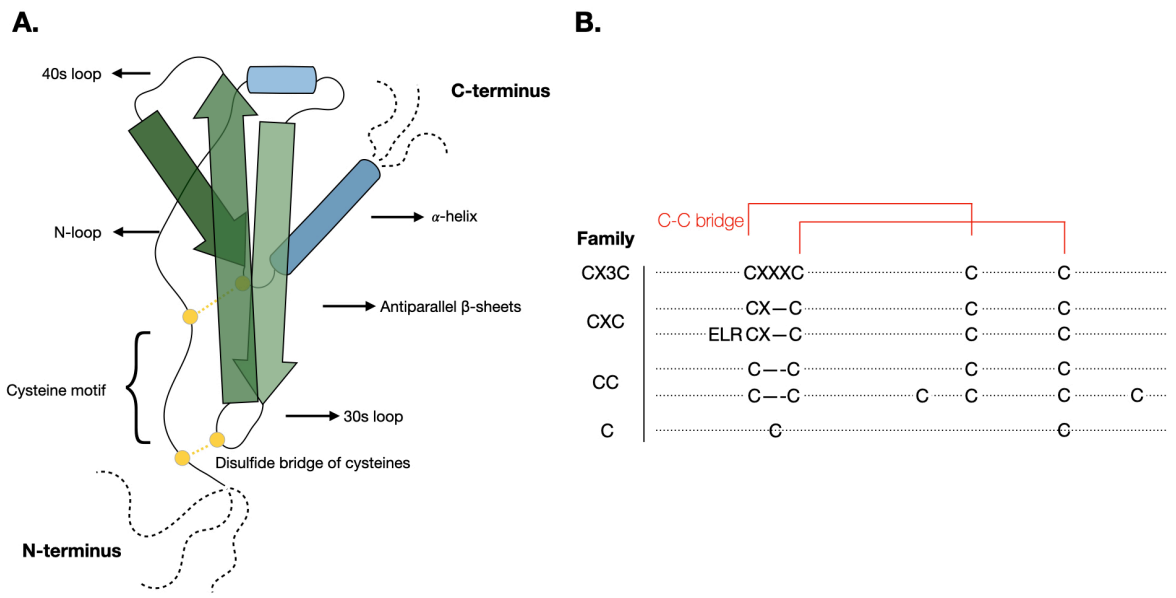


Figure 5 | Schematic representation of the structure and classification of chemokines. (A) Chemokines consist of a flexible N-terminus, followed by three anti-parallel β-sheets, and ends in a C-terminal α-helix and flexible C-terminus. The overall tertiary structure is held together by loops, including N, 30s and 40s loop, and is stabilized by intramolecular disulfide bonds. (B) Schematic classification of chemokines is based on the number and arrangement of their preserved cysteines, C. Linked cysteine residues, by red line, form disulfide bridges. ELR represents the glutamic acid-leucine-arginine motif and X one particular of the 21 amino acids.

The classical chemokine receptors are further classified as CCR, CXCR, XCR, and CX3CR based on the family of chemokines they recognize (**figure 6**). The atypical chemokine receptors form a separate subfamily due to their distinct features that differ from the classical chemokine receptors (explained in section 2.4.2). Each chemokine receptor can bind one or several chemokines and, similarly, a chemokine can bind to one or several CKRs. Moreover, CKRs can interact with various G proteins, while others recruit a particular G protein [35, 36].

2.2. Chemokines and their receptors are multifunctional regulators

The discovery of interleukin-8 (IL-8 or CXCL8)-mediated neutrophil migration revealed the primary function of the chemokine family, namely, to set leukocytes in motion [37]. Consequently, most researchers have focused their effort on elucidating the chemokine-chemokine receptors-axis that positions leukocytes in homeostasis and inflammatory conditions [38]. Due to the dynamic, spatial, and temporal expression of chemokines and their receptors, these are divided into two groups based on their contextual expression: homeostatic or inflammatory conditions [39-41]. Homeostatic chemokines and their receptors are constitutively expressed under normal conditions. They regulate the trafficking of leukocytes to specific tissues to be prepared for upcoming threats. T cells that express CCR7, for example, are recruited to the lymph nodes by CCL19 and CCL20, and CXCL12 attracts CXCR4+ hematopoietic stem cells (HSCs) in the bone marrow to create HSCs rich regions. Inflammatory chemokines are only upregulated at the site of

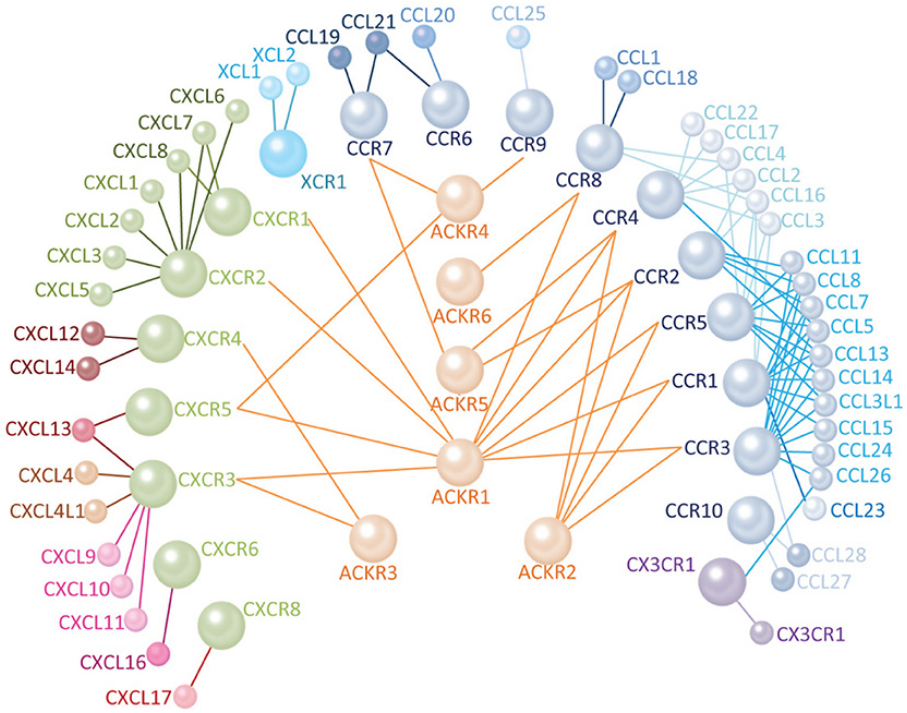


Figure 6 | Schematic overview of the chemokine receptors and their respective chemokines ligands. Adapted from Sommer, F, *et al,* 2020.

inflammation after activation by pro-inflammatory cytokines. Together with their induced CKR expressed on inflammation-regulating leukocytes, inflammatory chemokines attract immune cells to the damaged tissue to mediate the inflammation. Upregulation of CCL2 in inflamed airways, for instance, causes monocytes extravasation from the bone marrow and migrate to the inflamed region. Similarly, activated T cells and neutrophils follow a CXCL9-11 and CXCL1-CXCL8 chemokine gradient towards the damaged or septic tissue. Dual-type chemokines with inflammatory and homeostatic features are also observed. However, depending on the context in which their cognate receptor is expressed, only one feature manifests [37, 42].

Additional functions of chemokines and their receptors, other than the induction of chemotaxis, have also been observed, including the induction of cell adhesion, proliferation, differentiation, angiogenesis, survival, exo-, and endocytosis. All these functions help to maintain immune surveillance, support, and mediate precise immune responses and are also involved in the development of lymphoid tissues [37, 42].

2.3. Chemokine/chemokine receptor interactions

2.3.1. Binding mode of chemokines on chemokine receptors

Before chemokine-receptor structures were obtained, in vitro experiments using mutagenesis, peptides derived from receptor N-termini, or soluble scaffolds containing chemokine receptor elements revealed the importance of the chemokine's N-terminus and global fold. The chemokine's N-terminus interacts with specific AA in the orthosteric site and

the chemokine's globular core and the receptor's extracellular domain form an interplay that determines the overall binding affinity and selectivity of the chemokine necessary to activate the receptor. Based on these observations, a two-site/two-step activation model was proposed. First, the receptor's N-terminus binds the globular core of the chemokines and initiates the chemokine's binding to the extracellular domains of the receptor (interaction is referred to as chemokine recognition site 1, CRS1, and first step). Secondly, the correct binding of the globular core of the chemokine to the receptor orients the chemokine's N-terminus into the helical domain that activates the receptor (referred to as chemokine recognition site 2, CRS2, and second step) [43-45].

The determination of chemokine-receptor structures, including US28-CX3CL1 and CCR5–[5P7-CCL5], and CXCR4-vMIP-2, confirmed and refined the known CRS [46-49]. The distinct binding site for the receptor's N-termini on the chemokine or the CRS1-site could be defined by a groove created by the N-loop and the 40s loop near the 3rd antiparallel β-sheet, despite lacking the full structure density of the N-terminus. Specific binding sites of the chemokine's globular core with the receptor's ECLs were additionally observed. Moreover, each complex showed distinct binding modes suggesting that each chemokine interacts specifically with the extracellular domains of its cognate receptor to ensure their correct positioning. These structures also confirmed the CRS2-site; the interaction of the chemokine's N terminus in the binding pocket of the helical bundle of the receptor. Interestingly, a new chemokine recognition site CRS1.5, positioned in-between CRS1 and CRS2, was unraveled. This CRS1.5 site is proposed to act as a pivot to position the chemokines on the receptor and to amplify the interactions of the receptor's N-terminus with the chemokine's body and the disulfide bond of the receptor N-terminus with ECL3, which aligns the receptor N terminus in the groove of CRS1. Indeed, the superposition of the current structures revealed that all chemokines sit differently on the extracellular parts of the receptor [46-48, 50]. Furthermore, another chemokine recognition site, CRS0.5, has been proposed after the modelling of the ACKR3:CXCL12 complex. This complex showed a distinct interaction site for the end of receptors N terminus with the β1-strand of the CXC chemokines. CRS0.5 is suggested to mimic the CXC chemokine dimerization and could not be detected in the current structural complexes due to the low structural density of this region [51, 52].

Furthermore, to date, various chemokine receptor structures with their natural binding partners, including CCR5-CCL5[6P4]-Gαi, CXCR2-CXCL8-Gαi and CCR6-CCL20-Gαo complex, are available. All these structural data reveal distinct activation processes for each chemokine-receptor partner and provide more insight into the binding mode of different G proteins towards chemokine receptors and GPCRs in general [49, 53, 54].

A recent study functionally investigated the two-site/two-step model using CCR1 N-terminus peptides and chimeras of cognate and non-cognate ligands CCL7 and CCL2,

respectively. Based on the results, a three-step activation model was proposed, in which all two previously described CRS sites contribute to the binding affinity, but a third step is required for the activation of the receptor (**figure 7**). This three-step model considers different kinds of ligands, including cognate and non-cognate ligands, which can interact with the chemokine receptor [55].

Of note, a particular molecular signature of chemokine receptors is the disulfide bridge that connects the N-terminus and ECL3 of the receptor. This pseudo-ECL4 participates in guiding the chemokine into the ligand-binding pocket and in receptor activation [56]. For instance, mutation of the pseudo-ECL4 cysteine residues of chemokine receptor CCR6 impaired its response to CCL20 activation [57].

2.3.2. Chemokine receptor activation and desensitization cycle

The activation of chemokine receptors starts with the binding of a chemokine. This interaction induces conformational changes in the receptor that enables the activation of its associated heterotrimeric G proteins through the exchange of GDP for GTP in its catalytic site. Following the nucleotide exchange, the G protein dissociates into the Gα and Gβγ subunit, each initiating distinct downstream signaling pathways. Chemokine receptors mainly activate Gαi and Gαo proteins. This subunit inhibits the adenylyl cyclase enzyme, which leads to a decrease in the cellular cAMP levels. Together with the Gβγ subunit, increases in the intracellular calcium levels and activation of various signaling pathways, including MAPK/ERK, PKA, and Akt pathways, are observed [58]. Other chemokine receptors, including CXCR1, CXCR3 and CCR5, also activate the Gαq protein following ligand binding. Using the Gαi inhibitor PTX, CXCR1 could not fully reduce the calcium influx

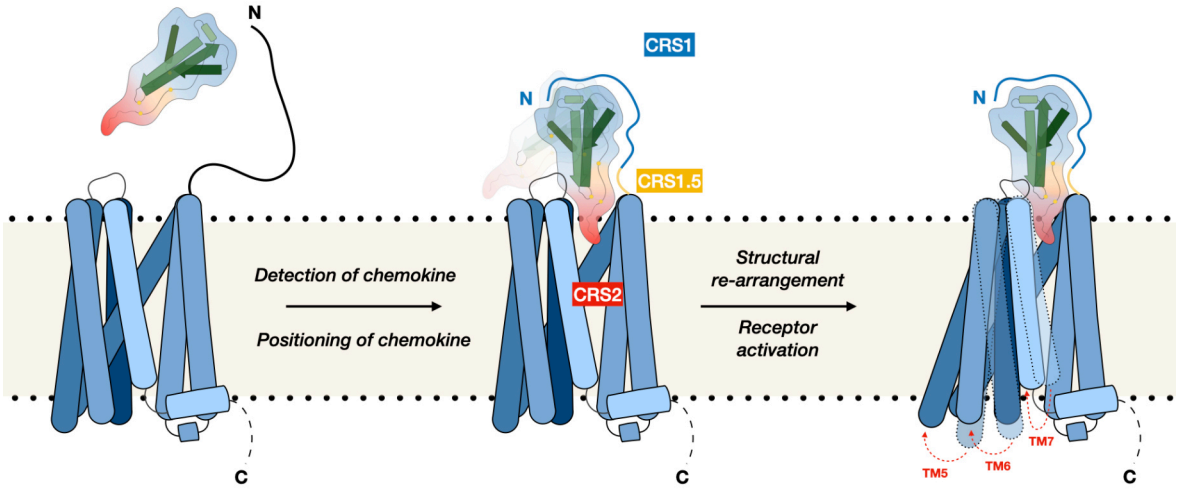


Figure 7 | Mechanistic model of the three site/three step model of chemokine-receptor binding. First, the fast but low affinity binding of the chemokine to the receptor's N-terminus occurs. Next, the chemokine binds slowly and oriented itself on the receptor, which results in high affinity and selective binding. In the last step, a conformational state change occurs that activates the receptor. CRS1 comprises all of the interactions made by the N-terminus of the receptor with the globular core of the chemokine. CRS1.5 site results of the interactions of the residues near the cysteine motif of the chemokine with the residues near the proline in the receptor's N-terminus. CRS2 consists of the binding interactions of the chemokine's N-termini in the receptor's binding pocket.

after IL-8 binding, suggesting a Gαq dependent signaling [59]. In memory CD4+ T cells, activation of CCR5 increases GTP-γS binding to Gαq protein but not in naïve CD4+ T cells in which CCR5 is not expressed [60]. Also, CCR7 and CXCR4 have been shown to induce Gαq dependent signaling events in DCs [61].

The desensitization of activated chemokine receptors is primarily mediated by (GRKs) and arrestins. GRKs, more specifically GRK2, GRK3, GRK5 and GRK6, phosphorylate the chemokine C-terminus, which allows β-arrestins to bind to the receptor, uncouple G proteins and trigger receptor internalization. Once internalized, the chemokine receptors can be targeted for degradation or recycling back to the cell surface after being dissociated from arrestins and dephosphorylated [15].

Chemokine receptors can be internalized even in absence of ligand, such as ACKR3 [62]. The binding of a chemokine to its receptor, however, enhances its internalization and trafficking in the intracellular compartments. The receptors' endocytosis can be mediated in a clathrin or clathrin-independent manner, where the latter is regulated by lipid raft/ caveolae. However, the primary mechanism used by chemokine receptors for their endocytosis is clathrin mediated. This internalization process is dependent on two major adaptor molecules: adaptin-2 and β-arrestin. Once the receptor is associated with these two proteins, clathrin is recruited to form clathrin-coated pits, which will detach from the cell plasma membrane with the help of dynamin. Once the pits are detached, the clathrin molecules will be removed from the vesicle before the receptor enters the endosomal compartments [63]. Recently, the need of β-arrestins to internalize receptors has been challenged for ACKR2, ACKR3 and ACKR4. Using cells lacking those effector proteins, the ACKRs were still able to scavenge extracellular chemokines indicating a β-arrestin independent internalization of the receptors. However, the uptake of the chemokine was less pronounced suggesting that β-arrestins could enhance their scavenging activities [64-67].

Rabs are GTPase proteins that regulate several trafficking events, including chemokine receptor trafficking, and affiliate with the endocytic compartments. One Rab is attached to a distinct endocytic compartment. For example: Rab5 mediates the trafficking of early endosomes, Rab7 regulates the movement of late endosomes towards the lysosome, and Rab11 or Rab4 arbitrates the slow or rapid recycling of endosomal compartments, respectively. Each of these endosomal compartments, together with their associated Rabs, play important roles in the trafficking of the chemokine receptor [63].

2.3.3. Biased signaling of chemokine receptors

The chemokine receptors and their ligands form a highly intricate network, in which many chemokines can bind different chemokine receptors and vice versa. The number of chemokines for the number of chemokine receptors hints towards redundancy. However,

the growing body of evidence on chemokines spatial and temporal expression in homeostasis and during inflammation, their binding potency and efficacy implies that this promiscuity ensures a certain degree of specificity to regulate the chemokines-receptor interactions and to fine-tune the immune response using functional selectivity or biased agonism, that is ligand, receptor, or cellular context-dependent [68, 69].

For instance, the chemokine receptor CCR7 binds the chemokines CCL19 and CCL21. Although both chemokines have been reported to be able to induce G protein activation and arrestin recruitment upon activation, only CCL19 was able to internalize the receptor [70, 71]. Other ligand biases have been described for CCR1, CCR5, CCR10, CXCR1, CXCR2, and CXCR3. CCL23 was shown to be efficient in internalizing CCR1 compared to CCL3 or CCL5, although showing similar G protein signaling and arrestin recruitment profiles. Likewise, both CCL27 and CCL28 reduce cAMP levels through CCR10, but only CCL27 can recruit β-arrestins and trigger receptor internalization [72]. Moreover, chemokine receptors bind different Gαi/o proteins isoforms. CCR5 binds to the Gαi1-3 and Gαoa and Gαob G protein isoforms. CCL5 was shown to have a higher efficacy to activate Gαi1-3 and Gαob isoform in contrast to CCL3 and CCL4 that activates Goa with higher efficacy [73].

Biased signaling can also be chemokine receptor dependent. Chemokine receptor bias has been best exemplified by the classical and atypical chemokine receptors. CXCL12 and CXCL11 trigger G protein activation and arrestin recruitment towards their kindred chemokine receptors, CXCR4 and CXCR3 respectively, yet, both chemokines also bind the atypical chemokine receptors ACKR3, a chemokine receptor that is unable to trigger G protein-mediated signaling but still recruits β-arrestins [74-76].

Tissue biases have also been reported. For example, DCs migrate to secondary lymph nodes, following a CCL19-mediated gradient, through CCR7 activation, while T-cells expressing CCR7 remain unresponsive to CCL19 in the same tissue [77]. This cellular effect has been attributed to a Gαq protein-dependent chemotactic migration of DC, but not of CD4+ T-cells. Additionally, bone marrow-derived neutrophils also showed Gαq protein-dependent chemotactic migration for CCR1, but not for CXCR1 or CXCR2, after chemokine activation [61].

2.4. Chemokine regulation and its impact on receptor activation

2.4.1. Chemokine-receptors post-translational modification

2.4.1.1. N and C terminal truncations of chemokines

Truncation of chemokines can occur at their N or C terminus or chemokines can be cleaved internally. The formers are mediated by various chemokine-modifying proteases, such as dipeptidyl peptidases and members of the matrix metalloproteinases (MMP) family,

while the latter is done by endopeptidases. These proteolytic truncations have been extensively observed in vivo and studied in vitro [78].

The first discovered chemokine, CXCL8, showed high N-terminus heterogeneity in vivo, and functional investigation of these truncated forms revealed enhanced chemotactic activities [88-92]. For many chemokines, like CXCL8, the removal of N-terminal residues results in enhanced activity, including CXCL1, CXCL5, CXCL7, CCL3, CCL4, CCL14, and CCL23. In the case of CXCL7 and CCL14, the cleavage of their N-terminus is a prerequisite to exert their biological role. Each protease recognizes a specific amino acid sequence within the chemokine's N terminus resulting in the cleavage of peptides with different lengths. For example, dipeptidyl peptidases 4, a serine exopeptidase, generate X-proline or X-alanine dipeptides, MMP9 cleaves after lysine or serine and MMP2 can break peptide bonds after arginine or in between a serine and lysine. However, for other chemokines, including CXCL11, this N-terminal post-translational modification results in an impaired chemokine activity on CXCR3, but not for ACKR3 [79-84].

The dipeptidyl peptidase-4 (DDP4 or CD26) is one of the major chemokine modifying proteases. This enzyme removes predominantly the two first N-terminal residues of many known human chemokines, at the X-proline motif site, which are important for the activation of the chemokine receptor. Hence, this protease lowers the activity of most chemokines, including CXCR3 agonists [94-96]. Interestingly, chemokines, including CCL7 and CCL13 can be protected from CD26 cleavage by N-terminal cyclization of their glutamine into pyroglutamic acid [85].

In addition to truncation of the chemokine's N-terminus by matrix metallopeptidases, C-terminal cleavage by these proteases has also been described. For example, the MMP-8 and MMP-9 cleave the C-terminus of the ELR- CXC chemokines CXCL9 and CXCL10 resulting in lower activity [99].

Interestingly, these various chemokine modifying proteases were shown to be upregulated during inflammatory conditions suggesting an important role in mediating the activity and bioavailability of inflammatory chemokines [82, 86].

2.4.1.2. Glycosylation and citrullination of chemokines

The addition of sugars moieties or glycosylation to chemokines has been observed, yet its biological role is still largely unknown. O-glycosylation of CCL11, CCL5, CCL14, or CCL2 has been described, but this post-translational modification did not affect the chemotactic activity for CCL11 or CCL5. However, the glycosylation of CCL2 in its C-terminal region showed an impaired, but more lasting, activity to induce chemotaxis suggesting an improvement in its functional stability [87-91].

Citrullination, or the conversion of arginine to citrulline, impacts the chemokine interaction with their kindred receptor and glycosaminoglycans (GAGs) due to its alternation in the amino acids' charge. Citrullination of CXCL5, CXCL8, CXCL10, CXCL11, and CXCL12 has been detected in vivo, and while this post-translational modification potentiates the chemotactic activity of CXCL8, it impairs the activity of CXCL5, CXCL10, CXCL11, and CXCL12 [92-98].

2.4.1.3. Chemokine receptor post-translational modifications

Chemokine receptors are also subjected to post-translational modifications. These include the formation of cysteine bridges, glycosylation, sulfation, phosphorylation, and ubiquitination. In general, these modifications alter the recognition of the receptor by its cognate chemokine or the ability of the receptor to interact with intracellular effectors and regulators, which results in modified signaling events [78].

The N- or O-glycosylation of the N-terminus of the chemokine receptors CCR5, CCR7, CXCR2, or CXCR4, and their influence on chemokine binding have been described [99-102]. The glycosylation of CCR5, for example, increases the binding affinity of its chemokines, yet it does not alter its binding with HIV-1 [99, 102]. Different leukocyte subsets show distinct glycosylation profiles of the chemokine receptor CCR7, each differently affecting its signaling [100].

The most common post-translational modification of chemokine receptor's N-terminus is the addition of a sulfate group to the extracellular tyrosine. In fact, the addition of this negative charge has been described for many chemokine receptors, including CCR3 [103], CCR5 [104], CXCR3 [105], and CXCR4 [106], which resulted in enhanced chemokine-receptor interaction and activity. Furthermore, it ensures specificity for the different endogenous ligands and has an impact on the dimerization of chemokines. For example, CXCR3's tyrosines at positions 27 and 29 required to be sulfated for the binding and activation of the receptor by CXCL9, CXCL10 as well as CXCL11 [105, 107].

The formation of disulfide bridges between two sulfhydryl-groups from cysteine residues is key to ensure proper folding of the receptors and, consequently, their functionality. The formation of the disulfide bond between the cysteines positioned at the end of the ECL1 and the middle of ECL2, known for most GPCRs from the rhodopsin family, and the chemokine receptor specific disulfide bridge between the receptor's N terminus and ECL3 are key post-translational modifications that in their absence impairs the receptor's activity and chemokine binding [57, 108]. Moreover, in ACKR3 an additional cysteine disulfide bridge, formed by the cysteines located in its N terminus at positions 21 and 26, gives rise to a 4 AA N-terminal loop. This N-terminal loop is unique for the ACKR3 chemokine receptors, although CXCR3 has two adjacent cysteines at position 37 and 38

(see chapter 4). All presently known chemokine receptors do not have analogous cysteines [51, 56, 109, 110].

Further post-translational modifications are the phosphorylation of the C-terminal tails of chemokine receptors, which is needed to bind arrestin and induce receptor desensitization. Palmitoylation and ubiquitination of the receptor also influence the receptors' function and trafficking [63]. CCR5, for instance, has been shown to have palmitoylated cysteines in its C terminus, which plays important roles in receptor distribution. Substitution of palmitoylated cysteines with alanines resulted in CCR5 rich clusters within the endoplasmic reticulum instead of being diffused that resulted in a lower trafficking rate and, ultimately, extracellular expression [111, 112]. Similarly, ubiquitination of ACKR3's C terminus ensures the receptors correct trafficking to and from the plasma membrane. This ubiquitination is reversibly removed following receptor activation with its endogenous ligand CXCL12 [62].

2.4.2. Chemokine oligomerization and implication of GAGs

Chemokines not only bind chemokine receptors to trigger signaling but also can interact with GAGs. This interaction is established by electrostatic interactions formed by positively charged AA from the chemokines and negatively charged residues from GAGs that shape a robust chemokine gradient providing a directional framework to guide immune and non-immune cells. Additionally, chemokines tend to dimerize and form higher-order oligomers on GAGs, due to their cooperative stabilizing properties, which increase the local chemokine concentration resulting from limited chemokine spatial diffusion [33, 113, 114].

Strikingly, the GAG and CKR binding epitopes of chemokines are overlapping, suggesting a negative cooperation. Moreover, the buried N-terminus of CC chemokine in dimers and the low affinity of the N terminus of the monomeric form of CXC chemokines suggest that the GAG-bound chemokines, in dimer or oligomeric form, are unable to bind to their cognate CKR. These properties stand perpendicular to the above-mentioned GAG-chemokine-dependent cell migration [113]. However, studies have described the synergic effects of GAGs and chemokines [115, 116]. For example, the presence of CCL19 and CCL21, ligands for CCR7, have been shown to induce CCR2+ monocyte migration and cellular response at lower CCR2 agonist concentration [117]. This synergetic effect was proposed to be indeed GAG dependent through competitive binding of chemokines. Displacement of chemokines in the proximity of cells expressing its cognate receptor by non-cognate chemokines present in the environment would increase the chemokine specific concentration and its activity on the receptor to fine-tune the chemokine-gradient induced leukocyte migration [113]. The latter has been suggested for the CXCL13 chemokine that mediates the activity of the CCL19, CCL21, and CCL25 for ACKR4 through competitive GAG binding effects [116].

Chemokines are also prone to be proteolyzed by various proteases present in inflamed tissues (explained in section 2.4.3). These proteolyzed chemokines have modified activities towards their kindred receptors. GAGs have been described to protect chemokines from these proteases due to steric hindrance of the chemokine-GAG interaction with the protease binding site or by the formation of chemokine oligomers that shield the protease cleavage site [118-121].

2.4.3. Atypical chemokine receptors

Atypical chemokine receptors behave differently than classical chemokine receptors. As briefly mentioned before, after ligand stimulation, atypical chemokine receptors fail to induce G protein-dependent signaling pathways. Currently, four chemokine receptors (ACKR1, ACKR2, ACKR3, and ACKR4) are members of the atypical chemokine receptor family based on their atypical signaling properties. Although each ACKR has different modes of action they are able to take up chemokines from their environment to modulate the chemokine availability and, in turn, the cognate classical chemokine receptor's activity [122].

ACKR1, previously known as duffy antigen receptor for chemokines (DARC), has the most peculiar features compared to the other ACKRs. Its AA sequence is the most distinct compared to the classical chemokine receptor AA sequence, lacks the DRYLAIV motif, and besides being expressed on endothelial cells (ECs), is the only chemokine receptor expressed on non-nucleated cells (erythrocytes). ACKR1 binds more than 20 inflammatory chemokines from the CC and CXC family and its expression on ECs guides leukocytes to follow the formed gradient across the ECs, referred to as chemokine transcytosis, and regulates angiogenesis by internalizing ELR+ chemokines. ACKR1 positive erythrocytes act as a "sink" or "buffer" allowing ACKR1 to keep a steady chemokine gradient under inflammatory conditions and protect leukocytes from overstimulation in these conditions [123-125]. On venular endothelial cells, ACKR1 acts as a presenter chemokine receptor by binding chemokines and presenting them to classical chemokine receptors [124, 126-128].

ACKR2, formerly known as D6 or CCBP2, was described to majorly recognize the inflammatory CC chemokines [129]. However, its chemokine binding catalog has been recently extended with the binding of the inflammatory CXCL10 chemokine (**chapter 6**). ACKR2 is expressed on ECs and leukocytes, where it is mainly found intracellularly, and internalizes chemokines from the extracellular space. After being internalized and the chemokines targeted for degradation, the receptor recycles back to the plasma membrane. Interestingly, ACKR2 can adapt its scavenging properties to an extent that it can increase its presence at the plasma membrane without affecting its internalization rate [130-133].

ACKR3, formerly CXCR7, binds the homeostatic chemokine CXCL12 and the inflammatory chemokine CXCL11 [134]. This atypical chemokine receptor is also expressed

on ECs and immune cells, and, like ACKR2, it is found intracellularly. ACKR3 has been shown to recycle from its intracellular compartments to the plasma membrane, even in the absence of ligand stimulation, to shape the CXCL12 gradient through its scavenging properties [135]. ACKR3 has also been described to bind non-chemokine ligands. For example, the Kaposi's sarcoma-associated herpesvirus (HHV-8)-encoded CC chemokine vMIP/vCCL2 and various opioid ligands were shown to induce β-arrestin after binding to ACKR3 [136-138]. Activation of ACKR3 by CXCL12 does not induce typical Gαi mediated signaling events, including GTP hydrolysis or calcium mobilization. Yet, in cortical astrocytes and Schann cells, which express CXCR4, ACKR3 was able to induce ERK1/2 and Akt signaling following CXCL12 binding. This response has been attributed to the β-arrestin mediated signaling, as depletion of arrestin by siRNA or blocking of ACKR3 with antagonist attenuated the cellular response [76, 139]. However, these signaling results require further confirmation.

ACKR4, or previously named CCX-CKR, binds and has been shown to scavenge the homeostatic chemokines CCL19 and CCL21. By regulating the CCL19/CCL21 chemokine gradient, ACKR4 regulates the homing of CCR7+ DCs to lymphoid tissues during inflammation and the trafficking and positioning of T cells [140-143]. ACKR4 is mainly expressed on lymphatic ECs, but its expression on other cell types, including epithelial cells and keratinocytes, has been observed [140, 144]. Recently, the binding of CCL20 and CCL22 to ACKR4 has been shown to induce β-arrestin, hereby expanding its chemokine binding partners (**chapter 5**) [145, 146].

Three additional receptors, ACKR5 and ACKR6, also known as CCRL2 and PITPNM3 respectively, and GPR182 have shown characteristics of the atypical chemokine receptors but need to be functionally confirmed before being classified in the ACKR family [122, 147]. However, one cannot exclude the possibility of the existence of other atypical chemokine receptors (**chapter 2**).

2.5. Chemokine receptors are immuno-oncology targets

Although chemokine receptors are primarily found on immune cells, they are also present on non-hematopoietic cells. In homeostatic and in inflammatory state, they mediate the trafficking and positioning of immune cells to ensure immune-surveillance or to the inflamed tissue to regulate the infection [148].

Impaired coordination, due to alternation in chemokine receptor expression or chemokine secretion, could derail their beneficial effect and instead worsen the inflammatory state to a condition of autoimmune diseases or cancer development and, in later stages, metastasis. Chemokine receptors are found on various cancer cells, including breast, pancreas, and melanoma cancer cells [42]. These cancer cells hijack the chemokine/receptor network to their advantage. Together with other elements within the

tumor microenvironment, they orchestrate the tumor immunity, indirectly and directly, by turning infiltrating leukocytes into immune-tolerant allies, attracting regulatory T cells, and impairing the immature DC to migrate to the lymph node. Additionally, they influence cancer progression and spreading and, as a result, the outcome of therapy [149-153]. Generally, the hijacked chemokine/receptor network helps maintaining a pro-tumoral environment. From all the human chemokines, some chemokines and their participation in shaping the tumor immunity are the best studied, including CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, and CXCL12. Therefore, due to their presence in the tumor environment and their biological function, chemokine receptors are potential targets for cancer therapy [154].

2.5.1. Targeting chemokine receptors

Reports on the involvement of chemokines and their receptors in human diseases as well as their impact on various pathologies studied through genetic deletion in mice have provided abundant evidence to mark them as valuable therapeutic targets. However, chemokine receptors are difficult targets. Many and extensive drug discovery studies that target chemokine receptors have been undertaken, yet the final success rate is not high.

Many factors contribute to the challenging drugability of chemokine receptors; including the spacial and temporal chemokine expression in vivo [155], the overall complexity of the chemokine receptor network, where different receptors that recognize a shared ligand can be present at the same time on the same cell, the complex and often redundant functions of the chemokine receptors [156], the difference in the chemokine/ receptor network between humans and mice [157], and the open, hydrophilic binding pocket. The latter is an important hurdle to overcome as hydrophobicity and size of the binding pocket are major features for the drug-ability of small molecules [50]. Yet, a limited number of drugs have passed the clinical trials and hit the market.

2.5.1.1. Small molecules

Only two small molecules that target chemokine receptors, Maraviroc and Plerixafor, have been put on the market. Maraviroc, is an antagonist that prevents the entry of HIV by blocking the interaction of CCR5 and the virus envelope protein gp120 [158]. The CXCR4 partial agonist, Plerixafor, mobilizes bone marrow-derived hemopoietic stem cells to the bloodstream and is used for autologous stem-cell transplantation in cancer patients [159]. Other small antagonist molecules have been generated and went to clinical trials, but many (most) have been discontinued [156]. Only a few are presently in clinical trials, such as CCX507 targeting CCR9 in inflammatory bowel disease, CCX354 for the treatment of rheumatoid arthritis through CCR1 and the ACKR3 antagonist ACT-1004-1239 for the modulation of plasma concentrations of its ligands CXCL11 and CXCL12 in the fields of immunology and oncology [160-164].

2.5.1.2. Modified chemokines

Modification of the target chemokine was one of the possible leads to overcome the hurdles of targeting chemokine receptors with small molecules, i.e. open and large hydrophilic binding pocket while retaining chemokine receptor specificity. As the N-terminus of chemokines is key to activate their receptors, modification in this section could turn the natural agonist into an antagonist or a chemokine with altered receptor activation. The generation of a CXCL8 mutant, peptide analogs of the CXCL12 protein or fusion protein with CCL2 have been developed and promising data have been generated, but once entered in clinical trials, they were all discontinued or terminated [165]. The major HIV co-receptor CCR5 has also been targeted by modified CCL5 chemokines. The development of 6P4-CCL5, 5P12-CCL5, and 5P14-CCL5 are promising molecules to prevent HIV entry through CCR5 *in vitro* and in a rhesus vaginal challenge model [166, 167].

2.5.1.3. Conventional antibodies

Other biologicals that target specifically GPCRs are antibodies. The anti-CCR4 antibody, Mogamulizumab, was the first chemokine receptor-mediated antibody that entered clinical trials and successfully went to the market. This humanized, fucosylated mouse antibody binds to the N-terminus of the CCR4 chemokine receptor and induces antibody-dependent cell-mediated cytotoxicity. Currently, Mogamulizumab is used for the treatment of CCR4+ cutaneous T cell lymphoma and is now explored as a treatment of HTLV-1–associated myelopathy [168]. At present, five antibodies targeting chemokine receptors, CCR2, CCR5, CXCR2, CXCR4, and CX3CR1, are in clinical trials with two in phase III [157].

2.5.1.4. Heavy-chain only antibodies (VHH) - Nanobodies

In Camelids, besides the conventional antibodies, heavy-chain only antibodies occur naturally (**figure 8**) [169]. The monomeric variable domain of heavy-chain only antibodies (VHH), also referred to as Nanobodies, has some distinct properties. They show high thermal and chemical stability and their elongated CDR1 and CDR3 loops adopt alternative structures that enable them to bind (conformational) epitopes in clefts with high affinity. Additionally, their small size (12-15 kDa) allows them to penetrate difficult to access tissues and are considered to have a low immunogenic effect due to their high homology with human VH-fragments [170]. These beneficial features allow them to be used in various applications, including molecular imaging, crystallography, and GPCR-study tool [171], as well as their potential to become a drug, like the recently approved FDA and EMA drug Caplacizumab.

Since 2010, two CXCR4-specific VHHs have been reported to prevent the binding of CXCL12. Injection of the VHHs in monkeys resulted in the deployment of hematopoietic stems cell due to the disruption of the CXCR4/CXCL12 axis. Additionally, an ACKR3 specific nanobody with antagonistic properties, which show a beneficial effect in vivo in a

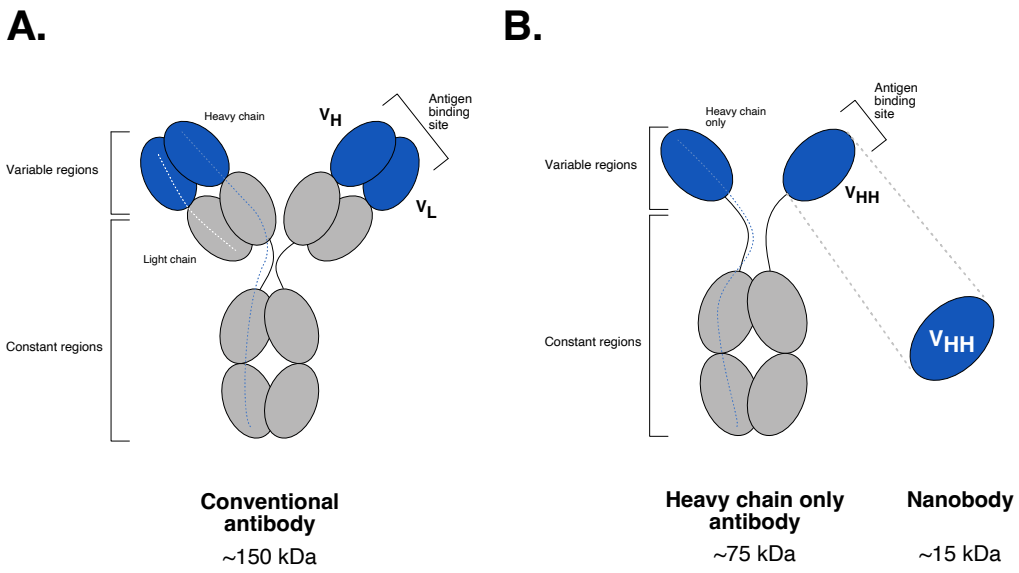


Figure 8 | Illustration of conventional and heavy-chain only antibodies. (A) Conventional antibodies, found in humans and camelids, are formed by a light and a heavy chain, which consists of one variable and three constant regions. (B) Heavy-chains only antibodies are primarily found in camelids and cartilaginous fish and are devoid of the light chain and one constant region compared to the conventional antibodies. The isolated variable domain, which binds antigens, is referred to as VHH or Nanobody.

head and neck cancer xenograft model [172], and VHH targeting the chemokines CXCL12, CXCL11, and CCL5, which prevent the binding to and activation of their cognate receptors, have been developed [173]. Furthermore, VHH antibody fragments have been reported for ACKR1. These recognize the N-terminus of the receptor, displace the CXCL8 chemokine, and prevent the infection of erythrocytes by Plasmodium vivax. Therefore, this nanobody could serve as a potential basis for the development of a new therapeutic drug against malaria [174]. Additionally, two classes of monovalent nanobodies against CXCR2 have been generated. The class 1 monovalent nanobody, recognizing the N-terminus, binds the receptors with high potency but is unable to modulate the receptor's activity. The other class monovalent nanobody, binding a conformational epitope formed by extracellular loops 1 and 3, showed lower potency, yet was able to inhibit CXCR2-mediate signaling by CXCL1 and CXCL8. Interestingly, the generation of a bivalent and biparatopic nanobody, using both classes of monovalent nanobodies, showed better properties compared to their monovalent peers [175]. Due to the nanobody's comparable size to chemokines, their ability to bind into cavities with high affinity, and the optimistic results in vivo models, they could be promising therapeutic alternatives to target chemokine receptors.

Recently, two nanobodies, VUN103 and the bivalent molecule VUN100bv, have been developed that target the constitutive active viral protein US28; a viral chemokine receptor that has a high homology with human chemokine receptors and is expressed during beta-herpesvirus human cytomegalovirus (HCMV) latency. VUN103 recognizes the US28 receptor’s ICL2 loop, which hinders the binding of the Gαq protein and β-arrestins without affecting the affinities for its endogenous ligands CX3CL1 and CCL5. This reduced US28 signaling inhibited the spheroid growth of glioblastoma cells and could be used as a tool to develop and identify other therapeutic drugs that stabilize this US28-VUN103 conformation.

The bivalent nanobody VUN100bv binds the viral chemokine receptor US28 extracellularly and acts as a partial inverse agonist. The reduced US28 signaling triggers the expression of HCMV antigens in latently virus-infected cells without fully reactivating viral replication, which, in turn, reactivates anti-HCMV cytotoxic T lymphocytes resulting in the killing of these latently virus-infected cells [176, 177].

3. CXCR3 chemokine receptor

3.1. The CXCR3 chemokine receptor and its ligands

The CXCR3 chemokine receptor is one of the 18 presently known classical chemokine receptors. CXCR3 was initially described as, and still is, an inducible chemokine receptor as its expression is limited to IL-2 activated T lymphocytes and is not present on other immune cells in a homeostatic state [178]. Nowadays, the expression of CXCR3 on other activated immune cells, including cytotoxic (CD8+) and helper 1 (Th1) T cells, natural killer (NK) cells, and dendritic cells (DCs), and non-immune cells, such as lung epithelial and endothelial cells, has been reported [179-185].

CXCR3 belongs to the CXC subclass of chemokine receptors and binds the CXCL9, CXCL10, and CXCL11 chemokines [178, 186]. These ERL- CXC chemokines are upregulated by the inflammatory cytokine interferon-gamma (IFN-y), and, weakly, by tumor necrosis factor (TNF)-alpha, and are secreted by various cells, including activated macrophages and endothelial cells [75, 186-189]. They are, therefore, mainly present at the site of inflammation where they recruit CXCR3 expressing immune cells to regulate the inflammation [190]. Consequently, CXCR3 and its ligands are involved in various inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, allograft rejection, atherosclerosis, and cancers [179, 190-194].

Although CXCR3 can be activated by three chemokines, CXCL11 shows the highest potency and efficacy, followed by CXCL10 and CXCL9 [195]. Binding studies using a CXCR3 chimeric receptor demonstrated that each CXCR3 chemokine binds differently at the receptor’s extracellular domain. CXCL11 and CXCL10 need to interact with the N terminus and first extracellular loop to activate CXCR3, while these were dispensable for CXCL9. On the other hand, CXCL9, as well as CXCL10, needs to connect with the third extracellular loop to trigger receptor activation. Moreover, all CXCR3 ligands showed to interact heavily with the second extracellular loop (ECL2), which is indispensable for receptor activation [196]. Yet, CXCL11 seems to be less susceptible to changes in the ECL2 [197]. Overall, each CXCR3 chemokine activates and interacts differently with the receptor suggesting allosteric features for these endogenous ligands. Indeed, investigation of CXCR3 ligands described CXCL11 as the most potent chemokine to induce G protein-mediated signaling events, arrestin recruitment and, consequently, receptor internalization.

CXCL10 and CXCL9 show preferential activation of G proteins, as low receptor internalization for CXCL10 and none for CXCL9 is observed [53,185].

GAG-chemokine interactions are vital to induce and guide leukocyte migration [113]. All CXCR3 ligands interact with GAGs, which in vivo are needed to mediate the migration of e.g. DCs [183]. Although each CXCR3 chemokine interacts with GAGs differently, the residues involved in these interactions do not play part in the binding to CXCR3 as mutations of these specific amino acids do not change their potency. Interestingly, from all CXCR3 chemokines, CXCL9 might be the less potent CXCR3 ligand but it is the most efficient GAG binder [198-200].

3.2. CXCR3 variants

In humans, three variants of the chemokine receptor CXCR3 have been described (**figure 9**). These CXCR3 variants, CXCR3-A, CXCR3-B, or CXCR3-Alt, result from alternative splicing of CXCR3 mRNA, transcribed from the cxcr3 gene located on

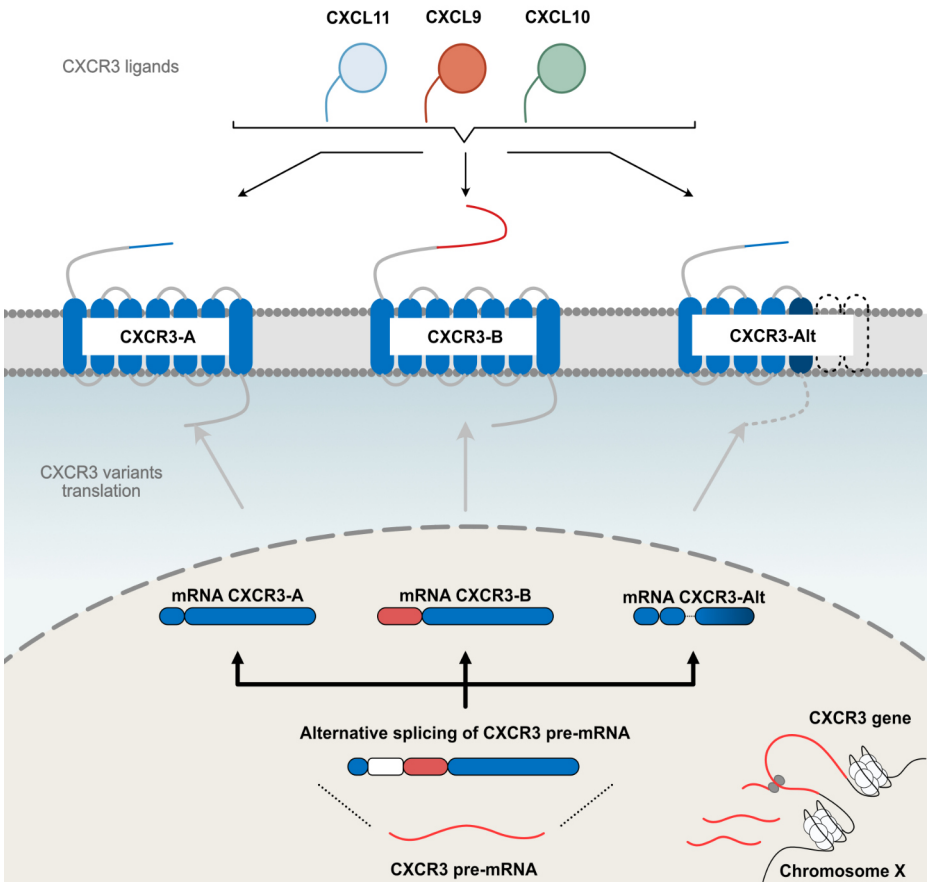


Figure 9 | Alternative splicing of the CXCR3 variants. The cxcr3 gene is located in the X chromosome and is comprised of three exons and one intron. After the transcription of the cxcr3 gene, the pre-mRNA can be alternatively spliced resulting in the generation of three CXCR3 variants: CXCR3-A, CXCR3-B, and CXCR3-Alt. The CXCR3-B variant, which consist of exon2 and exon3, display a 51 AA N terminus extension compared to CXCR3-A. CXCR3-Alt variant is from through the removal of the intron, exon2 and a 337-bp region in exon3, which results in the removal of two transmembrane regions and different a fifth TM and a C-terminus compared to CXCR3-A and CXCR3-B. At the plasma membrane, all CXCR3 variants bind the inflammatory ligands CXCL9, CXCL10 and CXCL11. Although, each of these endogenous ligands bind differently to the CXCR3 variants and have distinct activation potentials.

chromosome X and differ from each other by their structure, expression profile, binding, and activation efficacy of their ligands [201, 202].

The CXCR3-B variant has a 51 AA N-terminal extension and CXCR3-Alt consists of five transmembrane helixes, from which the fifth transmembrane helix and C-terminus differ from CXCR3-A [201, 202].

CXCR3-A is primarily expressed on leukocytes, while CXCR3-B has been primarily detected on endothelial cells and pericytes, although its expression could also be found on immune cells yet less abundant than CXCR3-A [178, 201]. Fewer data is available about the expression of the CXCR3-Alt variant, although this variant has been described to be present on T cells from patients with Crohn's disease and bladder cancer [203, 204].

Besides their structural and expression profile differences, all CXCR3 variants induce distinct signaling pathways. CXCR3-A is reported to be mainly coupled to Gαi and Gαq that, ultimately, mediate cell migration and proliferation [205]. Additionally, in T lymphocytes CXCR3-A is predominantly coupled to Gαi2 proteins but remains in competition with the Gαi3 proteins, which leads to the fine-tuning of Gαi2-mediated downstream signaling events [206]. Downstream G protein activation, CXCR3-A signals via the p44/42 MAP kinase (MAPK) pathway and partially via PI3K, as pertussis toxin, an adenylate cyclase inhibitor, completely inhibited the MAPK pathway and wortmannin, a PI3K inhibitor, diminished the CXCL11 induced Akt phosphorylation [205, 207]. Moreover, activation of CXCR3-A by CXCL10 and CXCL9 polarizes CD4+ T cells into Th1/Th17 effector cells, while CXCL11 turns these cells into T cells with regulatory functions, including Th2 and Tr1. These differences in CD4+ T cell polarization following CXCR3 ligands roots from the activation of different downstream signaling pathways, with CXCL9/10 triggering STAT1, STAT4, and STAT5 phosphorylation and CXCL11 mediating STAT3 and STAT6-dependent pathways [208].

CXCR3-B signaling events remain unclear. Initial reports on CXCR3-B described the coupling of Gαs protein due to increased cAMP levels following ligand stimulation, yet no calcium flux modulation was observed [201]. With CXCR3-B solely being expressed in pericytes, reports on CXCR3-B’s activation with CXCR3 ligands on these cell types showed activation of the ERK and p38 downstream signaling, which was corroborated by CXCR3-B expressing HEK cells although to a lesser extent. Furthermore, activation of CXCR3-B initiated cell migration, which was inhibited using ERK signaling inhibitor. Although no direct G protein coupling was investigated, no protein kinase A activation was observed upon CXCR3-B activation suggesting little or no cAMP production [209-211]. More confusion is brought by recent reports that described weak Gαi protein activation after CXCR3 ligand stimulation for CXCR3-B although a transient ERK signaling profile could be detected [212, 213].

Fewer reports on the CXCR3-Alt variant's biology have been reported. Receptor internalization but no G protein activation nor β-arrestin recruitment have been described upon activation of its cognate ligands, CXCL11, CXCL10, and CXCL9 [213].

The binding of CXCR3 endogenous ligands also induces receptor desensitization. The binding of CXCL11 to CXCR3-A and -B induced β-arrestin-1 and 2 recruitment. However, arrestin recruitment after CXCL10 stimulation is only observed for CXCR3-A and CXCL9 seems to be unable to recruit arrestins [212, 213]. Moreover, CXCR3-A is internalized after ligand stimulation, yet different features of the receptor influence receptor internalization. For example, CXCR3-A internalization is majorly mediated by the ICL3 and not by β-arrestins, as CXCR3-A chimeras lacking the C-terminus were still able to be internalized. The least potent chemokines, CXCL10 and CXCL9, are also able to internalize CXCR3-A, albeit to a lesser extent, and were shown to be mediated by the C-terminal tail of the receptor and β-arrestins [72, 214].

Of note, CXCR3-A and -B have been described to bind the platelet-derived CXCL4 [201]. This chemokine was able to trigger G protein-mediated signaling after CXCR3-A binding and inhibit endothelial cell proliferation [201, 205, 215].

3.3. Role of CXCR3 in inflammatory conditions

The role of CXCR3 in various disease models, including atherosclerosis, inflammatory bowel disease, and infectious diseases have been extensively described [192, 216-218]. While CXCR3 knock-out (KO) mice do not show any specific phenotype in normal conditions, deficiencies in NK cells [219], reduced migration, and differences in the organization of T cells were observed upon infection [220]. Indeed, CXCR3 has been shown to mediate the positioning of memory CD8+ T lymphocytes in secondary lymphoid organs after viral infection, to inflamed and harmful tissues, and to facilitate the interaction with activated DC to promote T lymphocyte activation and differentiation [221-226].

In the tumor microenvironment, CD8+ T cells play a crucial role to mediate tumor prognosis due to their production and release of cytotoxic enzymes and cytokines [227]. However, the tumors shape the environment to be immunosuppressive by inducing high expression levels of PD-1, which inhibit the T cell receptor and CD28 signaling, rendering T lymphocytes in this environment non-active, and thus promoting tumor progression [228, 229]. Immunotherapy, specifically immune checkpoint therapy, using anti-PD-1, anti-PD-L1, and anti-CTLA-4, lead towards a better prognosis of the disease [230, 231]. Recent studies showed that the CXCR3 axis plays an important role in the effectiveness of anti-PD-1 immunotherapy. Deletion of CXCR3 on activated CD8+ T cells showed impaired T lymphocytes infiltration in the tumor microenvironment resulting in increased tumor size, however, its importance in early T cell trafficking might need further investigation. In murine tumor models, the initial CXCR3-mediated signaling within the tumor microenvironment is

essential to promote an anti-PD-1 anti-tumoral effect. Indeed, anti-PD-1 treatment upregulate CD103+ DCs-derived and macrophage-mediated CXCL9 secretion. This chemokine is required for CXCR3+ CD8+ tumor-specific T cell infiltration in the tumor bed and enhances the proliferation and function of these T lymphocytes residing within the tumor microenvironment [232-234]. An overview of the role of CXCR3 variants in the tumor environment is described in **chapter 1** of this thesis.

3.4. CXCR3 modulators

At present, no CXCR3 modulator is available on the market. Several classes of CXCR3 antagonists have been generated, but only two of those classes have been optimized for drug development. Only particular small molecules within the 8-azaquinalozinones class, such as AMG487 [235], showed promise and were evaluated in clinical trials for psoriasis. This molecule reached phase IIa trials but failed to show significant effectiveness and further development of the molecule was terminated [236, 237]. An anti-CXCL10 antibody, named Eldelumab, showed also promise for the treatment of ulcerative colitis and Crohn's disease. However, its evaluation in phase II clinical trials also did not show significant effectiveness for both diseases [238, 239].

References

1. Sommer, F., V. Torraca, and A.H. Meijer, Chemokine Receptors and Phagocyte Biology in Zebrafish. Front Immunol, 2020. 11: p. 325.
2. Venkatakrishnan, A.J., et al., Molecular signatures of G-protein-coupled receptors. Nature, 2013. 494(7436): p. 185-94.
3. Seyedabadi, M., M.H. Ghahremani, and P.R. Albert, Biased signaling of G protein coupled receptors (GPCRs): Molecular determinants of GPCR/transducer selectivity and therapeutic potential. Pharmacol Ther, 2019. 200: p. 148-178.
4. Latorraca, N.R., A.J. Venkatakrishnan, and R.O. Dror, GPCR Dynamics: Structures in Motion. Chem Rev, 2017. 117(1): p. 139-155.
5. Smith, J.S., R.J. Lefkowitz, and S. Rajagopal, Biased signalling: from simple switches to allosteric microprocessors. Nat Rev Drug Discov, 2018. 17(4): p. 243-260.
6. Hauser, A.S., et al., Trends in GPCR drug discovery: new agents, targets and indications. Nat Rev Drug Discov, 2017. 16(12): p. 829-842.
7. Oberai, A., et al., A limited universe of membrane protein families and folds. Protein Sci, 2006. 15(7): p. 1723-34.
8. Katritch, V., V. Cherezov, and R.C. Stevens, Structure-function of the G protein-coupled receptor superfamily. Annu Rev Pharmacol Toxicol, 2013. 53: p. 531-56.
9. Wacker, D., R.C. Stevens, and B.L. Roth, How Ligands Illuminate GPCR Molecular Pharmacology. Cell, 2017. 170(3): p. 414-427.
10. Flock, T., et al., Selectivity determinants of GPCR-G-protein binding. Nature, 2017. 545(7654): p. 317-322.
11. Wootten, D., et al., Mechanisms of signalling and biased agonism in G protein-coupled receptors. Nat Rev Mol Cell Biol, 2018. 19(10): p. 638-653.
12. Okashah, N., et al., Variable G protein determinants of GPCR coupling selectivity. Proc Natl Acad Sci U S A, 2019. 116(24): p. 12054-12059.
13. Latorraca, N.R., et al., How GPCR Phosphorylation Patterns Orchestrate Arrestin-Mediated Signaling. Cell, 2020. 183(7): p. 1813-1825 e18.
14. Mayer, D., et al., Distinct G protein-coupled receptor phosphorylation motifs modulate arrestin affinity and activation and global conformation. Nat Commun, 2019. 10(1): p. 1261.
15. Weis, W.I. and B.K. Kobilka, The Molecular Basis of G Protein-Coupled Receptor Activation. Annu Rev Biochem, 2018. 87: p. 897-919.
16. Shenoy, S.K. and R.J. Lefkowitz, Seven-transmembrane receptor signaling through beta-arrestin. Sci STKE, 2005. 2005(308): p. cm10.
17. Nygaard, R., et al., The dynamic process of beta(2)-adrenergic receptor activation. Cell, 2013. 152(3): p. 532-42.
18. O'Hayre, M., et al., Genetic evidence that beta-arrestins are dispensable for the initiation of beta2-adrenergic receptor signaling to ERK. Sci Signal, 2017. 10(484).
19. Grundmann, M., et al., Lack of beta-arrestin signaling in the absence of active G proteins. Nat Commun, 2018. 9(1): p. 341.
20. Luttrell, L.M., et al., Manifold roles of beta-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. Sci Signal, 2018. 11(549).
21. Gurevich, V.V. and E.V. Gurevich, Arrestins and G proteins in cellular signaling: The coin has two sides. Sci Signal, 2018. 11(549).
22. Smith, J.S. and S. Rajagopal, The beta-Arrestins: Multifunctional Regulators of G Protein-coupled Receptors. J Biol Chem, 2016. 291(17): p. 8969-77.
23. Jakowiecki, J., et al., Structural diversity in ligand recognition by GPCRs, in GPCRs. 2020, Elsevier. p. 43-63.
24. Bonner, G., F. Meng, and H. Akil, Selectivity of mu-opioid receptor determined by interfacial residues near third extracellular loop. Eur J Pharmacol, 2000. 403(1-2): p. 37-44.
25. Lee, S.-M., J.M. Booe, and A.A. Pioszak, Structural insights into ligand recognition and selectivity for classes A, B, and C GPCRs. European journal of pharmacology, 2015. 763(Pt B): p. 196-205.
26. Liang, Y.L., et al., Cryo-EM structure of the active, Gs-protein complexed, human CGRP receptor. Nature, 2018. 561(7724): p. 492-497.
27. Zhang, Y., et al., Cryo-EM structure of the activated GLP-1 receptor in complex with a G protein. Nature, 2017. 546(7657): p. 248-253.
28. Bassilana, F., M. Nash, and M.G. Ludwig, Adhesion G protein-coupled receptors: opportunities for drug discovery. Nat Rev Drug Discov, 2019. 18(11): p. 869-884.
29. Schulte, G. and S.C. Wright, Frizzleds as GPCRs - More Conventional Than We Thought! Trends Pharmacol Sci, 2018. 39(9): p. 828-842.
30. Markovic, D. and R.A. Challiss, Alternative splicing of G protein-coupled receptors: physiology and pathophysiology. Cell Mol Life Sci, 2009. 66(20): p. 3337-52.
31. Marti-Solano, M., et al., Combinatorial expression of GPCR isoforms affects signalling and drug responses. Nature, 2020. 587(7835): p. 650-656.
32. Azam, F., I.A. Ibn-Rajab, and A.A. Alruiad, Adenosine A2A receptor antagonists as novel anti-Parkinsonian agents: a review of structure-activity relationships. Pharmazie, 2009. 64(12): p. 771-95.
33. Miller, M.C. and K.H. Mayo, Chemokines from a Structural Perspective. Int J Mol Sci, 2017. 18(10).
34. Hughes, C.E. and R.J.B. Nibbs, A guide to chemokines and their receptors. FEBS J, 2018. 285(16): p. 2944-2971.
35. Murphy, P.M., 10 - Chemokines and Chemokine Receptors, in Clinical Immunology (Fifth Edition), R.R. Rich, et al., Editors. 2019, Content Repository Only!: London. p. 157-170.e1.
36. Stone, M.J., et al., Mechanisms of Regulation of the Chemokine-Receptor Network. Int J Mol Sci, 2017. 18(2).
37. Lopez-Cotarelo, P., et al., Beyond Chemoattraction: Multifunctionality of Chemokine Receptors in Leukocytes. Trends Immunol, 2017. 38(12): p. 927-941.
38. Legler, D.F. and M. Thelen, Chemokines: Chemistry, Biochemistry and Biological Function. Chimia (Aarau), 2016. 70(12): p. 856-859.
39. Moser, B., et al., Chemokines: multiple levels of leukocyte migration control. Trends Immunol, 2004. 25(2): p. 75-84.
40. Schulz, O., et al., Chemokines and Chemokine Receptors in Lymphoid Tissue Dynamics. Annu Rev Immunol, 2016. 34: p. 203-42.
41. Farnsworth, R.H., et al., The Interplay Between Lymphatic Vessels and Chemokines. Front Immunol, 2019. 10: p. 518.
42. Chen, K., et al., Chemokines in homeostasis and diseases. Cell Mol Immunol, 2018. 15(4): p. 324-334.
43. 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, 2003. 278(7): p. 5179-87.
44. Allen, S.J., S.E. Crown, and T.M. Handel, Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol, 2007. 25: p. 787-820.
45. Arimont, M., et al., Chemokine Receptor Crystal Structures: What Can Be Learned from Them? Mol Pharmacol, 2019. 96(6): p. 765-777.
46. Burg, J.S., et al., Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. Science, 2015. 347(6226): p. 1113-7.
47. Qin, L., et al., Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. Science, 2015. 347(6226): p. 1117-22.
48. 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, 2017. 46(6): p. 1005-1017 e5.

49. Wasilko, D.J., et al., Structural basis for chemokine receptor CCR6 activation by the endogenous protein ligand CCL20. Nat Commun, 2020. 11(1): p. 3031.
50. Kufareva, I., et al., What Do Structures Tell Us About Chemokine Receptor Function and Antagonism? Annu Rev Biophys, 2017. 46: p. 175-198.
51. Gustavsson, M., et al., Structural basis of ligand interaction with atypical chemokine receptor 3. Nat Commun, 2017. 8: p. 14135.
52. Katancik, J.A., A. Sharma, and E. de Nardin, Interleukin 8, neutrophil-activating peptide-2 and GRO-alpha bind to and elicit cell activation via specific and different amino acid residues of CXCR2. Cytokine, 2000. 12(10): p. 1480-8.
53. Isaikina, P., et al., Structural basis of the activation of the CC chemokine receptor 5 by a chemokine agonist. Sci Adv, 2021. 7(25).
54. Liu, K., et al., Structural basis of CXC chemokine receptor 2 activation and signalling. Nature, 2020. 585(7823): p. 135-140.
55. 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, 2019. 294(10): p. 3464-3475.
56. Szpakowska, M., D. Perez Bercoff, and A. Chevigne, Closing the ring: a fourth extracellular loop in chemokine receptors. Sci Signal, 2014. 7(341): p. pe21.
57. Ai, L.S. and F. Liao, Mutating the four extracellular cysteines in the chemokine receptor CCR6 reveals their differing roles in receptor trafficking, ligand binding, and signaling. Biochemistry, 2002. 41(26): p. 8332-41.
58. Legler, D.F. and M. Thelen, New insights in chemokine signaling. F1000Res, 2018. 7: p. 95.
59. Hall, D.A., et al., Signalling by CXC-chemokine receptors 1 and 2 expressed in CHO cells: a comparison of calcium mobilization, inhibition of adenylyl cyclase and stimulation of GTPgammaS binding induced by IL-8 and GROalpha. Br J Pharmacol, 1999. 126(3): p. 810-8.
60. Sato, K., et al., An abortive ligand-induced activation of CCR1-mediated downstream signaling event and a deficiency of CCR5 expression are associated with the hyporesponsiveness of human naive CD4+ T cells to CCL3 and CCL5. J Immunol, 2002. 168(12): p. 6263-72.
61. Shi, G., et al., Identification of an alternative G{alpha}q-dependent chemokine receptor signal transduction pathway in dendritic cells and granulocytes. J Exp Med, 2007. 204(11): p. 2705-18.
62. Canals, M., et al., Ubiquitination of CXCR7 controls receptor trafficking. PLoS One, 2012. 7(3): p. e34192.
63. Neel, N.F., et al., Chemokine receptor internalization and intracellular trafficking. Cytokine Growth Factor Rev, 2005. 16(6): p. 637-58.
64. McCulloch, C.V., et al., Multiple roles for the C-terminal tail of the chemokine scavenger D6. J Biol Chem, 2008. 283(12): p. 7972-82.
65. Montpas, N., et al., Ligand-specific conformational transitions and intracellular transport are required for atypical chemokine receptor 3-mediated chemokine scavenging. J Biol Chem, 2018. 293(3): p. 893-905.
66. Saaber, F., et al., ACKR3 Regulation of Neuronal Migration Requires ACKR3 Phosphorylation, but Not beta-Arrestin. Cell Rep, 2019. 26(6): p. 1473-1488 e9.
67. Comerford, I., et al., The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. Eur J Immunol, 2006. 36(7): p. 1904-16.
68. Dyer, D.P., Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine-mediated leukocyte recruitment. Immunology, 2020. 160(4): p. 336-344.
69. Mantovani, A., The chemokine system: redundancy for robust outputs. Immunol Today, 1999. 20(6): p. 254-7.
70. Kohout, T.A., et al., Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. J Biol Chem, 2004. 279(22): p. 23214-22.
71. Zidar, D.A., et al., Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. Proc Natl Acad Sci U S A, 2009. 106(24): p. 9649-54.
72. Rajagopal, S., et al., Biased agonism as a mechanism for differential signaling by chemokine receptors. J Biol Chem, 2013. 288(49): p. 35039-48.
73. Corbisier, J., et al., Biased signaling at chemokine receptors. J Biol Chem, 2015. 290(15): p. 9542-54.
74. Bleul, C.C., et al., The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature, 1996. 382(6594): p. 829-33.
75. Tensen, C.P., et al., Human IP-9: A keratinocyte-derived high affinity CXC-chemokine ligand for the IP-10/Mig receptor (CXCR3). J Invest Dermatol, 1999. 112(5): p. 716-22.
76. Rajagopal, S., et al., Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. Proc Natl Acad Sci U S A, 2010. 107(2): p. 628-32.
77. Steen, A., et al., Biased and g protein-independent signaling of chemokine receptors. Front Immunol, 2014. 5: p. 277.
78. Vanheule, V., et al., How post-translational modifications influence the biological activity of chemokines. Cytokine, 2018. 109: p. 29-51.
79. Brandt, E., J. Van Damme, and H.D. Flad, Neutrophils can generate their activator neutrophil-activating peptide 2 by proteolytic cleavage of platelet-derived connective tissue-activating peptide III. Cytokine, 1991. 3(4): p. 311-21.
80. Brandt, E., et al., The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. J Leukoc Biol, 2000. 67(4): p. 471-8.
81. 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, 1998. 28(4): p. 1262-71.
82. Heutinck, K.M., et al., Serine proteases of the human immune system in health and disease. Mol Immunol, 2010. 47(11-12): p. 1943-55.
83. Oravecz, T., et al., 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, 1997. 186(11): p. 1865-72.
84. 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, 2018. 175(9): p. 1419-1438.
85. Van Coillie, E., et al., Functional comparison of two human monocyte chemotactic protein-2 isoforms, role of the amino-terminal pyroglutamic acid and processing by CD26/dipeptidyl peptidase IV. Biochemistry, 1998. 37(36): p. 12672-80.
86. Parks, W.C., C.L. Wilson, and Y.S. Lopez-Boado, Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol, 2004. 4(8): p. 617-29.
87. Ruggiero, P., et al., Glycosylation enhances functional stability of the chemotactic cytokine CCL2. Eur Cytokine Netw, 2003. 14(2): p. 91-6.
88. 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, 1998. 160(8): p. 4034-41.
89. Kameyoshi, Y., et al., Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. J Exp Med, 1992. 176(2): p. 587-92.
90. Noso, N., et al., Delayed production of biologically active O-glycosylated forms of human eotaxin by tumor-necrosis-factor-alpha-stimulated dermal fibroblasts. Eur J Biochem, 1998. 253(1): p. 114-22.
91. Richter, R., et al., Posttranslationally processed forms of the human chemokine HCC-1. Biochemistry, 2000. 39(35): p. 10799-805.
92. Loos, T., et al., Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation. Blood, 2008. 112(7): p. 2648-56.

93. Proost, P., et al., Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. J Exp Med, 2008. 205(9): p. 2085-97.
94. Pritzker, L.B., et al., Deimination of myelin basic protein. 2. Effect of methylation of MBP on its deimination by peptidylarginine deiminase. Biochemistry, 2000. 39(18): p. 5382-8.
95. Yoshida, K., et al., Citrullination of epithelial neutrophil-activating peptide 78/CXCL5 results in conversion from a non-monocyte-recruiting chemokine to a monocyte-recruiting chemokine. Arthritis Rheumatol, 2014. 66(10): p. 2716-27.
96. Mortier, A., et al., Posttranslational modification of the NH2-terminal region of CXCL5 by proteases or peptidylarginine Deiminases (PAD) differently affects its biological activity. J Biol Chem, 2010. 285(39): p. 29750-9.
97. Loos, T., et al., Citrullination of CXCL8 increases this chemokine's ability to mobilize neutrophils into the blood circulation. Haematologica, 2009. 94(10): p. 1346-53.
98. Struyf, S., et al., Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding with loss of inflammatory and anti-HIV-1 activity via CXCR4. J Immunol, 2009. 182(1): p. 666-74.
99. Bannert, N., et al., Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. J Exp Med, 2001. 194(11): p. 1661-73.
100. Hauser, M.A., et al., Distinct CCR7 glycosylation pattern shapes receptor signaling and endocytosis to modulate chemotactic responses. J Leukoc Biol, 2016. 99(6): p. 993-1007.
101. Ludwig, A., et al., Identification of distinct surface-expressed and intracellular CXC-chemokine receptor 2 glycoforms in neutrophils: N-glycosylation is essential for maintenance of receptor surface expression. J Immunol, 2000. 165(2): p. 1044-52.
102. Chabot, D.J., et al., N-linked glycosylation of CXCR4 masks coreceptor function for CCR5-dependent human immunodeficiency virus type 1 isolates. J Virol, 2000. 74(9): p. 4404-13.
103. Zhu, J.Z., et al., Tyrosine sulfation influences the chemokine binding selectivity of peptides derived from chemokine receptor CCR3. Biochemistry, 2011. 50(9): p. 1524-34.
104. Farzan, M., et al., Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. Cell, 1999. 96(5): p. 667-76.
105. Colvin, R.A., et al., CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. Mol Cell Biol, 2006. 26(15): p. 5838-49.
106. Farzan, M., et al., The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1 alpha association and HIV-1 entry. J Biol Chem, 2002. 277(33): p. 29484-9.
107. Raghuwanshi, S.K., et al., The chemokine receptors CXCR1 and CXCR2 couple to distinct G protein-coupled receptor kinases to mediate and regulate leukocyte functions. J Immunol, 2012. 189(6): p. 2824-32.
108. Rummel, P.C., et al., Extracellular disulfide bridges serve different purposes in two homologous chemokine receptors, CCR1 and CCR5. Mol Pharmacol, 2013. 84(3): p. 335-45.
109. Benredjem, B., et al., Mutational Analysis of Atypical Chemokine Receptor 3 (ACKR3/CXCR7) Interaction with Its Chemokine Ligands CXCL11 and CXCL12. J Biol Chem, 2017. 292(1): p. 31-42.
110. Szpakowska, M., et al., Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. Biochem Pharmacol, 2012. 84(10): p. 1366-80.
111. Blanpain, C., et al., Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. J Biol Chem, 2001. 276(26): p. 23795-804.
112. Percherancier, Y., et al., Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. J Biol Chem, 2001. 276(34): p. 31936-44.
113. Proudfoot, A.E.I., et al., Glycosaminoglycan Interactions with Chemokines Add Complexity to a Complex System. Pharmaceuticals (Basel), 2017. 10(3).
114. Monneau, Y., F. Arenzana-Seisdedos, and H. Lortat-Jacob, The sweet spot: how GAGs help chemokines guide migrating cells. J Leukoc Biol, 2016. 99(6): p. 935-53.
115. Gouwy, M., et al., Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration. J Leukoc Biol, 2004. 76(1): p. 185-94.
116. Verkaar, F., et al., Chemokine cooperativity is caused by competitive glycosaminoglycan binding. J Immunol, 2014. 192(8): p. 3908-3914.
117. Kuscher, K., et al., Synergy-inducing chemokines enhance CCR2 ligand activities on monocytes. Eur J Immunol, 2009. 39(4): p. 1118-28.
118. Sadir, R., et al., Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. J Biol Chem, 2004. 279(42): p. 43854-60.
119. Ziarek, J.J., et al., Heparin oligosaccharides inhibit chemokine (CXC motif) ligand 12 (CXCL12) cardioprotection by binding orthogonal to the dimerization interface, promoting oligomerization, and competing with the chemokine (CXC motif) receptor 4 (CXCR4) N terminus. J Biol Chem, 2013. 288(1): p. 737-46.
120. Liang, W.G., et al., Structural basis for oligomerization and glycosaminoglycan binding of CCL5 and CCL3. Proc Natl Acad Sci U S A, 2016. 113(18): p. 5000-5.
121. Ellyard, J.I., et al., Eotaxin selectively binds heparin. An interaction that protects eotaxin from proteolysis and potentiates chemotactic activity in vivo. J Biol Chem, 2007. 282(20): p. 15238-47.
122. Vacchini, A., M. Locati, and E.M. Borroni, Overview and potential unifying themes of the atypical chemokine receptor family. J Leukoc Biol, 2016. 99(6): p. 883-92.
123. Bonecchi, R., et al., Chemokine decoy receptors: structure-function and biological properties. Curr Top Microbiol Immunol, 2010. 341: p. 15-36.
124. Ulvmar, M.H., E. Hub, and A. Rot, Atypical chemokine receptors. Exp Cell Res, 2011. 317(5): p. 556-68.
125. Nibbs, R.J. and G.J. Graham, Immune regulation by atypical chemokine receptors. Nat Rev Immunol, 2013. 13(11): p. 815-29.
126. Pruenster, M., et al., The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. Nat Immunol, 2009. 10(1): p. 101-8.
127. Neote, K., et al., Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. Blood, 1994. 84(1): p. 44-52.
128. Novitzky-Basso, I. and A. Rot, Duffy antigen receptor for chemokines and its involvement in patterning and control of inflammatory chemokines. Frontiers in Immunology, 2012. 3(266).
129. Fra, A.M., et al., Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. J Immunol, 2003. 170(5): p. 2279-82.
130. Lee, K.M., R.J. Nibbs, and G.J. Graham, D6: the 'crowd controller' at the immune gateway. Trends Immunol, 2013. 34(1): p. 7-12.
131. Weber, M., et al., The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. Mol Biol Cell, 2004. 15(5): p. 2492-508.
132. Bonecchi, R., et al., Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation. Blood, 2008. 112(3): p. 493-503.
133. Borroni, E.M., et al., beta-arrestin-dependent activation of the cofilin pathway is required for the scavenging activity of the atypical chemokine receptor D6. Sci Signal, 2013. 6(273): p. ra30 1-11, S1-3.
134. Burns, J.M., et al., A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell

adhesion, and tumor development. J Exp Med, 2006. 203(9): p. 2201-13.
135. Levoye, A., et al., CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. Blood, 2009. 113(24): p. 6085-93.
136. Szpakowska, M., et al., Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7. Biochem Pharmacol, 2016. 114: p. 14-21.
137. Meyrath, M., et al., The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides. Nat Commun, 2020. 11(1): p. 3033.
138. Meyrath, M., et al., Proadrenomedullin N-Terminal 20 Peptides (PAMPs) Are Agonists of the Chemokine Scavenger Receptor ACKR3/CXCR7. ACS Pharmacol Transl Sci, 2021. 4(2): p. 813-823.
139. Santagata, S., et al., CXCR4 and CXCR7 Signaling Pathways: A Focus on the Cross-Talk Between Cancer Cells and Tumor Microenvironment. Front Oncol, 2021. 11: p. 591386.
140. Heinzel, K., C. Benz, and C.C. Bleul, A silent chemokine receptor regulates steady-state leukocyte homing in vivo. Proc Natl Acad Sci U S A, 2007. 104(20): p. 8421-6.
141. Ulvmar, M.H., et al., The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. Nat Immunol, 2014. 15(7): p. 623-30.
142. Lucas, B., et al., CCRL1/ACKR4 is expressed in key thymic microenvironments but is dispensable for T lymphopoiesis at steady state in adult mice. Eur J Immunol, 2015. 45(2): p. 574-83.
143. Bryce, S.A., et al., ACKR4 on Stromal Cells Scavenges CCL19 To Enable CCR7-Dependent Trafficking of APCs from Inflamed Skin to Lymph Nodes. J Immunol, 2016. 196(8): p. 3341-53.
144. Comerford, I., et al., The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in circulation and tissues and suppresses Th17 responses. Blood, 2010. 116(20): p. 4130-40.
145. Matti, C., et al., CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4. J Leukoc Biol, 2020. 107(6): p. 1137-1154.
146. Meyrath, M., et al., Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22. J Leukoc Biol, 2021. 109(2): p. 373-376.
147. Le Mercier, A., et al., GPR182 is an endothelium-specific atypical chemokine receptor that maintains hematopoietic stem cell homeostasis. Proc Natl Acad Sci U S A, 2021. 118(17).
148. Vilgelm, A.E. and A. Richmond, Chemokines Modulate Immune Surveillance in Tumorigenesis, Metastasis, and Response to Immunotherapy. Front Immunol, 2019. 10: p. 333.
149. Balkwill, F., Cancer and the chemokine network. Nat Rev Cancer, 2004. 4(7): p. 540-50.
150. Zou, W., Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol, 2006. 6(4): p. 295-307.
151. Viola, A., et al., The pros and cons of chemokines in tumor immunology. Trends Immunol, 2012. 33(10): p. 496-504.
152. Sarvaiya, P.J., et al., Chemokines in tumor progression and metastasis. Oncotarget, 2013. 4(12): p. 2171-85.
153. Nagarsheth, N., M.S. Wicha, and W. Zou, Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nat Rev Immunol, 2017. 17(9): p. 559-572.
154. Mollica Poeta, V., et al., Chemokines and Chemokine Receptors: New Targets for Cancer Immunotherapy. Front Immunol, 2019. 10: p. 379.
155. Schall, T.J. and A.E. Proudfoot, Overcoming hurdles in developing successful drugs targeting chemokine receptors. Nat Rev Immunol, 2011. 11(5): p. 355-63.
156. Solari, R., J.E. Pease, and M. Begg, "Chemokine receptors as therapeutic targets: Why aren't there more drugs?". Eur J Pharmacol, 2015. 746: p. 363-7.
157. Zhao, S., B. Wu, and R.C. Stevens, Advancing Chemokine GPCR Structure Based Drug Discovery. Structure, 2019. 27(3): p. 405-408.
158. Dean, L., Maraviroc Therapy and CCR5 Genotype, in Medical Genetics Summaries, V.M. Pratt, et al., Editors. 2012: Bethesda (MD).
159. Plerixafor: AMD 3100, AMD3100, JM 3100, SDZ SID 791. Drugs R D, 2007. 8(2): p. 113-9.
160. Walters, M.J., et al., CCR9 inhibition does not interfere with the development of immune tolerance to oral antigens. Immunol Lett, 2013. 151(1-2): p. 44-7.
161. Seitz, L., et al., Orally Administered CCR9 Inhibitor CCX507 Effectively Blocks CCR9 in Circulating Human Leukocytes: 1678. Official journal of the American College of Gastroenterology | ACG, 2014. 109: p. S497.
162. Dairaghi, D.J., et al., Pharmacokinetic and pharmacodynamic evaluation of the novel CCR1 antagonist CCX354 in healthy human subjects: implications for selection of clinical dose. Clin Pharmacol Ther, 2011. 89(5): p. 726-34.
163. Tak, P.P., et al., Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. Ann Rheum Dis, 2013. 72(3): p. 337-44.
164. Huynh, C., et al., A Multipurpose First-in-Human Study With the Novel CXCR7 Antagonist ACT-1004-1239 Using CXCL12 Plasma Concentrations as Target Engagement Biomarker. Clin Pharmacol Ther, 2021. 109(6): p. 1648-1659.
165. Blanchet, X., et al., Touch of chemokines. Front Immunol, 2012. 3: p. 175.
166. Gaertner, H., et al., Highly potent, fully recombinant anti-HIV chemokines: reengineering a low-cost microbicide. Proc Natl Acad Sci U S A, 2008. 105(46): p. 17706-11.
167. Veazey, R.S., et al., Topically applied recombinant chemokine analogues fully protect macaques from vaginal simian-human immunodeficiency virus challenge. J Infect Dis, 2009. 199(10): p. 1525-7.
168. Ueda, R., Clinical Application of Anti-CCR4 Monoclonal Antibody. Oncology, 2015. 89 Suppl 1: p. 16-21.
169. Hamers-Casterman, C., et al., Naturally occurring antibodies devoid of light chains. Nature, 1993. 363(6428): p. 446-8.
170. Muyldermans, S., et al., Camelid immunoglobulins and nanobody technology. Vet Immunol Immunopathol, 2009. 128(1-3): p. 178-83.
171. De Groof, T.W.M., et al., Nanobodies: New avenues for imaging, stabilizing and modulating GPCRs. Mol Cell Endocrinol, 2019. 484: p. 15-24.
172. Maussang, D., et al., Llama-derived single variable domains (nanobodies) directed against chemokine receptor CXCR7 reduce head and neck cancer cell growth in vivo. J Biol Chem, 2013. 288(41): p. 29562-72.
173. Blanchetot, C., et al., Neutralizing nanobodies targeting diverse chemokines effectively inhibit chemokine function. J Biol Chem, 2013. 288(35): p. 25173-82.
174. Smolarek, D., et al., A recombinant dromedary antibody fragment (VHH or nanobody) directed against human Duffy antigen receptor for chemokines. Cell Mol Life Sci, 2010. 67(19): p. 3371-87.
175. Bradley, M.E., et al., Potent and efficacious inhibition of CXCR2 signaling by biparatopic nanobodies combining two distinct modes of action. Mol Pharmacol, 2015. 87(2): p. 251-62.
176. De Groof, T.W.M., et al., Targeting the latent human cytomegalovirus reservoir for T-cell-mediated killing with virus-specific nanobodies. Nat Commun, 2021. 12(1): p. 4436.
177. De Groof, T.W.M., et al., Selective targeting of ligand-dependent and -independent signaling by GPCR conformation-specific anti-US28 intrabodies. Nat Commun, 2021. 12(1): p. 4357.
178. Loetscher, M., et al., Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med, 1996. 184(3): p. 963-9.
179. Qin, S., et al., The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest, 1998. 101(4): p. 746-54.

180. Bonecchi, R., et al., Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med, 1998. 187(1): p. 129-34.
181. Garcia-Lopez, M.A., et al., CXCR3 chemokine receptor distribution in normal and inflamed tissues: expression on activated lymphocytes, endothelial cells, and dendritic cells. Lab Invest, 2001. 81(3): p. 409-18.
182. Romagnani, P., et al., Interferon-inducible protein 10, monokine induced by interferon gamma, and interferon-inducible T-cell alpha chemoattractant are produced by thymic epithelial cells and attract T-cell receptor (TCR) alphabeta+ CD8+ single-positive T cells, TCRgammadelta+ T cells, and natural killer-type cells in human thymus. Blood, 2001. 97(3): p. 601-7.
183. Kohrgruber, N., et al., Plasmacytoid dendritic cell recruitment by immobilized CXCR3 ligands. J Immunol, 2004. 173(11): p. 6592-602.
184. Aksoy, M.O., et al., CXCR3 surface expression in human airway epithelial cells: cell cycle dependence and effect on cell proliferation. Am J Physiol Lung Cell Mol Physiol, 2006. 290(5): p. L909-18.
185. Kelsen, S.G., et al., The chemokine receptor CXCR3 and its splice variant are expressed in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol, 2004. 287(3): p. L584-91.
186. Cole, K.E., et al., Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. J Exp Med, 1998. 187(12): p. 2009-21.
187. Farber, J.M., A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. Proc Natl Acad Sci U S A, 1990. 87(14): p. 5238-42.
188. Loos, T., et al., TLR ligands and cytokines induce CXCR3 ligands in endothelial cells: enhanced CXCL9 in autoimmune arthritis. Lab Invest, 2006. 86(9): p. 902-16.
189. Kaplan, G., et al., The expression of a gamma interferon-induced protein (IP-10) in delayed immune responses in human skin. J Exp Med, 1987. 166(4): p. 1098-108.
190. Reynders, N., et al., The Distinct Roles of CXCR3 Variants and Their Ligands in the Tumor Microenvironment. Cells, 2019. 8(6).
191. Sorensen, T.L., et al., Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. J Clin Invest, 1999. 103(6): p. 807-15.
192. Mach, F., et al., Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. J Clin Invest, 1999. 104(8): p. 1041-50.
193. Tokunaga, R., et al., CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation - A target for novel cancer therapy. Cancer Treat Rev, 2018. 63: p. 40-47.
194. Fulton, A.M., The chemokine receptors CXCR4 and CXCR3 in cancer. Curr Oncol Rep, 2009. 11(2): p. 125-31.
195. Cox, M.A., et al., Human interferon-inducible 10-kDa protein and human interferon-inducible T cell alpha chemoattractant are allotopic ligands for human CXCR3: differential binding to receptor states. Mol Pharmacol, 2001. 59(4): p. 707-15.
196. Xanthou, G., T.J. Williams, and 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, 2003. 33(10): p. 2927-36.
197. Nedjai, B., et al., Small molecule chemokine mimetics suggest a molecular basis for the observation that CXCL10 and CXCL11 are allosteric ligands of CXCR3. Br J Pharmacol, 2012. 166(3): p. 912-23.
198. 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, 2015. 290(35): p. 21292-304.
199. Campanella, G.S., et al., Oligomerization of CXCL10 is necessary for endothelial cell presentation and in vivo activity. J Immunol, 2006. 177(10): p. 6991-8.
200. Severin, I.C., et al., Characterization of the chemokine CXCL11-heparin interaction suggests two different affinities for glycosaminoglycans. J Biol Chem, 2010. 285(23): p. 17713-24.
201. Lasagni, L., et al., An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. J Exp Med, 2003. 197(11): p. 1537-49.
202. Ehlert, J.E., et al., Identification and partial characterization of a variant of human CXCR3 generated by posttranscriptional exon skipping. J Immunol, 2004. 173(10): p. 6234-40.
203. Manousou, P., et al., Expression of a splice variant of CXCR3 in Crohn's disease patients; indication for a lymphocyte--epithelial cell interaction. J Gastroenterol Hepatol, 2008. 23(12): p. 1823-33.
204. Vollmer, T., et al., The intratumoral CXCR3 chemokine system is predictive of chemotherapy response in human bladder cancer. Sci Transl Med, 2021. 13(576).
205. Korniejewska, A., et al., Expression and agonist responsiveness of CXCR3 variants in human T lymphocytes. Immunology, 2011. 132(4): p. 503-15.
206. Thompson, B.D., et al., Inhibition of G alpha i2 activation by G alpha i3 in CXCR3-mediated signaling. J Biol Chem, 2007. 282(13): p. 9547-9555.
207. Smit, M.J., et al., CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. Blood, 2003. 102(6): p. 1959-65.
208. Zohar, Y., et al., CXCL11-dependent induction of FOXP3-negative regulatory T cells suppresses autoimmune encephalomyelitis. J Clin Invest, 2014. 124(5): p. 2009-22.
209. Bonacchi, A., et al., Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. J Biol Chem, 2001. 276(13): p. 9945-54.
210. Petrai, I., et al., Activation of p38(MAPK) mediates the angiostatic effect of the chemokine receptor CXCR3-B. Int J Biochem Cell Biol, 2008. 40(9): p. 1764-74.
211. Bodnar, R.J. and A. Wells, Differential regulation of pericyte function by the CXC receptor 3. Wound Repair Regen, 2015. 23(6): p. 785-96.
212. Smith, J.S., et al., C-X-C Motif Chemokine Receptor 3 Splice Variants Differentially Activate Beta-Arrestins to Regulate Downstream Signaling Pathways. Mol Pharmacol, 2017. 92(2): p. 136-150.
213. Berchiche, Y.A. and T.P. Sakmar, CXC Chemokine Receptor 3 Alternative Splice Variants Selectively Activate Different Signaling Pathways. Mol Pharmacol, 2016. 90(4): p. 483-95.
214. Colvin, R.A., et al., Intracellular domains of CXCR3 that mediate CXCL9, CXCL10, and CXCL11 function. J Biol Chem, 2004. 279(29): p. 30219-27.
215. Metzemaekers, M., et al., Overview of the Mechanisms that May Contribute to the Non-Redundant Activities of Interferon-Inducible CXC Chemokine Receptor 3 Ligands. Front Immunol, 2017. 8: p. 1970.
216. Flier, J., et al., Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. J Pathol, 2001. 194(4): p. 398-405.
217. Glass, W.G., et al., Mechanisms of host defense following severe acute respiratory syndrome-coronavirus (SARS-CoV) pulmonary infection of mice. J Immunol, 2004. 173(6): p. 4030-9.
218. Campanella, G.S., et al., Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. Proc Natl Acad Sci U S A, 2008. 105(12): p. 4814-9.
219. Hancock, W.W., et al., Requirement of the chemokine receptor CXCR3 for acute allograft rejection. J Exp Med, 2000. 192(10): p. 1515-20.
220. Muller, M., et al., CXCR3 signaling reduces the severity of experimental autoimmune

encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. J Immunol, 2007. 179(5): p. 2774-86.
221. Hu, J.K., et al., Expression of chemokine receptor CXCR3 on T cells affects the balance between effector and memory CD8 T-cell generation. Proc Natl Acad Sci U S A, 2011. 108(21): p. E118-27.
222. Kastenmuller, W., et al., Peripheral prepositioning and local CXCL9 chemokine-mediated guidance orchestrate rapid memory CD8+ T cell responses in the lymph node. Immunity, 2013. 38(3): p. 502-13.
223. Schmueck-Henneresse, M., et al., Peripheral blood-derived virus-specific memory stem T cells mature to functional effector memory subsets with self-renewal potency. J Immunol, 2015. 194(11): p. 5559-67.
224. Hickman, H.D., et al., CXCR3 chemokine receptor enables local CD8(+) T cell migration for the destruction of virus-infected cells. Immunity, 2015. 42(3): p. 524-37.
225. Mikucki, M.E., et al., Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. Nat Commun, 2015. 6: p. 7458.
226. Groom, J.R., et al., CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. Immunity, 2012. 37(6): p. 1091-103.
227. Martinez-Lostao, L., A. Anel, and J. Pardo, How Do Cytotoxic Lymphocytes Kill Cancer Cells? Clin Cancer Res, 2015. 21(22): p. 5047-56.
228. Speiser, D.E., P.C. Ho, and G. Verdeil, Regulatory circuits of T cell function in cancer. Nat Rev Immunol, 2016. 16(10): p. 599-611.
229. Hashimoto, M., et al., CD8 T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. Annu Rev Med, 2018. 69: p. 301-318.
230. Sharma, P. and J.P. Allison, Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell, 2015. 161(2): p. 205-14.
231. Ribas, A. and J.D. Wolchok, Cancer immunotherapy using checkpoint blockade. Science, 2018. 359(6382): p. 1350-1355.
232. Chheda, Z.S., et al., Chemoattractant Receptors BLT1 and CXCR3 Regulate Antitumor Immunity by Facilitating CD8+ T Cell Migration into Tumors. J Immunol, 2016. 197(5): p. 2016-26.
233. Chow, M.T., et al., Intratumoral Activity of the CXCR3 Chemokine System Is Required for the Efficacy of Anti-PD-1 Therapy. Immunity, 2019. 50(6): p. 1498-1512 e5.
234. House, I.G., et al., Macrophage-Derived CXCL9 and CXCL10 Are Required for Antitumor Immune Responses Following Immune Checkpoint Blockade. Clin Cancer Res, 2020. 26(2): p. 487-504.
235. Johnson, M., et al., Discovery and optimization of a series of quinazolinone-derived antagonists of CXCR3. Bioorg Med Chem Lett, 2007. 17(12): p. 3339-43.
236. Andrews, S.P. and R.J. Cox, Small Molecule CXCR3 Antagonists. J Med Chem, 2016. 59(7): p. 2894-917.
237. Ribeiro, S. and R. Horuk, The clinical potential of chemokine receptor antagonists. Pharmacol Ther, 2005. 107(1): p. 44-58.
238. Sandborn, W.J., et al., Eldelumab [Anti-IP-10] Induction Therapy for Ulcerative Colitis: A Randomised, Placebo-Controlled, Phase 2b Study. J Crohns Colitis, 2016. 10(4): p. 418-28.
239. Sandborn, W.J., et al., Eldelumab [anti-interferon-gamma-inducible protein-10 antibody] Induction Therapy for Active Crohn's Disease: a Randomised, Double-blind, Placebo-controlled Phase IIa Study. J Crohns Colitis, 2017. 11(7): p. 811-819.

Aims of the thesis

The CXCR3 chemokine receptor, together with its cognate ligands, CXCL9, CXCL10, and CXCL11, plays important roles in the guidance and positioning of leukocytes to ensure immune surveillance but also contribute to the development of various pathologies, including cancer development and metastasis. The cxcr3 chemokine receptor gene can be transcribed into three variants, CXCR3-A, CXCR3-B, and CXCR3-Alt. Each variant differs in its amino acid sequences and demonstrates different cellular effects. CXCR3-A is considered the reference receptor, as it was originally found on activated immune cells, and mediates the migration of immune cells through $G_{\alpha i/o}$ protein activation. The CXCR3-B variant has an extension of 51 AA at its N terminus compared to CXCR3-A and is mainly present on endothelial cells. This CXCR3 variant display opposite cellular effects to CXCR3-A, but its underlying mechanism is still unclear. Conflicting data about its function, expression, and downstream signaling have been reported making this variant still an enigmatic receptor. CXCR3-Alt lacks two transmembrane helices and a modified C terminal region in contrast to CXCR3-A. Fewer data about its biology is currently available. However, some reports showed that all CXCR3 chemokines induce CXCR3-Alt receptor internalization and that this receptor, together with CXCL11, are important players for the attenuation of bladder cancer upon chemotherapy.

Targeting the CXCR3 variants, to regulate their activity in the tumor bed, remains a challenge. One molecule, AMG487, has entered the clinical stages but failed to demonstrate efficacy for the treatment of psoriasis resulting in discontinuation of the clinical trials. Additionally, the lack of CXCR3 variant-specific probes and tools that modulate or distinguish the CXCR3 variants makes it difficult to study these receptors in vitro and in vivo.

This thesis aimed at deciphering the biology and signaling of the CXCR3 variants, CXCR3-A and CXCR3-B, and linking their functions to the observed cellular effects in the tumor microenvironment. Furthermore, this thesis strived at developing new variant-specific probes to be able to distinguish and modulate the CXCR3 variants to further investigate these receptors in vitro and in vivo.

In the tumor microenvironment, CXCR3-A and CXCR3-B show opposing cellular effects. CXCR3-A enhances cancer cell proliferation but also inhibits tumor progression by attracting immune cells to the inflamed bed. Similarly, CXCR3-B also attenuates tumor progress although the underlying mechanism remains unclear. To fully map the different cellular outcomes induced by CXCR3-A, CXCR3-B, or their ligands in the tumor bed, we first performed an elaborate literature review (**chapter 1**). We were able to assign distinct functions to each player of the CXCR3 axis in the tumor bed. These newly acquired insights prompt us to conduct an in-depth pharmacological comparison of both CXCR3- variants to elucidate their signaling properties, which would explain their cellular effects in the tumor bed (**chapter 2**). We were able to attribute different modes of action for both CXCR3 variants and their shared ligands by studying the receptors-mediated signaling events, their distribution in the absence and presence of ligands, and the capability to take up endogenous ligands. The obtained results majorly explain their cellular effects detected in the tumor microenvironment.

The currently available CXCR3 antibodies cannot distinguish both variants as a common linear epitope was used during their development. Considering the different cellular effects of CXCR3-A and CXCR3-B, we assumed that their distinct characteristics probably lie in adapting different conformational states following receptor activation. Therefore, we aimed at developing CXCR3 variant-specific conformational nanobodies, which could be used to further investigate these receptors in vitro and in vivo. Due to the sanitary pandemic (COVID-19), this part of the PhD had to be put on hold and left uncompleted despite spending much time and effort. Consequently, this work will not be presented or discussed in this thesis.

Previous studies on ACKR3 conducted in our group showed that this atypical receptor displays a different ligand binding mode compared to CXCR4 and CXCR3 and has a strong activation potential. We questioned whether these atypical attributes could be assigned to the intra-N terminal 4 AA loop formed by a disulphide bridge; a feature only observed in this chemokine receptor. In line with ACKR3, CXCR3 also has two adjacent cysteines in its N terminus that possibly forms a disulphide bridge. In **chapter 3,** we generated variants of ACKR3 and CXCR3-A with alternated residues in these cysteine regions to investigate the impact of this N terminal loop and the disulphide bridge on the binding of their ligands and the activation of the receptors. We were able to show that ACKR3's N terminal arch impacted the binding of its endogenous ligands, but not its functionality, and that this receptor shows some degree of structural plasticity in absence of extracellular disulphide bridges. The substitution of CXCR3's N terminal cysteines with serines increased its expression at the cell surface but did not alter receptor activation.

The implementation of a highly sensitive β-arrestin recruitment, based on the NanoBiT technology, in our group also lead to the discovery of new ligand-receptor pairings. The

systematic reassessment of the chemokine-atypical chemokine receptors ACKR2 and ACKR4 pairing allowed us the identify and characterize new ligands of these two ACKRs: **Chapter 4** describes the identification of two CC chemokines, CCL20 and CCL22, that bind and induce arrestin recruitment to ACKR4, and **chapter 5** reports CXCL10 as a new agonist of ACKR2.

Chapter 1

The distinct roles of CXCR3 variants and their ligands in the tumor microenvironment

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Contribution:

Literature research and writing of the manuscript

1. Abstract

First thought to orchestrate exclusively leukocyte trafficking, chemokines are now acknowledged for their multiple roles in the regulation of cell proliferation, differentiation, and survival. Dysregulation of their normal functions contributes to various pathologies, including inflammatory diseases and cancer. The two chemokine receptor 3 variants CXCR3-A and CXCR3-B, together with their cognate chemokines (CXCL11, CXCL10, CXCL9, CXCL4, and CXCL4L1), are involved in the control but also in the development of many tumors. CXCR3-A drives the infiltration of leukocytes to the tumor bed to modulate tumor progression (paracrine axis). Conversely, tumor-driven changes in the expression of the CXCR3 variants and their ligands promote cancer progression (autocrine axis). This review summarizes the anti- and pro-tumoral activities of the CXCR3 variants and their associated chemokines with a focus on the understanding of their distinct biological roles in the tumor microenvironment.

2. Introduction

Chemokines, or chemotactic cytokines, are small, secretory proteins that form the largest group within the cytokine family [1]. They bind to their cognate chemokine receptors, which belong to the G protein-coupled receptor (GPCR) superfamily, and mediate cellular responses as a result of downstream signaling pathways initiated by the activation of G proteins and regulated by the recruitment of arrestins [1–3]. To date, 48 chemokines and 23 receptors have been identified in humans. The chemokine–receptor network is highly intricate; a chemokine can bind one or many receptors, while a receptor usually recognizes several chemokines [1,4]. Moreover, a recent paradigm shift brought to light the concept known as biased signaling according to which GPCRs, including chemokine receptors, depending on the ligand or cellular context, trigger distinct signaling pathways [3,5,6]. Chemokines primarily arbitrate various immune responses through the migration of chemokine-receptor-expressing leukocytes to the target tissues, while they also orchestrate the development and maintenance of lymphoid or non-lymphoid tissues by inducing the migration, proliferation, and differentiation of stromal and immune cells [7–12]. In addition, chemokines play key roles in the tumor microenvironment (TME) as they are secreted by and act on the different cellular components comprising tumor cells, T- and B-lymphocytes, natural killer (NK) cells, macrophages, dendritic cells (DCs), fibroblasts, and vascular and lymphatic endothelial cells and their associated pericytes. Together with other soluble factors such as growth factors, inflammatory cytokines, and extracellular matrix enzymes, they contribute to cancer development and the formation of a cancer-specific immunity, influencing the maturation, activation, and trafficking of tumor-infiltrating immune cells [11,13–19].

The interferon (IFN)-gamma inducible chemokines CXCL11, CXCL10, and CXCL9, and the platelet-derived chemokines CXCL4 and its non-allelic variant CXCL4L1, exert their

biological function by binding to the IFN-gamma inducible chemokine receptor CXCR3 [6,20– 22]. Interestingly, due to alternative splicing of the CXCR3 mRNA, three variants have been reported, CXCR3-A, CXCR3-B, and CXCR3-Alt, which exhibit differences in their expression profiles, their N or C terminus, and the number of transmembrane domains (Figure 1). These structural differences alter the physiological characteristics of the receptors, including ligand- binding properties, signaling pathways, and cellular responses [23]. CXCR3-A and CXCR3-B are the two variants that have been best studied. They differ by a 52-amino acid (AA) extension at the N terminus of CXCR3-B and exert opposing cellular effects [24]. On the one hand, binding of CXCL11, CXCL10, and CXCL9 to the CXCR3-A variant induces chemotaxis and proliferation of cells by activating G_i and G_q proteins and triggering their downstream signaling pathways, including the intracellular Ca^{2+} release, ERK1/2, and Akt pathways [5,23,25–34]. The activation of CXCR3-A by CXCL4 and its variant CXCL4L1 remains to be elucidated [30,31,35]. On the other hand, stimulation of CXCR3-B by these chemokines inhibits cell migration and proliferation and induces apoptosis. The mechanism behind this opposite cellular response driven by CXCR3-B is still unclear; however, one study suggests a coupling to the G_s protein [24].

While CXCR3 and its endogenous ligands are mainly involved in inflammation and wound healing [8,36], they have also been described to have a dual role in tumor progression and immunity. This review aims to outline the impact of the CXCR3 ligand–

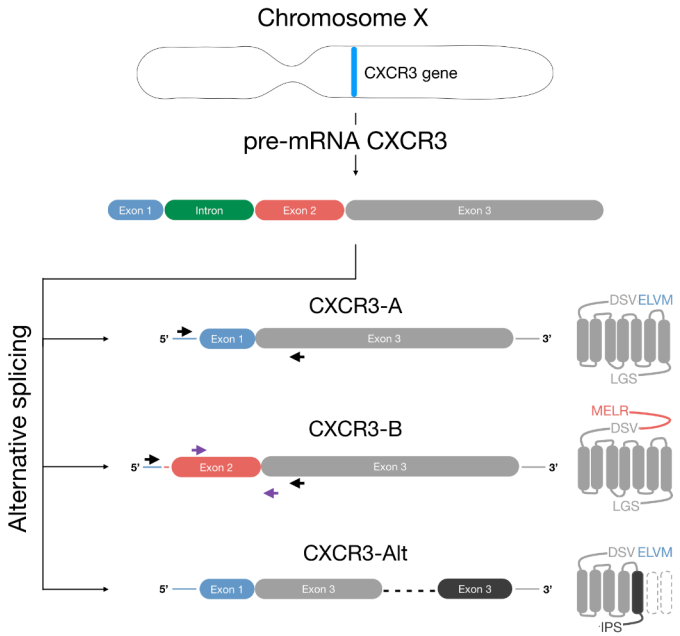

Figure 1 | Schematic representation of CXCR3 variants. Due to alternative splicing of the pre-mRNA of the CXCR3 gene, located in chromosome X, three CXCR3 variants can be generated. The CXCR3-A variant is the product of the splicing of the exon 1 and exon 3 within the CXCR3 gene. The assembly of exon 2 and exon 3 results in the CXCR3-B variant which has an N terminus longer by 52 AA compared with CXCR3-A. The removal of the intron, exon 2 and a 337-bp region within the third exon during RNA splicing results in the CXCR3-Alt variant that comprises the N terminus and the first four transmembrane domains identical to CXCR3-A as well as a possible fifth transmembrane region and a C terminus, which are different from CXCR3-A or -B. The primers used to detect CXCR3-A, which also recognize CXCR3-B, and CXCR3-B are represented by the black and purple arrows, respectively.

receptor axis and its expression changes on the TME with a focus on the CXCR3-A and CXCR3-B variants.

3. The cross talk of the CXCR3 variants and their chemokine ligands with the tumor microenvironment

Chemokines are abundantly present in the TME and play key roles in inducing proliferation of benign and malignant cells, leukocyte migration, and angiogenesis [11,12,37,38]. These processes can be initiated and maintained in paracrine and autocrine fashions.

3.1. CXCR3-A on leukocytes mediates their migration to the TME to attenuate tumor progression

The presence of tumor-infiltrating leukocytes (TIL) in the TME is known to influence tumor development [39]. Following the discovery of CXCR3-A on activated T-lymphocytes [25] and the anti-tumoral activity of CXCL10 [40], the possibility of an anti-tumoral response through the migration of CXCR3-A+ leukocytes to the TME was proposed [41]. The importance of CXCR3-dependent anti-tumoral activity was confirmed by Hensbergen et al. where CXCL11-producing EL4 lymphoma cells, injected in mice, were rejected due to the infiltration of CXCR3+ CD8+ T lymphocytes and macrophages [42]. Similar observations were reported in murine models of renal cell carcinoma (RCC) (RENCA) and spontaneous melanoma (B16F10) where the reduced tumor growth resulted from an increased presence of CXCR3-A-expressing CD4+ and CD8+ lymphocytes and NK cells in the tumor bed [43–45].

In another study, melanoma (B16F10) or breast cancer (E0771) cells injected in $CXCR3^{-/-}$ mice showed a significant increase in tumor growth compared to wild type (WT) mice, which was associated with a lower prevalence of CD8+ and CD4+ T cells as well as NK cells. This TIL-dependent anti-tumoral activity was furthermore validated in B16F10 tumor-bearing $Rag2^{-/-}$ mice, which showed a significantly increased tumor growth when transferred with $CXCR3^{-/-}$ cytotoxic T-lymphocytes (CTLs) compared to WT CTLs [46]. Such anti-tumoral activity of CXCR3-A+ leukocytes was also detected in human breast and gastric cancers as well as in RMA lymphoma [47–49]. Interestingly, the regressive characteristic of melanoma or certain melanocytic lesions was proposed to depend on the increased attraction of CXCR3- A+ cytotoxic lymphocytes to the TME [50].

In contrast, the higher prevalence of CXCR3+ regulatory T cells (Tregs) in human ovarian carcinomas was suggested to dampen the effector cell response, thus favoring the progression of the tumor [51]. This pro-tumoral effect of CXCR3+ Tregs was also observed in hepatocellular carcinoma (HCC), where a correlation could be made between CXCR3–CXCL10-dependent Treg infiltration and increased tumor growth and HCC recurrence after liver transplantation [52]. Moreover, in a chemically inducible murine model of skin

carcinoma, CXCR3 knockout (KO) mice developed fewer tumors compared to WT mice. This observation was linked to a reduced presence of CXCR3-expressing CD3+ T cells, suggesting a cell proliferative effect on epidermal cells and an pro-tumoral activity of these TIL [53].

Taken together, these data suggest that the expression of CXCR3-A on leukocytes is needed to attract them to the TME and allows an anti-tumoral activity that diminishes tumor growth. However, the attraction of Tregs or other T-lymphocytes to the TME could also have a pro-tumoral effect by inducing cell proliferation and inhibiting the antitumor activity of effector leukocytes.

Of note, an adequate recruitment of CTLs in the tumor bed is not always observed. Correlated to a poor survival, it was for instance demonstrated that only 16% of patients with esophageal squamous cell carcinomas (ESCCs) or adenocarcinomas showed detectable CD8+ T cell infiltration within the tumor [54]. In fact, insufficient recruitment of activated T lymphocytes to the tumor bed is also recognized as one of the major hurdles in the current immunotherapeutic approaches [55]. Notably, the relevance of CXCR3-driven responses in immunotherapy has been demonstrated in several studies. In a B16-OVA mouse model, Mikucki et al. identified CXCR3-mediated trafficking at the tumor vasculature as important for effective T-cell-based cancer therapy by showing that CXCR3 is required for the extravasation of CTL to the tumor bed [45]. Similarly, in a B16 melanoma mouse model, the deletion of CXCR3 in CTLs lead to an impaired anti-tumoral response due to the failure of CTL infiltration in the TME upon anti-PD-1 treatment, suggesting CXCR3-dependent anti- PD-1 based anti-tumoral response [46]. Moreover, the blockade of PD-1 on naïve T cells enhanced CXCR3 expression and their absolute number in the spleen [56]. These studies show that CXCR3 is a potential target to increase the effectiveness of current immunotherapies.

3.2. CXCR3-A on malignant cells in the TME contributes to tumor growth and dissemination

As described above, the expression of CXCR3-A on leukocytes mediates their migration to the TME to exert their anti- and pro-tumoral activity; however, the CXCR3-A variant is also known to induce proliferation of various cell types, including human mesangial cells [24]. Therefore, the presence of CXCR3-A on tumor cells can induce an adverse effect by promoting and sustaining tumor development. Indeed, an increased proliferation of malignant glioma cells compared with normal astrocytes [57] and an anti-apoptotic effect in CXCR3-A- overexpressing human myeloma cell lines (HMCLs) was observed after CXCL10 treatment [58]. The increased expression of CXCR3-A on stage II colorectal cancer (CRC) and papillary thyroid cancer (PTC) cells was also associated with increased tumor development and negative prognosis [59,60]. In line with this,

immunohistochemical analysis of localized clear cell RCC patient samples showed strong staining for CXCR3, which was correlated with a negative disease prognosis [61].

In addition to tumor development, expression of CXCR3-A on tumor cells promotes their dissemination. B16F10 melanoma injected into the footpads of mice showed macroscopic metastatic tumors in the lymph nodes already after one week, which could be attenuated with antisense CXCR3-A RNA [62]. An immunohistological analysis of primary invasive cutaneous melanoma samples from patients also supported the implication of CXCR3-A in tissue invasion and metastasis of malignant cells [63]. Furthermore, a metastatic potential conferred by CXCR3-A was observed in other human diseases, including epidermotropic and other B-cell disorders [64,65], glioblastoma (GBM) [66,67], CRC [68,69], high-grade serous ovarian cancer (HGSC) [70], lung adenocarcinoma [71] and breast cancer [72,73].

In conclusion, the expression of CXCR3-A on malignant cells leads to an increased proliferation and dissemination.

3.3. CXCR3-B has an anti-proliferative effect in the tumor microenvironment

In contrast to CXCR3-A, the CXCR3-B variant was shown to inhibit cell migration and proliferation [24]. CXCR3-B was first detected on human microvascular endothelial cells (HuMVECs) [24], but has also been found on CD4+ T lymphocytes [74], airway epithelial cells [75], and pericytes [76]. Although CXCR3-B is often expressed concomitantly with CXCR3-A, distinct CXCR3 variant expression levels can be observed on diverse cell types. Endothelial cells and pericytes abundantly express CXCR3-B [24,76], whereas CXCR3-A is not detectable [76,77], and activated T lymphocytes show higher CXCR3-A expression levels compared with CXCR3-B [31]. Various cancers evolve mechanisms to shift the CXCR3 variant expression levels towards ratios that are more beneficial for tumor maintenance or progression. For instance, human RCC downregulate CXCR3-B, which results in the upregulation of the anti-apoptotic heme oxygenase-1 (HO-1) and an increased tumor cell proliferation and dissemination [78,79]. Similarly, lower CXCR3-B mRNA levels were observed in ovarian and breast cancer cells compared with non-cancerous epithelial cells [80,81]. Moreover, decreasing the expression of CXCR3-B in breast cancer cells in vitro, using CXCR3-B-specific siRNA, resulted in increased cell proliferation and expression of anti- apoptotic proteins [81,82]. In addition to the inhibitory effect of CXCR3-B on cell proliferation, it has been observed that a rise in CXCR3-B expression could lead to increased tumor necrosis and lower the metastatic ability of tumor cells [83,84]. Of note, cancer stem cell-like properties upon CXCR3-B overexpression were reported in vitro in a breast cancer cell line, which could be prevented by silencing its mRNA [84].

Overall, it seems that cancer cells lower their expression of CXCR3-B to promote tumor proliferation, survival and dissemination and that this cellular effect can be reversed by enhancing CXCR3-B expression.

3.4. Is the CXCR3-B variant on endothelial cells involved in angiogenesis regulation?

Tumors require adequate blood supply to obtain the sufficient oxygen, nutrients and other metabolic components to ensure their growth and survival. In contrast to normal angiogenesis, in which new blood vessels mature rapidly and show slow cell turn-over, tumor angiogenesis is an uncontrolled process where new blood vessels are formed in an irregular fashion [85,86]. Due to the high expression of CXCR3-B on endothelial cells and the angiostatic effect of CXCL10, CXCL9, and CXCL4 [24,87–90], it was proposed that the CXCR3-B variant could be responsible for this negative impact on tumor angiogenesis. Arenberg et al. indeed reported a decrease in angiogenesis and neovascularization in squamous cell carcinoma (SCC) after addition of CXCL10 in the TME that resulted in an attenuation in tumor growth and dissemination to the lung [91]. A similar observation was made following injection of CXCL9 to the tumor site in a mouse RENCA model [43]. In contrast, a rise in CXCL11 had no impact on the angiogenesis of mouse EL4 lymphoma cells nor human CRC [42,92]. Considering the expression of CXCR3 variants was not analyzed in these studies, one can only speculate that the inhibitory effect of these chemokines on tumor angiogenesis is driven through CXCR3-B present on endothelial cells, a phenomenon which may be cancer-type-dependent.

Although the above-mentioned data point to CXCR3-B-dependent angiostatic and inhibitory effects on cell proliferation, it has also been suggested that these are instead GAG- mediated. Indeed, both WT and CXCR3 KO endothelial cells showed an equivalent inhibitory effect on cell proliferation in the presence of CXCL10, which could not be reversed by neutralizing anti-CXCR3 antibodies. Moreover, the generation of a CXCL10 mutant devoid of CXCR3 signaling properties could also inhibit human umbilical cord endothelial cell (HUVEC) proliferation [93].

3.5. The CXCR3 ligands are upregulated in the TME and act in an autocrine and paracrine manner

Tumor cells as well as endothelial and immune cells secrete chemokines in the TME, which induce the recruitment of immune cells, modulate tumor immunity and promote tumor cell proliferation as well as metastasis [12]. Cancer cells also control the production and the activity of chemokines through the transcriptional silencing [94–96] or the post-translational modifications, via dipeptidyl peptidase 4 (DPP4 or CD26) [97,98]. These modifications undermine the attraction of tumor-invading lymphocytes, which impairs their anti-tumor effect [99,100] and the success of immunotherapy. In addition, CXCR3-endogenous ligands have been described to activate distinct signaling pathways, leading to specific cellular

responses. For example, CXCL9 and CXCL10 induce effector Th1/Th17 cells and CXCL11 drives a Treg and Th2-biased effector cell polarization upon activation of CXCR3 on CD4+ T cells, while they upregulate tumor cell-related proteins that are beneficial for their survival, such as PD-L1 [21,101,102]. Here, we give an outline on the impact of the TME on the expression and secretion of CXCL11, CXCL10, CXCL9, CXCL4 and its variant CXCL4L1, and the effect of these chemokines on the different cellular components present within the TME.

3.5.1. CXCL11

Although CXCL11 is the most potent CXCR3 ligand [103], its role in CXCR3-driven processes is sometimes overlooked due to its impaired production in certain mouse strains; such as in C57BL/6 mice where a 2-bp insertion after the start codon results in a premature stop codon and ultimately CXCL11 null mutant mice [101,104,105]. CXCL11 is also less available compared with CXCL9 and CXCL10, whether in health or disease state [106–108]. For CXCL11, being the full agonist of CXCR3 [109], its production may also be submitted to stricter regulation. In addition, ACKR3, formerly known as CXCR7, is a scavenger receptor for CXCL11 that may reduce the availability of CXCL11 *in vivo* and in the tumor bed. Indeed, ACKR3 has been shown to be upregulated on various tumor cells as well as on tumor- associated vasculature, therefore potentially interfering with the CXCR3–CXCL11 interactions [103,110–112].

Nevertheless, several studies pinpointed the importance of CXCL11–CXCR3 axis within the TME. It has been reported that mice challenged with EL4 T-cell lymphoma cells genetically modified to produce murine CXCL11 attracted more CD8+ lymphocytes and macrophages with anti-tumoral activity to the TME compared to WT EL4 T-cell lymphoma cells [42]. On the other hand, a study on human colon adenocarcinoma showed enhanced tumor growth and invasiveness after injection of CXCL11 to the TME [92]. It has also been described that the repression of CXCL11 in CRC tissues diminished the tumor cell growth and metastasis [113]. In addition, the observed increased expression of CXCL11 in HCC cells was linked with the upregulation of stem cell-related genes and the acquisition and maintenance of self-renewal and tumorigenic properties of tumor-initiating cells through an autocrine axis [114].

3.5.2. CXCL10

Although CXCL10 is only a partial agonist of CXCR3, its impact on many human pathologies including cancer has been extensively studied. In glioma cells, for example, the expression of CXCL10 is significantly upregulated when compared with healthy astrocytes and has been shown to increase cell proliferation by acting on CXCR3 in an autocrine manner [57]. This pro-tumoral CXCL10 feed-forward loop was also observed in breast

cancer; albeit, CXCL10 also attracted more CD4+ and CD8+ lymphocytes to the TME, in a paracrine manner, which attenuated tumor progression [115].

This pro- and anti-tumoral activity of CXCL10 could also be observed in CRC. Stage IV CRC patients showed an increased presence of CXCL10 in the TME, which was associated with a poor prognosis and promoted metastasis to the liver [116,117]. In contrast, higher secretion of CXCL10 in CRC increased the infiltration of CD8+ T lymphocytes and inhibited the ability of cancer cells to metastasize [118]. In addition, patients with ESCC showing higher CXCL10 expression had a better overall survival rate when compared with CXCL10-negative patients [119], while enhanced proliferation and metastatic behavior of melanoma tumor cells could be linked to higher CXCL10 secretion [120].

CXCL10 having opposing effects on TME that may occur simultaneously, the overall outcome of the disease seems to be tumor-type dependent and thus hard to predict.

3.5.3. CXCL9

Similar to CXCL10, CXCL9 is one of the most extensively studied CXCR3 chemokines in cancer and also plays a dual role in the TME. CXCL9 gene upregulation in CRC and non- small-cell-lung-cancer (NSCLC) patient samples correlated with an increased overall survival, suggesting a beneficial impact of higher secretion of this chemokine in the TME [121,122]. In line with this observation, higher secretion of CXCL9 in HGSC and fibrosarcoma tumors also resulted in an increased overall survival presumable due to an enhanced attraction of leukocytes, in particular T and NK cells, to the tumor bed [95,123]. The anti-tumoral activity of CXCL9 could also be observed in lymphocyte-rich gastric carcinoma (GC) and regressing Burkitt's lymphoma tumors. These regressing tumors showed a higher prevalence of CXCL9 in the TME compared with conventional tumors and exhibited an increased presence of TIL and tumor necrosis rate [124,125]. In contrast, it has been reported that the higher CXCL9 levels in melanoma—secreted by the melanoma endothelial cells—disrupt the endothelial barrier, which in turn leads to a higher dissemination of melanoma cells. This transendothelial migration of the tumor cells was diminished upon addition of anti-CXCL9 monoclonal antibody (mAb) [126]. The CXCL9-mediated metastasis was also observed in tongue squamous cell carcinoma (TSCC). TSCC cell show an increased CXCL9 and CXCR3 expression, which results in cytoskeleton alterations and decreased cell adhesion molecules that promotes tumor cell invasion and migration to the lymph nodes [127]. Interestingly, in follicular lymphoma (FL), elevated levels of CXCL9 result in lower event-free survival after treatment with immunotherapy implying a potential negative role of this chemokine after cancer treatment [128]. Thus, depending on the tumor cell type, CXCL9 can have either an adverse or a beneficial effect. This beneficial effect appears to rely on leukocyte attraction to the TME in a paracrine

fashion; however, the adverse effect of CXCL9 on the malignant cell might not be direct as for CXCL10.

3.5.4. CXCL4 and its CXCL4L1 Variant

While CXCL4 is mainly released after activation of platelets, it can also be secreted by stromal cells and tumor cells. In the two most frequent types of human lung adenocarcinomas (KRAS and TP53) and in CRC, an increase in CXCL4 expression was detected and correlated to lower patient survival probably due to its proliferative effect on tumor cells and anti- proliferative effect on CTLs [129–131]. The CXCL4L1 non-allelic variant has also been described to bind to CXCR3-A and -B [132]. The production of CXCL4L1 in melanoma, lung carcinoma and pancreatic adenocarcinoma (PDAC) inhibits tumor growth as well as neovascularization. Furthermore, CXCL4L1, as well as CXCL4, are able to recruit T cells, NK cells and DC to the TME, which may contribute to their anti-tumoral activity [132–135].

4. Technical Hurdles to Studying the CXCR3 Variants

The identification of the CXCR3-B variant, with its opposite cellular responses, raised new issues pertaining to the study and validation of the exact expression profile, signaling and the differential contribution of the two isoforms in CXCR3-mediated processes. Variant-specific tools were thus needed to be able to attribute the various cellular responses on the different cell types to CXCR3-A or CXCR3-B.

Nucleotide probes, or primers, were the first easily accessible tools to detect and distinguish endogenous expression of CXCR3-A- and CXCR3-B-specific transcripts in tissue and cells. The most frequently used primers, described by Lasagni et al., detect the spliced mRNA encoding both CXCR3-A and CXCR3-B as they bind to sequences that are common to the two variants. Nevertheless, the PCR products can be distinguished based on their different DNA band size. Primers designed for specific detection of CXCR3-A or CXCR3-B were also reported [24] (Figure 1). The CXCR3-A-specific primer aligns with the nucleotide sequence coding for the first seven amino acids present only in this variant [136] while the primer specific to CXCR3-B aligns within its extended N terminus. Besides PCR-based approaches, another application of nucleotide probes is *in situ* hybridization. This technique was used to screen for mRNA expression of the CXCR3 variants directly in slices of fixed tissues allowing the direct visualization of the receptor distribution and identification of receptor-positive cell types [137].

Variant-specific nucleotide probes proved to be easily manufactured and useful tools to evaluate the gene expression levels. However, confirming the presence of CXCR3 at protein level and, in particular, on the cell surface turned out to be a more difficult task. For the time being, the anti-CXCR3-A antibodies also recognize the CXCR3-B variant due to the usage of CXCR3-A N-terminus-derived peptides for immunization. The production of

the first CXCR3- B-specific mAb raised against the extended 52-AA N terminus and its use in immunofluorescence and immunohistochemistry was first described by Lasagni et al. [24]. Meanwhile, other CXCR3-B-specific mAbs, using different CXCR3-B N-terminus-derived peptides as immunogen, are available but usually show an application spectrum limited to ELISA or Western Blot. To circumvent the staining of both CXCR3 isoforms, an alternative may be the use of conformational antibodies against each variant as previously generated for other GPCRs or chemokine receptors, including CXCR4 [138] CCR5 [139] and ACKR3 [109]. Indeed, the opposite cellular response of the two CXCR3 variants suggests that the presence of the N-terminal extension of CXCR3-B modifies the general tertiary structure of the receptor and might therefore expose different discontinued epitopes compared with CXCR3-A. Although their use would be limited to the detection of CXCR3 in its native conformation, it would help to confirm and quantify the presence of the two variants on the cell surface and understand the biology behind the selective receptor expression on the various cell types of the TME.

The diverse cellular effects of the CXCR3 variants were proposed to rely on the activation of different downstream signaling pathways. To investigate and identify CXCR3 variant- specific pathways, novel variant-selective modulators (antagonists, agonists or allosteric modulators) are needed as the currently used endogenous chemokines bind and activate both variants almost indifferently. Although CXCR3-A small-molecule modulators have been generated, their activity on CXCR3-B remains to be elucidated [106,140–142]. In addition, no CXCR3-B-specific small molecules have been reported so far. Therefore, the generation of small modulators is timely and would enable to unravel the CXCR3 variants signaling and could hold potential therapeutic value.

5. Conclusions, Challenges, and Future Directions

Over the last two decades, much effort has been made to understand the biology of the chemokine receptor CXCR3. Although the mechanism controlling the alternative splicing of both CXCR3 variants remains to be elucidated, numerous data on their signaling pathways and their respective contribution in tumor development and immunity have been generated.

On the one hand, it was shown that tumor cells can manipulate the expression of the CXCR3 variants in a way that is overall more beneficial for their maintenance. Indeed, by upregulating the expression of CXCR3-A and lowering the expression of CXCR3-B at their surface, tumor-initiating cells are able to enhance their proliferative potential and survival through autocrine stimulation loops that are further reinforced by the simultaneous upregulation of the expression of the CXCR3 ligands in the TME.

On the other hand, the increased secretion of CXCR3 ligands within the TME was shown to enhance the recruitment and the infiltration of various leukocytes into the tumor

bed in a paracrine fashion, thereby modulating tumor progression. In a similar manner, chemokines participate in the control of metastasis of CXCR3-expressing tumor cells (summarized in Figure 2).The demonstration of the impact of the CXCR3 axis in cancer points to its potential interest as a therapeutic target. However, the dual activity of CXCR3-A in the proliferative loops and the attraction of immune cells to the tumor bed, and the opposing roles of the CXCR3 variants ligand–receptor axis, as well as ligand and cell context bias, are important aspects that need to be considered for the development and use of anti-CXCR3 drugs or neutralizing antibodies. In addition, other players in the TME, including growth factors, inflammatory cytokines, and extracellular matrix enzymes, and the impact of the tumor on the microenvironment, such as induction of hypoxia, influence the CXCR3 axis and add yet another level of complexity to CXCR3-regulated processes, ultimately making the outcome of potential blockade hard to predict.

Therefore, a better understanding of all aspects of the biology of CXCR3 variants, including their exact signaling pathways and cellular responses in the tumor-microenvironment context, is urgently needed before envisaging the CXCR3 variants as potential anti-tumoral targets and the development of CXCR3 variant-specific drugs to be used for combined therapy, for example with anti-PD-1 immunotherapy, to turn cold to hot tumors.

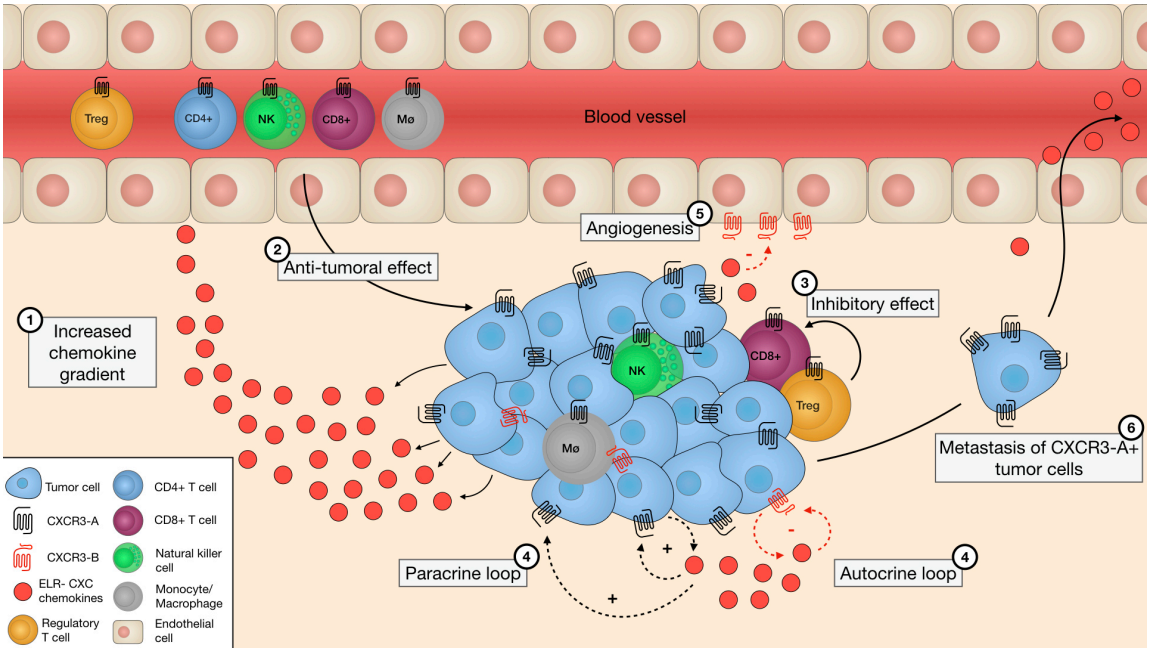

Figure 2 | Illustration of the roles of CXCR3 variants and their ligands in the tumor microenvironment. The increased secretion of CXCR3 ligands in the TME, originating from the tumor cells, results in an increased chemokine gradient (1) and the recruitment of CXCR3-A+ leukocytes towards the tumor (paracrine axis) (2). The Th1-related immune cells drive anti-tumoral responses, while the recruited CXCR3-A+ Tregs have a pro-tumoral effect by suppressing other immune cells. The secreted chemokines also bind to CXCR3-A or CXCR3-B, present on secreting (autocrine axis) and neighboring tumor cells, and depending on the cellular context, may induce a proliferative or inhibitory effect (3). Moreover, the chemokines in close proximity to the endothelial cells attenuate the angiogenesis by activating CXCR3-B (4). In addition, the increased expression of CXCR3-A, together with decreased CXCR3-B expression, on the tumor cells enable them to detach from the tumor mass and disseminate to a distant tissue (5).

References

1. Zlotnik, A.; Yoshie, O. The chemokine superfamily revisited. *Immunity* **2012**, *36*, 705–716.
2. Thelen, M. Dancing to the tune of chemokines. *Nat. Immunol.* **2001**, *2*, 129–134.
3. Kleist, A.B.; Getschman, A.E.; Ziarek, J.J.; Nevins, A.M.; Gauthier, P.-A.; Chevigné, A.; Szpakowska, M.; Volkman, B.F. New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. *Biochem. Pharmacol.* **2016**, *114*, 53–68.
4. Szpakowska, M.; Dupuis, N.; Baragli, A.; Counson, M.; Hanson, J.; Piette, J.; Chevigné, A. Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7. *Biochem. Pharmacol.* **2016**, *114*, 14–21.
5. Smith, J.S.; Alagesan, P.; Desai, N.K.; Pack, T.F.; Wu, J.-H.; Inoue, A.; Freedman, N.J.; Rajagopal, S. CXC motif chemokine receptor 3 splice variants differentially activate beta-arrestins to regulate downstream signaling pathways. *Mol. Pharmacol.* **2017**, *92*, 136–150.
6. Metzemaekers, M.; Vanheule, V.; Janssens, R.; Struyf, S.; Proost, P. Overview of the mechanisms that may contribute to the non-redundant activities of interferon-inducible CXC chemokine receptor 3 ligands. *Front. Immunol.* **2018**, *8*, 1970.
7. Moser, B.; Loetscher, P. Lymphocyte traffic control by chemokines. *Nat. Immunol.* **2001**, *2*, 123–128.
8. Griffith, J.W.; Sokol, C.L.; Luster, A.D. Chemokines and chemokine receptors: Positioning cells for host defense and immunity. *Annu. Rev. Immunol.* **2014**, *32*, 659–702.
9. Thelen, M.; Stein, J.V. How chemokines invite leukocytes to dance. *Nat. Immunol.* **2008**, *9*, 953–959.
10. Luster, A.D. Chemokines—Chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **1998**, *338*, 436–445.
11. Balkwill, F. Cancer and the chemokine network. *Nat. Rev. Cancer* **2004**, *4*, 540–550.
12. Nagarsheth, N.; Wicha, M.S.; Zou, W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. *Nat. Rev. Immunol.* **2017**, *17*, 559–572.
13. Relation, T.; Dominici, M.; Horwitz, E.M. Concise review: An (Im) Penetrable shield: How the tumor microenvironment protects cancer stem cells. *Stem Cells* **2017**, *35*, 1123–1130.
14. Binnewies, M.; Roberts, E.W.; Kersten, K.; Chan, V.; Fearon, D.F.; Merad, M.; Coussens, L.M.; Gabrilovich, D.I.; Ostrand-Rosenberg, S.; Hedrick, C.C. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat. Med.* **2018**, *24*, 541–550.
15. Zou, W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat. Rev. Cancer* **2005**, *5*, 263–274.
16. Franciszkiewicz, K.; Boissonnas, A.; Boutet, M.; Combadière, C.; Mami-Chouaib, F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. *Cancer Res.* **2012**, *72*, 6325–6332.
17. Witsch, E.; Sela, M.; Yarden, Y. Roles for growth factors in cancer progression. *Physiology* **2010**, *25*, 85–101.
18. Wang, M.; Zhao, J.; Zhang, L.; Wei, F.; Lian, Y.; Wu, Y.; Gong, Z.; Zhang, S.; Zhou, J.; Cao, K. Role of tumor microenvironment in tumorigenesis. *J. Cancer* **2017**, *8*, 761–773.
19. Balkwill, F.R.; Capasso, M.; Hagemann, T. The tumor microenvironment at a glance. *J. Cell Sci.* **2012**, *125*, 5591–5596.
20. Groom, J.R.; Luster, A.D. CXCR3 ligands: Redundant, collaborative and antagonistic functions. *Immunol. Cell Biol.* **2011**, *89*, 207–215.
21. Tokunaga, R.; Zhang, W.; Naseem, M.; Puccini, A.; Berger, M.D.; Soni, S.; McSkane, M.; Baba, H.; Lenz, H.-J. CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation—A target for novel cancer therapy. *Cancer Treat. Rev.* **2018**, *63*, 40–47.
22. Van Raemdonck, K.; Van den Steen, P.E.; Liekens, S.; Van Damme, J.; Struyf, S. CXCR3 ligands in disease and therapy. *Cytokine Growth Factor Rev.* **2015**, *26*, 311–327.
23. Berchiche, Y.A.; Sakmar, T.P. CXC chemokine receptor 3 alternative splice variants selectively activate different signaling pathways. *Mol. Pharmacol.* **2016**, *90*, 483–495.
24. Lasagni, L.; Francalanci, M.; Annunziato, F.; Lazzeri, E.; Giannini, S.; Cosmi, L.; Sagrinati, C.; Mazzinghi, B.; Orlando, C.; Maggi, E. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J. Exp. Med.* **2003**, *197*, 1537–1549.
25. Loetscher, M.; Gerber, B.; Loetscher, P.; Jones, S.A.; Piali, L.; Clark-Lewis, I.; Baggiolini, M.; Moser, B. Chemokine receptor specific for IP10 and mig: Structure, function, and expression in activated T-lymphocytes. *J. Exp. Med.* **1996**, *184*, 963–969.
26. Cole, K.E.; Strick, C.A.; Paradis, T.J.; Ogborne, K.T.; Loetscher, M.; Gladue, R.P.; Lin, W.; Boyd, J.G.; Moser, B.; Wood, D.E. Interferon–inducible T cell alpha chemoattractant (I-TAC): A novel Non-ELR CXC Chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J. Exp. Med.* **1998**, *187*, 2009–2021.
27. Loetscher, M.; Loetscher, P.; Brass, N.; Meese, E.; Moser, B. Lymphocyte-specific chemokine receptor CXCR3: Regulation, chemokine binding and gene localization. *Eur. J. Immunol.* **1998**, *28*, 3696–3705.
28. Thompson, B.D.; Jin, Y.; Wu, K.H.; Colvin, R.A.; Luster, A.D.; Birnbaumer, L.; Wu, M.X. Inhibition of Gai2 activation by Gai3 in CXCR3-mediated signaling. *J. Biol. Chem.* **2007**, *282*, 9547–9555.
29. Smit, M.J.; Verdijk, P.; van der Raaij-Helmer, E.M.; Navis, M.; Hensbergen, P.J.; Leurs, R.; Tensen, C.P. CXCR3-mediated chemotaxis of human T cells is regulated by a Gi-and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood* **2003**, *102*, 1959–1965.
30. Mueller, A.; Meiser, A.; McDonagh, E.M.; Fox, J.M.; Petit, S.J.; Xanthou, G.; Williams, T.J.; Pease, J.E. CXCL4-induced migration of activated T lymphocytes is mediated by the chemokine receptor CXCR3. *J. Leukoc. Biol.* **2008**, *83*, 875–882.
31. Korniejewska, A.; McKnight, A.J.; Johnson, Z.; Watson, M.L.; Ward, S.G. Expression and agonist responsiveness of CXCR3 variants in human T lymphocytes. *Immunology* **2011**, *132*, 503–515.
32. Watts, A.O.; Scholten, D.J.; Heitman, L.H.; Vischer, H.F.; Leurs, R. Label-free impedance responses of endogenous and synthetic chemokine receptor CXCR3 agonists correlate with G i-protein pathway activation. *Biochem. Biophys. Res. Commun.* **2012**, *419*, 412–418.
33. Romagnani, P.; Beltrame, C.; Annunziato, F.; Lasagni, L.; Luconi, M.; Galli, G.; Cosmi, L.; Maggi, E.; Salvadori, M.; Pupilli, C. Role for interactions between IP-10/Mig and CXCR3 in proliferative glomerulonephritis. *J. Am. Soc. Nephrol.* **1999**, *10*, 2518–2526.
34. Bonacchi, A.; Romagnani, P.; Romanelli, R.G.; Efsen, E.; Annunziato, F.; Lasagni, L.; Francalanci, M.; Serio, M.; Laffi, G.; Pinzani, M. Signal transduction by the chemokine receptor CXCR3: Activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. *J. Biol. Chem.* **2001**, *276*, 9945–9954.
35. Chen, Y.-P.; Wu, H.-L.; Boyé, K.; Pan, C.-Y.; Chen, Y.-C.; Pujol, N.; Lin, C.-W.; Chiu, L.-Y.; Billottet, C.; Alves, I.D. Oligomerization state of CXCL4 chemokines regulates G protein-coupled receptor activation. *Acs Chem. Biol.* **2017**, *12*, 2767–2778.
36. Billottet, C.; Quemener, C.; Bikfalvi, A. CXCR3, a double-edged sword in tumor progression and angiogenesis. *Biochim. Et Biophys. Acta (BBA) Rev. Cancer* **2013**, *1836*, 287–295.
37. Strieter, R.M.; Burdick, M.D.; Gomperts, B.N.; Belperio, J.A.; Keane, M.P. CXC chemokines in

angiogenesis. *Cytokine Growth Factor Rev.* **2005**, *16*, 593–609.
38. Mgrditchian, T.; Arakelian, T.; Paggetti, J.; Noman, M.Z.; Viry, E.; Moussay, E.; Van Moer, K.; Kreis, S.; Guerin, C.; Buart, S. Targeting autophagy inhibits melanoma growth by enhancing NK cells infiltration in a CCL5-dependent manner. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E9271–E9279.
39. Gooden, M.J.; de Bock, G.H.; Leffers, N.; Daemen, T.; Nijman, H.W. The prognostic influence of tumour-infiltrating lymphocytes in cancer: A systematic review with meta-analysis. *Br. J. Cancer* **2011**, *105*, 93–103.
40. Luster, A.D.; Leder, P. IP-10, a-CXC-chemokine, elicits a potent thymus-dependent antitumor response in vivo. *J. Exp. Med.* **1993**, *178*, 1057–1065.
41. Mullins, I.M.; Slingluff, C.L.; Lee, J.K.; Garbee, C.F.; Shu, J.; Anderson, S.G.; Mayer, M.E.; Knaus, W.A.; Mullins, D.W. CXC chemokine receptor 3 expression by activated $CD8^+$ T cells is associated with survival in melanoma patients with stage III disease. *Cancer Res.* **2004**, *64*, 7697–7701.
42. Hensbergen, P.J.; Wijnands, P.G.B.; Schreurs, M.W.; Scheper, R.J.; Willemze, R.; Tensen, C.P. The CXCR3 targeting chemokine CXCL11 has potent antitumor activity in vivo involving attraction of $CD8^+$ T lymphocytes but not inhibition of angiogenesis. *J. Immunother.* **2005**, *28*, 343–351.
43. Pan, J.; Burdick, M.D.; Belperio, J.A.; Xue, Y.Y.; Gerard, C.; Sharma, S.; Dubinett, S.M.; Strieter, R.M. CXCR3/CXCR3 ligand biological axis impairs RENCA tumor growth by a mechanism of immunoangiostasis. *J. Immunol.* **2006**, *176*, 1456–1464.
44. Reckamp, K.L.; Figlin, R.A.; Moldawer, N.; Pantuck, A.J.; Belldegrun, A.S.; Burdick, M.D.; Strieter, R.M. Expression of CXCR3 on mononuclear cells and CXCR3 ligands in patients with metastatic renal cell carcinoma in response to systemic IL-2 therapy. *J. Immunother.* **2007**, *30*, 417–424.
45. Mikucki, M.; Fisher, D.; Matsuzaki, J.; Skitzki, J.; Gaulin, N.; Muhitch, J.; Ku, A.; Frelinger, J.; Odunsi, K.; Gajewski, T. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nat. Commun.* **2015**, *6*, 7458.
46. Chheda, Z.S.; Sharma, R.K.; Jala, V.R.; Luster, A.D.; Haribabu, B. Chemoattractant receptors BLT1 and CXCR3 regulate antitumor immunity by facilitating $CD8^+$ T cell migration into tumors. *J. Immunol.* **2016**, *197*, 2016–2026.
47. Oghumu, S.; Varikuti, S.; Terrazas, C.; Kotov, D.; Nasser, M.W.; Powell, C.A.; Ganju, R.K.; Satoskar, A.R. CXCR 3 deficiency enhances tumor progression by promoting macrophage M2 polarization in a murine breast cancer model. *Immunology* **2014**, *143*, 109–119.
48. Li, K.; Zhu, Z.; Luo, J.; Fang, J.; Zhou, H.; Hu, M.; Maskey, N.; Yang, G. Impact of chemokine receptor CXCR3 on tumor-infiltrating lymphocyte recruitment associated with favorable prognosis in advanced gastric cancer. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 14725.
49. Wendel, M.; Galani, I.E.; Suri-Payer, E.; Cerwenka, A. Natural killer cell accumulation in tumors is dependent on IFN-γ and CXCR3 ligands. *Cancer Res.* **2008**, *68*, 8437–8445.
50. Wenzel, J.; Bekisch, B.; Uerlich, M.; Haller, O.; Bieber, T.; Tüting, T. Type I interferon–associated recruitment of cytotoxic lymphocytes: A common mechanism in regressive melanocytic lesions. *Am. J. Clin. Pathol.* **2005**, *124*, 37–48.
51. Redjimi, N.; Raffin, C.; Raimbaud, I.; Pignon, P.; Matsuzaki, J.; Odunsi, K.; Valmori, D.; Ayyoub, M. $CXCR3^+$ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity. *Cancer Res.* **2012**, *72*, 4351–4360.
52. Li, C.X.; Ling, C.C.; Shao, Y.; Xu, A.; Li, X.C.; Ng, K.T.-P.; Liu, X.B.; Ma, Y.Y.; Qi, X.; Liu, H. CXCL10/CXCR3 signaling mobilized-regulatory T cells promote liver tumor recurrence after transplantation. *J. Hepatol.* **2016**, *65*, 944–952.
53. Winkler, A.E.; Brotman, J.J.; Pittman, M.E.; Judd, N.P.; Lewis, J.S.; Schreiber, R.D.; Uppaluri, R. CXCR3 enhances a T cell dependent epidermal proliferative response and promotes skin tumorigenesis. *Cancer Res.* **2011**, *71*, 5707–5716.
54. Schumacher, K.; Haensch, W.; Röefzaad, C.; Schlag, P.M. Prognostic significance of activated $CD8^+$ T cell infiltrations within esophageal carcinomas. *Cancer Res.* **2001**, *61*, 3932–3936.
55. Sackstein, R.; Schatton, T.; Barthel, S.R. T-lymphocyte homing: An underappreciated yet critical hurdle for successful cancer immunotherapy. *Lab. Investig.* **2017**, *97*, 669–697.
56. Pauken, K.E.; Jenkins, M.K.; Azuma, M.; Fife, B.T. PD-1_+ but not PD-L1, expressed by islet-reactive $CD4^+$ T cells suppresses infiltration of the pancreas during type 1 diabetes. *Diabetes* **2013**, *62*, 2859–2869.
57. Maru, S.V.; Holloway, K.A.; Flynn, G.; Lancashire, C.L.; Loughlin, A.J.; Male, D.K.; Romero, I.A. Chemokine production and chemokine receptor expression by human glioma cells: Role of CXCL10 in tumour cell proliferation. *J. Neuroimmunol.* **2008**, *199*, 35–45.
58. Giuliani, N.; Bonomini, S.; Romagnani, P.; Lazzaretti, M.; Morandi, F.; Colla, S.; Tagliaferri, S.; Lasagni, L.; Annunziato, F.; Crugnola, M. CXCR3 and its binding chemokines in myeloma cells: Expression of isoforms and potential relationships with myeloma cell proliferation and survival. *Haematologica* **2006**, *91*, 1489–1497.
59. Bai, M.; Chen, X.; Ba, Y. CXCL10/CXCR3 overexpression as a biomarker of poor prognosis in patients with stage II colorectal cancer. *Mol. Clin. Oncol.* **2016**, *4*, 23–30.
60. Urra, S.; Fischer, M.C.; Martínez, J.R.; Véliz, L.; Orellana, P.; Solar, A.; Bohmwald, K.; Kalergis, A.; Riedel, C.; Corvalán, A.H. Differential expression profile of CXCR3 splicing variants is associated with thyroid neoplasia. Potential role in papillary thyroid carcinoma oncogenesis? *Oncotarget* **2018**, *9*, 2445–2467.
61. Klatte, T.; Seligson, D.B.; Leppert, J.T.; Riggs, S.B.; Yu, H.; Zomorodian, N.; Kabbinavar, F.F.; Strieter, R.M.; Belldegrun, A.S.; Pantuck, A.J. The chemokine receptor CXCR3 is an independent prognostic factor in patients with localized clear cell renal cell carcinoma. *J. Urol.* **2008**, *179*, 61–66.
62. Kawada, K.; Sonoshita, M.; Sakashita, H.; Takabayashi, A.; Yamaoka, Y.; Manabe, T.; Inaba, K.; Minato, N.; Oshima, M.; Taketo, M.M. Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes. *Cancer Res.* **2004**, *64*, 4010–4017.
63. Monteagudo, C.; Martin, J.M.; Jorda, E.; Llombart-Bosch, A. CXCR3 chemokine receptor immunoreactivity in primary cutaneous malignant melanoma: Correlation with clinicopathological prognostic factors. *J. Clin. Pathol.* **2007**, *60*, 596–599.
64. Magro, C.M.; Momtahen, S.; Lee, B.A.; Swanson, D.L.; Pavlovic, M.D. Epidermotropic B-cell lymphoma: A unique subset of CXCR3-positive marginal zone lymphoma. *Am. J. Dermatopathol.* **2016**, *38*, 105–112.
65. Trentin, L.; Agostini, C.; Facco, M.; Piazza, F.; Perin, A.; Siviero, M.; Gurrieri, C.; Galvan, S.; Adami, F.; Zambello, R. The chemokine receptor CXCR3 is expressed on malignant B cells and mediates chemotaxis. *J. Clin. Investig.* **1999**, *104*, 115–121.
66. Liu, C.; Luo, D.; Reynolds, B.A.; Meher, G.; Katritzky, A.R.; Lu, B.; Gerard, C.J.; Bhadha, C.P.; Harrison, J.K. Chemokine receptor CXCR3 promotes growth of glioma. *Carcinogenesis* **2010**, *32*, 129–137.
67. Pu, Y.; Li, S.; Zhang, C.; Bao, Z.; Yang, Z.; Sun, L. High expression of CXCR3 is an independent prognostic factor in glioblastoma patients that promotes an invasive phenotype. *J. Neuro-Oncol.* **2015**, *122*, 43–51.
68. Murakami, T.; Kawada, K.; Iwamoto, M.; Akagami, M.; Hida, K.; Nakanishi, Y.; Kanda, K.; Kawada, M.; Seno, H.; Taketo, M.M. The role of CXCR3 and CXCR4 in colorectal cancer metastasis. *Int. J. Cancer* **2013**, *132*, 276–287.
69. Kawada, K.; Hosogi, H.; Sonoshita, M.; Sakashita, H.; Manabe, T.; Shimahara, Y.; Sakai, Y.; Takabayashi, A.; Oshima, M.; Taketo, M. Chemokine receptor CXCR3

promotes colon cancer metastasis to lymph nodes. *Oncogene* **2007**, *26*, 4679–4688.
70. Windmüller, C.; Zech, D.; Avril, S.; Boxberg, M.; Dawidek, T.; Schmalfeldt, B.; Schmitt, M.; Kiechle, M.; Bronger, H. CXCR3 mediates ascites-directed tumor cell migration and predicts poor outcome in ovarian cancer patients. *Oncogenesis* **2017**, *6*, e331.
71. Duruisseaux, M.; Rabbe, N.; Antoine, M.; Vieira, T.; Poulot, V.; Cadranel, J.; Wislez, M. Pro-tumoural CXCL10/CXCR3-A autocrine loop in invasive mucinous lung adenocarcinoma. *ERJ Open Res.* **2017**, *3*.
72. Ma, X.; Norsworthy, K.; Kundu, N.; Rodgers, W.H.; Gimotty, P.A.; Goloubeva, O.; Lipsky, M.; Li, Y.; Holt, D.; Fulton, A. CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. *Mol. Cancer Ther.* **2009**, *8*, 490–498.
73. Zhu, G.; Yan, H.H.; Pang, Y.; Jian, J.; Achyut, B.R.; Liang, X.; Weiss, J.M.; Wiltrout, R.H.; Hollander, M.C.; Yang, L. CXCR3 as a molecular target in breast cancer metastasis: Inhibition of tumor cell migration and promotion of host anti-tumor immunity. *Oncotarget* **2015**, *6*, 43408–43419.
74. Romagnani, P.; Maggi, L.; Mazzinghi, B.; Cosmi, L.; Lasagni, L.; Liotta, F.; Lazzeri, E.; Angeli, R.; Rotondi, M.; Filì, L. CXCR3-mediated opposite effects of CXCL10 and CXCL4 on TH1 or TH2 cytokine production. *J. Allergy Clin. Immunol.* **2005**, *116*, 1372–1379.
75. Kelsen, S.G.; Aksoy, M.O.; Yang, Y.; Shahabuddin, S.; Litvin, J.; Safadi, F.; Rogers, T.J. The chemokine receptor CXCR3 and its splice variant are expressed in human airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2004**, *287*, L584–L591.
76. Bodnar, R.J.; Wells, A. Differential regulation of pericyte function by the CXC receptor 3. *Wound Repair Regen.* **2015**, *23*, 785–796.
77. Murdoch, C.; Monk, P.N.; Finn, A. Cxc chemokine receptor expression on human endothelial cells. *Cytokine* **1999**, *11*, 704–712.
78. Datta, D.; Contreras, A.G.; Grimm, M.; Waaga-Gasser, A.M.; Briscoe, D.M.; Pal, S. Calcineurin inhibitors modulate CXCR3 splice variant expression and mediate renal cancer progression. *J. Am. Soc. Nephrol.* **2008**, *19*, 2437–2446.
79. Datta, D.; Banerjee, P.; Gasser, M.; Waaga-Gasser, A.M.; Pal, S. CXCR3-B can mediate growth-inhibitory signals in human renal cancer cells by down-regulating the expression of heme oxygenase-1. *J. Biol. Chem.* **2010**, *285*, 36842–36848.
80. Furuya, M.; Yoneyama, T.; Miyagi, E.; Tanaka, R.; Nagahama, K.; Miyagi, Y.; Nagashima, Y.; Hirahara, F.; Inayama, Y.; Aoki, I. Differential expression patterns of CXCR3 variants and corresponding CXC chemokines in clear cell ovarian cancers and endometriosis. *Gynecol. Oncol.* **2011**, *122*, 648–655.
81. Balan, M.; Pal, S. A novel CXCR3-B chemokine receptor-induced growth-inhibitory signal in cancer cells is mediated through the regulation of Bach-1 protein and Nrf2 protein nuclear translocation. *J. Biol. Chem.* **2014**, *289*, 3126–3137.
82. Datta, D.; Flaxenburg, J.A.; Laxmanan, S.; Geehan, C.; Grimm, M.; Waaga-Gasser, A.M.; Briscoe, D.M.; Pal, S. Ras-induced modulation of CXCL10 and its receptor splice variant CXCR3-B in MDA-MB-435 and MCF-7 cells: Relevance for the development of human breast cancer. *Cancer Res.* **2006**, *66*, 9509–9518.
83. Gacci, M.; Serni, S.; Lapini, A.; Vittori, G.; Alessandrini, M.; Nesi, G.; Palli, D.; Carini, M. CXCR3-B expression correlates with tumor necrosis extension in renal cell carcinoma. *J. Urol.* **2009**, *181*, 843–848.
84. Li, Y.; Reader, J.C.; Ma, X.; Kundu, N.; Kochel, T.; Fulton, A.M. Divergent roles of CXCR3 isoforms in promoting cancer stem-like cell survival and metastasis. *Breast Cancer Res. Treat.* **2015**, *149*, 403–415.
85. De Palma, M.; Biziato, D.; Petrova, T.V. Microenvironmental regulation of tumour angiogenesis. *Nat. Rev. Cancer* **2017**, *17*, 457–474.
86. Bergers, G.; Benjamin, L.E. Angiogenesis: Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* **2003**, *3*, 401–410.
87. Maione, T.E.; Gray, G.S.; Petro, J.; Hunt, A.J.; Donner, A.L.; Bauer, S.I.; Carson, H.F.; Sharpe, R.J. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* **1990**, *247*, 77–79.
88. Luster, A.D.; Greenberg, S.M.; Leder, P. The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J. Exp. Med.* **1995**, *182*, 219–231.
89. Strieter, R.M.; Polverini, P.J.; Kunkel, S.L.; Arenberg, D.A.; Burdick, M.D.; Kasper, J.; Dzuiba, J.; Van Damme, J.; Walz, A.; Marriott, D. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* **1995**, *270*, 27348–27357.
90. Angiolillo, A.L.; Sgadari, C.; Taub, D.D.; Liao, F.; Farber, J.M.; Maheshwari, S.; Kleinman, H.K.; Reaman, G.H.; Tosato, G. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J. Exp. Med.* **1995**, *182*, 155–162.
91. Arenberg, D.A.; Kunkel, S.L.; Polverini, P.J.; Morris, S.B.; Burdick, M.D.; Glass, M.C.; Taub, D.T.; Iannettoni, M.D.; Whyte, R.I.; Strieter, R.M. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J. Exp. Med.* **1996**, *184*, 981–992.
92. Rupertus, K.; Sinistra, J.; Scheuer, C.; Nickels, R.M.; Schilling, M.K.; Menger, M.D.; Kollmar, O. Interaction of the chemokines I-TAC (CXCL11) and SDF-1 (CXCL12) in the regulation of tumor angiogenesis of colorectal cancer. *Clin. Exp. Metastasis* **2014**, *31*, 447–459.
93. Campanella, G.S.; Colvin, R.A.; Luster, A.D. CXCL10 can inhibit endothelial cell proliferation independently of CXCR3. *PLoS ONE* **2010**, *5*, e12700.
94. Nagarsheth, N.; Peng, D.; Kryczek, I.; Wu, K.; Li, W.; Zhao, E.; Zhao, L.; Wei, S.; Frankel, T.; Vatan, L. PRC2 epigenetically silences Th1-type chemokines to suppress effector T-cell trafficking in colon cancer. *Cancer Res.* **2016**, *76*, 275–282.
95. Bronger, H.; Singer, J.; Windmüller, C.; Reuning, U.; Zech, D.; Delbridge, C.; Dorn, J.; Kiechle, M.; Schmalfeldt, B.; Schmitt, M.; et al. CXCL9 and CXCL10 predict survival and are regulated by cyclooxygenase inhibition in advanced serous ovarian cancer. *Br. J. Cancer* **2016**, *115*, 553–563.
96. Bronger, H.; Kraeft, S.; Schwarz-Boeger, U.; Cerny, C.; Stöckel, A.; Avril, S.; Kiechle, M.; Schmitt, M. Modulation of CXCR3 ligand secretion by prostaglandin E_2 and cyclooxygenase inhibitors in human breast cancer. *Breast Cancer Res.* **2012**, *14*, R30.
97. Havre, P.A.; Abe, M.; Urasaki, Y.; Ohnuma, K.; Morimoto, C.; Dang, N.H. The role of CD26/dipeptidyl peptidase IV in cancer. *Front. Biosci.* **2008**, *13*, 1634–1645.
98. Mortier, A.; Van Damme, J.; Proost, P. Overview of the mechanisms regulating chemokine activity and availability. *Immunol. Lett.* **2012**, *145*, 2–9.
99. Proost, P.; Schutyser, E.; Menten, P.; Struyf, S.; Wuyts, A.; Opdenakker, G.; Detheux, M.; Parmentier, M.; Durinx, C.; Lambeir, A.-M. Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties. *Blood* **2001**, *98*, 3554–3561.
100. DaSilva,R.B.;Laird,M.E.;Yatim,N.;Fiette,L.;Ingersoll,M.A.;Albert,M.L.Dipeptidylpeptidase4inhibition enhances lymphocyte trafficking, improving both naturally occurring tumor immunity and immunotherapy. *Nat. Immunol.* **2015**, *16*, 850–858.
101. Karin,N.;Wildbaum,G.;Thelen,M.Biasedsignalingpathways viaCXCR3controlthedevelopmentand function of $CD4^+$ T cell subsets. *J. Leukoc. Biol.* **2016**, *99*, 857–862.

102. Zhang, C.; Li, Z.; Xu, L.; Che, X.; Wen, T.; Fan, Y.; Li, C.; Wang, S.; Cheng, Y.; Wang, X. CXCL9/10/11, a regulator of PD-L1 expression in gastric cancer. *BMC Cancer* **2018**, *18*, 462.
103. Szpakowska, M.; Nevins, A.M.; Meyrath, M.; Rhainds, D.; D'huys, T.; Guité-Vinet, F.; Dupuis, N.; Gauthier, P.A.; Counson, M.; Kleist, A. 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.* **2018**, *175*, 1419–1438.
104. Sierro, F.; Biben, C.; Martínez-Muñoz, L.; Mellado, M.; Ransohoff, R.M.; Li, M.; Woehl, B.; Leung, H.; Groom, J.; Batten, M. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 14759–14764.
105. Zohar, Y.; Wildbaum, G.; Novak, R.; Salzman, A.L.; Thelen, M.; Alon, R.; Barsheshet, Y.; Karp, C.L.; Karin, N. CXCL11-dependent induction of FOXP3-negative regulatory T cells suppresses autoimmune encephalomyelitis. *J. Clin. Investig.* **2014**, *124*, 2009–2022.
106. Heise,C.E.;Pahuja,A.;Hudson,S.C.;Mistry,M.S.;Putnam,A.L.;Gross,M.M.;Gottlieb,P.A.;Wade,W.S.; Kiankarimi, M.; Schwarz, D. Pharmacological characterization of CXC chemokine receptor 3 ligands and a small molecule antagonist. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 1263–1271.
107. Proost,P.;Verpoest,S.;VandeBorne,K.;Schutyser,E.;Struyf,S.;Put,W.;Ronsse,I.;Grillet,B.;Opdenakker,G.; Van Damme, J. Synergistic induction of CXCL9 and CXCL11 by Toll-like receptor ligands and interferon-γ in fibroblasts correlates with elevated levels of CXCR3 ligands in septic arthritis synovial fluids. *J. Leukoc. Biol.* **2004**, *75*, 777–784.
108. Sauty,A.;Colvin,R.A.;Wagner,L.;Rochat,S.;Spertini,F.; Luster,A.D.CXCR3internalizationfollowingT cell-endothelial cell contact: Preferential role of IFN-inducible T cell α chemoattractant (CXCL11). *J. Immunol.* **2001**, *167*, 7084–7093.
109. Szpakowska,M.;Meyrath,M.;Reynders,N.;Counson,M.;Hanson,J.;Steyaert,J.;Chevigné,A.Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists. *Biochem. Pharmacol.* **2018**, *153*, 299–309.
110. Burns,J.M.;Summers,B.C.;Wang,Y.;Melikian,A.;Berahovich,R.;Miao,Z.;Penfold,M.E.;Sunshine,M.J.; Littman, D.R.; Kuo, C.J. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J. Exp. Med.* **2006**, *203*, 2201–2213.
111. Massara,M.;Bonavita,O.;Mantovani,A.;Locati,M.;Bonecchi,R.Atypicalchemokinereceptorsincancer: Friends or foes? *J. Leukoc. Biol.* **2016**, *99*, 927–933.
112. Freitas, C.; Desnoyer, A.; Meuris, F.; Bachelerie, F.; Balabanian, K.; Machelon, V. The relevance of the chemokine receptor ACKR3/CXCR7 on CXCL12-mediated effects in cancers with a focus on virus-related cancers. *Cytokine Growth Factor Rev.* **2014**, *25*, 307–316.
113. Gao,Y.J.;DeLinLiu,S.L.;Yuan,G.F.;Li,L.;Zhu,H.Y.;Cao, G.Y.Down-regulationofcXcl11inhibitscolorectal cancer cell growth and epithelial-mesenchymal transition. *Oncotargets Ther.* **2018**, *11*, 7333–7343.
114. Zhang, Y.; Zhao, W.; Li, S.; Lv, M.; Yang, X.; Li, M.; Zhang, Z. CXCL11 promotes self-renewal and tumorigenicity of $\alpha2\beta1^{+}$ liver tumor-initiating cells through CXCR3/ERK1/2 signaling. *Cancer Lett.* **2019**, *449*, 163–171.
115. Mulligan, A.M.; Raitman, I.; Feeley, L.; Pinnaduwage, D.; Nguyen, L.T.; O'Malley, F.P.; Ohashi, P.S.; Andrulis, I.L. Tumoral lymphocytic infiltration and expression of the chemokine CXCL10 in breast cancers from the Ontario Familial Breast Cancer Registry. *Clin. Cancer Res.* **2013**, *19*, 336–346.
116. Toiyama, Y.; Fujikawa, H.; Kawamura, M.; Matsushita, K.; Saigusa, S.; Tanaka, K.; Inoue, Y.; Uchida, K.; Mohri, Y.; Kusunoki, M. Evaluation of CXCL10 as a novel serum marker for predicting liver metastasis and prognosis in colorectal cancer. *Int. J. Oncol.* **2012**, *40*, 560–566.
117. Zipin-Roitman,A.;Meshel,T.;Sagi-Assif,O.;Shalmon,B.;Avivi,C.;Pfeffer,R.M.;Witz,I.P.;Ben-Baruch,A. CXCL10 promotes invasion-related properties in human colorectal carcinoma cells. *Cancer Res.* **2007**, *67*, 3396–3405.
118. Zumwalt,T.J.;Arnold,M.;Goel,A.;Boland,C.R.Activesecretionof CXCL10andCCL5fromcolorectalcancermicroenvironments associates with $GranzymeB^{+}$ $CD8^{+}$ T-cell infiltration. *Oncotarget* **2014**, *6*, 2981–2991.
119. Sato,Y.;Motoyama,S.;Nanjo,H.;Wakita,A.;Yoshino,K.; Sasaki,T.;Nagaki,Y.;Liu,J.;Imai,K.;Saito,H. CXCL10 expression status is prognostic in patients with advanced thoracic esophageal squamous cell carcinoma. *Ann. Surg. Oncol.* **2016**, *23*, 936–942.
120. Wightman, S.; Uppal, A.; Pitroda, S.; Ganai, S.; Burnette, B.; Stack, M.; Oshima, G.; Khan, S.; Huang, X.; Posner, M. Oncogenic CXCL10 signalling drives metastasis development and poor clinical outcome. *Br. J. Cancer* **2015**, *113*, 327–335.
121. Wu,Z.;Huang,X.;Han,X.;Li,Z.;Zhu,Q.;Yan,J.;Yu,S.;Jin,Z.;Wang,Z.;Zheng,Q.ThechemokineCXCL9 expression is associated with better prognosis for colorectal carcinoma patients. *Biomed. Pharmacother.* **2016**, *78*, 8–13.
122. Cao,Y.;Huang,H.;Wang,Z.;Zhang,G.TheInflammatory CXCChemokines,GROαhigh,IP-10low,and MIGlow, in Tumor Microenvironment Can Be Used as New Indicators for Non-small Cell Lung Cancer Progression. *Immunol. Investig.* **2017**, *46*, 361–374.
123. Gorbachev,A.V.;Kobayashi,H.;Kudo,D.;Tannenbaum,C.S.;Finke,J.H.;Shu,S.;Farber,J.M.;Fairchild,R.L. CXC chemokine ligand 9/monokine induced by IFN-γ production by tumor cells is critical for T cell-mediated suppression of cutaneous tumors. *J. Immunol.* **2007**, *178*, 2278–2286.
124. Ohtani,H.;Jin,Z.;Takegawa,S.;Nakayama,T.;Yoshie,O. AbundantexpressionofCXCL9(MIG)bystromal cells that include dendritic cells and accumulation of $CXCR3^{+}$ T cells in lymphocyte-rich gastric carcinoma. *J. Pathol. A J. Pathol. Soc. Great Br. Irel.* **2009**, *217*, 21–31.
125. Sgadari, C.; Farber, J.M.; Angiolillo, A.L.; Liao, F.; Teruya-Feldstein, J.; Burd, P.R.; Yao, L.; Gupta, G.; Kanegane, C.; Tosato, G. Mig, the monokine induced by interferon-γ, promotes tumor necrosis in vivo. *Blood* **1997**, *89*, 2635–2643.
126. Amatschek,S.;Lucas,R.;Eger,A.;Pflueger,M.;Hundsberger,H.;Knoll,C.;Grosse-Kracht,S.;Schuett,W.; Koszik, F.; Maurer, D. CXCL9 induces chemotaxis, chemorepulsion and endothelial barrier disruption through CXCR3-mediated activation of melanoma cells. *Br. J. Cancer* **2011**, *104*, 469–479.
127. Li, Z.; Liu, J.; Li, L.; Shao, S.; Wu, J.; Bian, L.; He, Y. Epithelial mesenchymal transition induced by the CXCL9/CXCR3 axis through AKT activation promotes invasion and metastasis in tongue squamous cell carcinoma. *Oncol. Rep.* **2018**, *39*, 1356–1368.
128. Mir, M.A.; Maurer, M.J.; Ziesmer, S.C.; Slager, S.L.; Habermann, T.; Macon, W.R.; Link, B.K.; Syrbu, S.; Witzig, T.; Friedberg, J.W. Elevated serum levels of IL-2R, IL-1RA, and CXCL9 are associated with a poor prognosis in follicular lymphoma. *Blood* **2015**, *125*, 992–998.
129. Pucci, F.; Rickelt, S.; Newton, A.P.; Garris, C.; Nunes, E.; Evavold, C.; Pfirschke, C.; Engblom, C.; Mino-Kenudson, M.; Hynes, R.O. PF4 promotes platelet

production and lung cancer growth. *Cell Rep.* **2016**, *17*, 1764–1772.
130. Deng,S.;Deng,Q.;Zhang,Y.;Ye,H.;Yu,X.;Zhang,Y.;Han ,G.Y.;Luo,P.;Wu,M.;Yu,Y.Non-platelet-derived CXCL4 differentially regulates cytotoxic and regulatory T cells through CXCR3 to suppress the immune response to colon cancer. *Cancer Lett.* **2019**, *443*, 1–12.
131. Zhang,Y.;Gao,J.;Wang,X.;Deng,S.;Ye,H.;Guan,W.;W u,M.;Zhu,S.;Yu,Y.;Han,W.CXCL4mediates tumor regrowth after chemotherapy by suppression of antitumor immunity. *Cancer Biol. Ther.* **2015**, *16*, 1775–1783.
132. Struyf,S.;Salogni,L.;Burdick,M.D.;Vandercappellen,J.; Gouwy,M.;Noppen,S.;Proost,P.;Opdenakker,G.; Parmentier, M.; Gerard, C. Angiostatic and chemotactic activities of the CXC chemokine CXCL4L1 (platelet factor-4 variant) are mediated by CXCR3. *Blood* **2011**, *117*, 480–488.
133. Struyf,S.;Burdick,M.D.;Proost,P.;VanDamme,J.;Striet er,R.M.PlateletsreleaseCXCL4L1,anonallelic variant of the chemokine platelet factor-4/CXCL4 and potent inhibitor of angiogenesis. *Circ. Res.* **2004**, *95*, 855–857.
134. Struyf,S.;Burdick,M.D.;Peeters,E.;VandenBroeck,K.; Dillen,C.;Proost,P.;VanDamme,J.;Strieter,R.M. Platelet factor-4 variant chemokine CXCL4L1 inhibits melanoma and lung carcinoma growth and metastasis by preventing angiogenesis. *Cancer Res.* **2007**, *67*, 5940–5948.
135. Quemener, C.; Baud, J.; Boyé, K.; Dubrac, A.; Billottet, C.; Soulet, F.; Darlot, F.; Dumartin, L.; Sire, M.; Grepin, R. Dual roles for CXCL4 chemokines and CXCR3 in angiogenesis and invasion of pancreatic cancer. *Cancer Res.* **2016**, *76*, 6507–6519.
136. Karjalainen,M.K.;Ojaniemi,M.;Haapalainen,A.M.;Mahl man,M.;Salminen,A.;Huusko,J.M.;Määttä,T.A.; Kaukola, T.; Anttonen, J.; Ulvila, J. CXCR3 polymorphism and expression associate with spontaneous preterm birth. *J. Immunol.* **2015**, *195*, 2187–2198.
137. Wu,Q.;Dhir,R.;Wells,A.AlteredCXCR3isoformexpressi onregulatesprostatecancercellmigrationand invasion. *Mol. Cancer* **2012**, *11*, 3.
138. Baribaud,F.;Edwards,T.G.;Sharron,M.;Brelot,A.;Heve ker,N.;Price,K.;Mortari,F.;Alizon,M.;Tsang,M.; Doms, R.W. Antigenically distinct conformations of CXCR4. *J. Virol.* **2001**, *75*, 8957–8967.
139. Blanpain, C.; Vanderwinden, J.-M.; Cihak, J.; Wittamer, V.; Le Poul, E.; Issafras, H.; Stangassinger, M.; Vassart, G.; Marullo, S.; Schlöndorff, D.; et al. Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Mol. Biol. Cell* **2002**, *13*, 723–737.
140. Jopling, L.; Watt, G.; Fisher, S.; Birch, H.; Coggon, S.; Christie, M. Analysis of the pharmacokinetic/ pharmacodynamic relationship of a small molecule CXCR3 antagonist, NBI-74330, using a murine CXCR3 internalization assay. *Br. J. Pharmacol.* **2007**, *152*, 1260–1271.
141. Scholten, D.; Canals, M.; Wijtmans, M.; De Munnik, S.; Nguyen, P.; Verzijl, D.; De Esch, I.; Vischer, H.; Smit, M.; Leurs, R. Pharmacological characterization of a small-molecule agonist for the chemokine receptor CXCR3. *Br. J. Pharmacol.* **2012**, *166*, 898–911.
142. Andrews,S.P.;Cox,R.J. Small molecule CXCR3 antagonists.*J.Med.Chem.***2015**,*59*,2894–2917.

Chapter 2

Pharmacological comparison of CXCR3 variants reveals that CXCR3-B is an chemokine receptor with atypical attributes

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Contribution:

Literature research, experiment design and execution, and writing of the manuscript

1. Abstract

The chemokine receptor CXCR3 plays a critical role in immune cell recruitment and activation. CXCR3 exists as two main splice variants, CXCR3A and CXCR3B, differing only by the presence of an N-terminal 51-amino acid extension in CXCR3-B but having opposite functional effects. CXCR3-B variant is an elusive and enigmatic chemokine receptor and the mechanisms underlying its function and signaling remain poorly understood. To unravel these aspects, we undertook an in-depth cellular and molecular study of CXCR3-A and CXCR3-B, comparing their activation properties at different levels of the signaling cascades, including G protein coupling, β-arrestin recruitment and modulation of secondary messengers as well as related gene response elements. We also investigated the subcellular localization of the two variants, their trafficking under resting and stimulated conditions along with their ability to internalize CXCR3-related chemokines. Here, we show that the N-terminal extension of CXCR3-B drastically affects receptor features, modifying the receptor cellular localization and preventing G protein coupling, while preserving β-arrestin recruitment and chemokine uptake capacities. Moreover, we demonstrate that gradual truncation of the N terminus leads to progressive recovery of surface expression and recoupling to G proteins. Our study clarifies the molecular basis underlying the divergent effects of CXCR3 variants, proposing CXCR3-B as a β-arrestin-biased receptor and a new atypical chemokine receptor. Our results also suggest that the two CXCR3 variants may be regarded as opposing effectors in analogy to classical and atypical chemokine receptors.

2. Introduction

Chemokine receptors are class A seven transmembrane domain G protein-coupled receptors (GPCRs), that bind small, structurally conserved cytokines with chemotactic properties, referred to as chemokines. Chemokine receptors are classified into four subfamilies (CCR, CXCR, XCR, and CX3CR) according to distinct cysteine motifs within the N terminus of the chemokines that they recognize. Chemokines and their receptors form an intricate network in which a chemokine activates one or many receptors, and one receptor recognizes one or several chemokines [1, 2].

Over the last decades, a new subfamily of chemokine receptors, referred to as ‘atypical’ chemokine receptors (ACKRs), has emerged as important regulators of the chemokine network. Presently it comprises four members (ACKR1–4) that differ from the ‘classical’ chemokine receptors notably by their inability to elicit G protein-mediated signaling, while most of them conserved the ability to recruit β-arrestin in response to chemokine binding. The molecular basis of G protein decoupling remains elusive but has been partly attributed to alterations in the DRY motif and structural particularities in their intracellular pocket that preclude efficient G protein binding or activation [2, 3]. Despite the absence of G protein signaling upon activation, ACKRs modulate the chemokine-mediated

cell migration and physiological processes by regulating the chemokine availability for the classical chemokine receptors, among others through chemokine internalization. This activity was previously considered to mainly rely on β-arrestin, yet recent studies showed that other effectors may also drive the regulatory functions of ACKRs [3-11]. Other distinctive properties of ACKRs are their unconventional cellular localization, trafficking and expression profile. ACKRs are indeed mostly found in intracellular endosomal vesicles and are generally expressed on endothelial and epithelial cells of barrier organs as opposed to classical chemokine receptors that are mostly present at the cell surface of hematopoietic and immune cells [12, 13].

The chemokine receptor CXCR3 is mainly present on activated immune cells and mediates their migration towards sites of inflammation, but is also expressed on cancer cells within the tumor microenvironment [14, 15]. In humans, the gene encoding for CXCR3 can be transcribed to three alternative splice variants: CXCR3-A, CXCR3-B, and CXCR3-Alt, which gives rise to structurally distinct proteins. Compared to CXCR3-A, CXCR3-B bears an N-terminal 51-amino-acid extension, while CXCR3-Alt lacks two transmembrane regions and shows a modified C terminus (Supplementary Fig 1) [16, 17]. The three variants also exert different cellular functions in response to their chemokine ligands CXCL11, CXCL10, and CXCL9. The biology of CXCR3-Alt is not well investigated and although receptor internalization upon cognate chemokine binding has been described, no Gi protein activation nor β-arrestin recruitment could be observed [18]. More efforts have been put in investigating the remaining two variants, which have been attributed opposing cellular functions. Indeed, CXCR3-A, mainly present on leukocytes, mediates cell migration and proliferation through activation of Gi and calcium signaling [18-24]. In contrast CXCR3-B, which is reported to be abundantly expressed on barrier cells [25], including endothelial cells and pericytes, inhibits cell migration and proliferation, and induces apoptosis upon ligand stimulation [16]. However, the molecular mechanism underlying this cellular effect remains unclear as conflicting reports on CXCR3-B signaling and functional role exist [18, 19, 26].

Similar contrasting functions for CXCR3 splice variants have been documented in Zebrafish. In this organism, Cxcr3.2 and Cxcr3.3 variants, both expressed on macrophages, have been shown to coordinate their migration during bacterial infection by functioning antagonistically. The cxcr3.3 variant shows atypical properties and is not able to elicit G protein-mediated signaling upon ligand stimulation. It was therefore suggested to attenuate the signaling of Cxcr3.2 by scavenging the ligands the two variants share [27].

The divergent cellular effects and expression pattern of the human CXCR3-A and CXCR3-B variants, lead us to hypothesize that CXCR3-B could also exert a scavenging function similarly to atypical chemokine receptors [15, 28]. Therefore, we investigated the

properties of both CXCR3 variants and compared them to the distinct features of the classical and atypical chemokine receptors.

In this study, we show that the N-terminal extension of CXCR3-B considerably alters its properties, including the subcellular distribution and signaling capacity. In particular, CXCR3-B loses the classical chemokine receptor's ability of G-protein coupling and signaling and gains atypical features such as predominantly intracellular localization, preservation of β-arrestin recruitment capacity and chemokine uptake. Hence, we bring the attention to the functional diversity and biased signaling of the two CXCR3 splice variants, which could be regarded as opposing effectors in analogy to classical and atypical chemokine receptors.

3. Material and Methods

3.1. Chemokines and antibodies

Native chemokines: CXCL11, CXCL10, CXCL9, CXCL12, and CCL5 were purchased from Peprotech.

Fluorescent labeled chemokines: CXCL11, CXCL10, CXCL9, and CCL5 coupled to Alexa Fluor 647 were purchased from Protein Foundry, LLC.

Custom chemokines: The N-terminally truncated, P2G-mutated and N-loop chimera CXCL11 chemokines were produced as previously described [2, 29-31]. In brief, cells were grown in Terrific Broth and production of modified CXCL11 chemokines, cloned into pQE30 vectors, was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside before being harvested. Cell pellets were then lysed, centrifugated at 12 000 x g for 20 minutes and the supernatant and solubilized inclusion body pellets were added to nitrilotriacetic acid resin for one hour. Bound proteins were eluted with 6 M guanidinium chloride, 50 mM Na2PO4 (pH 7.4), 300 mM NaCl, 500 mM imidazole, 0.2% sodium azide and 0.1% β-mercaptoethanol, the eluate pooled and refolded via dilution overnight before cleavage of the His6SUMO fusion tag by Ulp1 protease for 4 hours. The His6SUMO fusion tag and chemokine were separated using cation-exchange and the eluate subjected to reverse-phase high-performance liquid chromatography as a final purification.

Chemokine processed by dipeptidyl peptidase 4: CXCL11, CXCL10, CXCL9 and CCL5 chemokines (9 µM) were incubated with recombinant dipeptidyl peptidase 4 (CD26) (100 U) in 20 µl Tris/HCl 50 mM pH7.5 + 1 mM EDTA for 90 minutes at 37°C.

Antibodies: Anti-CXCR3 (1C6) and anti-CXCR4 (12G5) mAbs were purchased from BD Biosciences, anti-ACKR3 (8F11-M16) from BioLegend, anti-ACKR3 (11G8) and anti-ACKR2 (196124) from R&D Systems. All the antibodies were phycoerythrin–conjugated.

3.2. Cell culture

HEK293T and U87 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (10 and 15% respectively) and penicillin/ streptomycin (100 units/ml and 100 µg/ml). HEK293T.CXCR3-A or HEK293T.CXCR3-B cell lines stably expressing CXCR3 variants were established using pIRES-hygromycin vector and antibiotic selection and were grown in DMEM medium supplemented with 100 µg/mL hygromycin. Stable cell lines HEK293T-ACKR3 [32] and HEK293T-ACKR2 [33] were grown in DMEM medium supplemented with 5 µg/mL puromycin and 200 µg/mL hygromycin, respectively. HEK293T.pGlo, stably expressing cAMP GloSensor (GloSensor-22F cAMP, Promega), were grown in DMEM medium supplemented with 150 µg/mL hygromycin.

3.3. Recruitment assays

NanoBiT assay: miniG protein (mG, engineered GTPase domain of Gα subunit), β-arrestin-1 and β-arrestin-2 recruitment to WT and chimeric chemokine receptors (CXCR3-A, CXCR3-B, CXCR3-B N-terminally truncated variants, CXCR4, and CXCR4-B) upon chemokine-stimulation, was monitored using a Nanoluciferase complementation-based assay (NanoBiT, Promega) [34-36]. 4 x 106 HEK293T cells or 1.5 x 106 U87 cells were plated in 10-cm dishes and cultured for 24 or 48 hours respectively before transfection with vectors encoding for intracellular effectors N-terminally fused to LgBiT and chemokine receptor variants C-terminally fused to SmBiT. 48 hours after transfection, cells were harvested, incubated for 20 minutes at 37°C with Nano-Glo Live Cell substrate in Opti-MEM, and distributed into white 96-well plates (1.5 x 105 cells/well). Chemokine ligands were then added and the luminescence generated upon Nanoluciferase complementation was measured with a Mithras LB940 luminometer (Berthold Technologies).

NanoBRET assay: β-arrestin-1 and β-arrestin-2 recruitment to CXCR3-A and CXCR3-B upon chemokine-stimulation was monitored using NanoBRET (Promega) [33, 37]. 4 x 106 HEK293T cells were plated in 10-cm dishes and cultured for 48 hours before transfection with vectors encoding for the human β-arrestin-1 or β-arrestin-2 N-terminally fused to Nanoluciferase and the chemokine receptor variants C-terminally fused to mNeonGreen. 48 hours after transfection, cells were harvested, incubated with Nano-Glo Live Cell substrate in Opti-MEM and immediately distributed into white 96-well plates (1.5 x 105 cells/well). The BRET signal generated was measured with a GloMax plate reader (Promega) using a 450 BP filter for the luminescence and a 530 LP filter for the fluorescent signal.

Ligand-mediated G protein dissociation was monitored using BRET-based G protein activity sensors [38]. These biosensors encode for a tricistronic plasmid which encodes Nanoluciferase-tagged Gα subunits together with the related Gβ and circularly permutated Venus tagged Gγ.. G protein activity is monitored through the reduction of BRET signal upon G protein subunit dissociation. 4 x 106 HEK293T cells were plated in 10-cm dishes, cultured for 24 hours before transfection with BRET sensors and untagged CXCR3-A or

CXCR3-B. 48 hours after transfection, cells were harvested, incubated with Nano-Glo Live Cell substrate in Opti-MEM and immediately distributed into white 96-well plates (105 cells/ well). The BRET signal generated was measured with a GloMax plate reader (Promega) using a 450 BP filter for the luminescence and a 530LP filter for the fluorescent signal.

3.4. cAMP measurements

cAMP measurements upon chemokine stimulation were performed using a luminescence GloSensor cAMP reporter assay (Promega) [39]. 4 x 106 HEK293T.pGlo cells were plated in 10-cm dishes and cultured for 24 hours before transfection with CXCR3-A or CXCR3-B-encoding pIRES vectors or empty vector. 48 hours later, cells were harvested, incubated for one hour at 37°C with Nano-Glo Live Cell substrate and IBMX (300 μM) in HBSS (120 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 10 mM HEPES, pH 7.4, 10 mM glucose) and distributed into white 96-well plates (1 x 105 cells per well). cAMP-dependent changes in luminescence in response to chemokines at concentrations ranging from 10 pM to 300 nM was measured with a Mithras LB940 luminometer (Berthold Technologies).

3.5. Ca2+ mobilization

To assess CXCR3-driven chemokine-induced calcium flux, an assay based on Nanoluciferase complementation (NanoBiT) and Ca2+-dependent calmodulin–MYLK2S protein association was used. HEK293T cells were plated in a 6-well plate (0.8 x 106 cells per well) and cultured for 24 hours before transfection with CXCR3-A- or CXCR3-B-encoding pIRES vectors and plasmids encoding for calmodulin C-terminally fused to SmBiT and MYLK2S N-terminally fused to LgBiT. 48 hours after transfection, cells were incubated in PBS supplemented with 1 mM CaCl2 and 0.5 mM MgCl2 for 10 minutes at 37°C. Nano-Glo Live Cell substrate was then added, cells distributed in a 96-well plate (105 cells per well) and incubated for 20 minutes at 37°C. The baseline signal was acquired for 2 minutes. Calcium flux upon stimulation with chemokines (100 nM) or the calcium ionophore A23187 (1 μM) were quantified using the changes in luminescence measured on a GloMax plate reader (Promega).

Indo-1 AM ratiometric fluorescent indicator was also used as additional readout. HEK293T, HEK293T cells stably expressing CXCR3-A or CXCR3-B were incubated with 1 μM of Indo-1 AM (Thermo Fisher Scientific) in PBS supplemented with 1 mM CaCl2 and 0.5 mM MgCl2 for one hour at 37°C. Cells were pelleted, resuspended in PBS/CaCl2/ MgCl2, distributed in a 96-well plate (105 cells per well) and incubated for 30 min. First the baseline signal was acquired for 2 minutes. Cells were then stimulated with chemokines (100 nM) and the calcium flux was measured for 2 min. The validity of the assay was confirmed using the calcium ionophore A23187 (1 μM). Fluorescence was acquired on a GloMax plate reader using the 365 nm excitation laser and 415–445 and 495–505 emission filters to evaluate calcium-bound and free Indo-1, respectively.

3.6. Transcriptional Nanoluciferase reporter assays

Activation of the MAPK/ERK, RhoA-dependent, and cAMP-dependent signaling pathways was evaluated using a serum response element (SRE), Serum Response Factor Response Element (SRF-RE), and a cAMP response element (CRE) Nanoluciferase reporter assay, respectively (Promega). 1 x 106 HEK293T cells were seeded in a 6-well plate, cultured for 24 hours, and co-transfected with the pNL3.2.SRE, pNL3.2.NFAT-RE, pNL3.2.SRF-RE or pNL3.2.CRE, and CXCR3-A- or CXCR3-B-encoding pIRES vectors. 24 hours after transfection, 50 x 103 cells/well were seeded in a white clear-bottom 96-well plate and 24 hours later the medium was replaced by serum-free DMEM and incubated for 30 minutes at 37°C. Chemokines (100 nM) and corresponding positive control (50 nM phorbol 12-myristate 13-acetate (PMA), 10 % FBS or 50 nM PMA, 1 µM ionomycin, 10 % FBS and 50 µM forskolin) were then added and incubated for six hours. Nano-Glo Live Cell substrate (Promega) was then added and luminescence was read over 20 minutes on a Mithras LB940 plate reader (Berthold Technologies).

3.7. Receptor cellular distribution assays

Fluorescent microscopy: 11.5 x 104 HEK293T cells transiently transfected with CXCR3-A, CXCR3-B, ACKR3, or ACKR2 C-terminally fused to mNeonGreen were seeded in a poly-lysine-coated µ-Slide 8 well-chambered coverslip (Ibidi). After 24 hours, cells were washed twice with PBS and fixed with 3.5% (w/v) paraformaldehyde solution for 20 minutes at RT. Cells were washed three times with PBS and incubated with anti-CXCR3 (1C6), anti-ACKR3 (8F11-M16), and anti-ACKR2 (196124) mAb for one hour at 4°C. Cells were again washed twice with PBS and incubated for 20 minutes at RT with Hoechst 33342 dye (1 µg/mL). Cells were washed twice with PBS before adding mounting media and acquiring images on a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss).

To detect chemokine receptors at the cell surface, HEK293T cells were incubated 45 minutes at 4°C with receptor-specific mAbs, washed once with FACS buffer (PBS, 0.1% Sodium azide, 1% BSA) and then incubated for 20 minutes at 4°C with Zombie Green viability dye, before measuring the fluorescence on a Quanteon Flow Cytometer (NovoCyte).

To monitor receptor cellular cycling, 1.5 x 105 HEK293T.CXCR3-A, HEK293T.CXCR3-B, HEK293T.ACKR3 [32] or HEK293T.ACKR2 [33] cells were seeded in a 96-well plate and incubated for 3 hours with 0.1 mg/mL proteinase K to remove all extracellular epitopes. Cells were washed twice with PBS and incubated for one additional hour at 37°C in DMEM supplemented with 50 µM cycloheximide to measure re-surfacing receptors. Cells were then washed twice with PBS and an excess of anti-CXCR3 (1C6), anti-ACKR3 (8F11-M16), or anti-ACKR2 (196124) mAb was added and incubated for 45

minutes at 4°C. Cells were then washed once with PBS and incubated for 20 minutes at 4°C with Zombie Green viability dye (BioLegend). After two PBS washes surface receptor expression was measured on a Quanteon Flow Cytometer (NovoCyte).

To follow receptor cellular distribution after chemokine stimulation, 1.5 x 105 HEK293T.CXCR3-A, HEK293T.CXCR3-B, HEK293T.ACKR2 or HEK293T.ACKR3 cells were seeded in a 96-well plate, incubated or not with the V ATPase inhibitor, Bafilomycin A1 (1.5μM), for 40 minutes and then stimulated with chemokines (100 nM) for 10, 20, 40 or 60 minutes at 37°C in medium containing 50 μM cycloheximide. Cells were then washed twice with PBS and incubated for 40 minutes at 37°C, in the absence of chemokines to allow possible receptors re-surfacing. Cells were washed once with a low-pH buffer (50 mM Glycine, 150 mM NaCl, pH 3.5) and twice with PBS. An excess of receptor-specific antibody was then added and incubated for 45 minutes at 4°C. Cells were then washed once with PBS and incubated for 20 minutes at 4°C with Zombie Green viability dye (BioLegend). The receptor extracellular expression was measured on a Quanteon Flow Cytometer (NovoCyte).

Surface Nanoluciferase complementation (HiBiT, Promega): Receptor cellular distribution, in basal conditions and upon ligand stimulation, was monitored by nanoluciferase complementation assay. In brief, chemokine-induced changes in surface receptor levels were monitored with the use of Nano-Glo HiBiT extracellular detection system (Promega). 4 x 106 HEK293T cells were plated in 10-cm dishes and cultured for 24 hours before transfection with pHiBiT vectors encoding for CXCR3 variants N-terminally fused to HiBiT. 48 hours later, cells were distributed in white 96-well plates (5 x 104 cells per well) and stimulated with chemokines (100 nM) for 0, 5, 10, 20, 40 minutes at 37°C. Luminescence was then recorded over 30 minutes with a GloMax plate reader (Promega). In un-stimulated conditions, surface and total receptor expression was determined using Nano-Glo HiBiT extracellular detection system (Promega) and Nano-Glo HiBiT lytic detection system (Promega), respectively.

3.8. Chemokine uptake

Flow Cytometry: To monitor chemokine uptake and binding 1.5x 105 HEK293T cells transiently transfected with vectors encoding CXCR3-A or CXCR3-B C-terminally fused to mNeonGreen were incubated for 1 hour at 37 °C or 4°C in the presence of Alexa Fluor 647-labelled chemokines (33 nM). Cells were washed twice with FACS buffer and subjected or not to proteinase K treatment (0.1 mg/mL) for 3 hours at 4°C to evaluate and compare unspecific chemokine binding to the cell surface. Cells were washed twice with FACS buffer and then treated with Zombie NIR viability dye (BioLegend) to exclude dead cells. After two PBS washes, the fluorescent chemokine uptake was quantified using a Quanteon Flow Cytometer (NovoCyte).

Confocal Microscopy: 3 x 104 HEK293T cells transiently transfected with vectors encoding CXCR3-A or CXCR3-B C-terminally fused to mNeonGreen were seeded in a poly-lysine-coated µ-Slide 8-well-chambered coverslips (Ibidi) and grown for 24 hours. Cells were incubated for one hour at 37°C with 100 nM of Alexa Fluor 647-labelled chemokines (Protein Foundry) and co-incubated one additional hour with 750 nM LysoTracker™ Red DND-99 (ThermoFisher). Cells were then washed twice with PBS, fixed with 3.5% (w/v) paraformaldehyde for 20 minutes at RT, and washed twice. Afterward, nuclear staining was performed with Hoechst 33342 dye (1 µg/mL) for 20 minutes at RT. Cells were washed 3 times with PBS before mounting media was added. Images were acquired with a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss).

3.9. Data and statistical analysis

Concentration-response curves were fitted to the three-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 8.4.3) to provide EC_{50} values and standard errors of the mean. All curves were fitted to data points generated from the mean of at least three independent experiments. The statistical tests two-way ANOVA, and post hoc analysis were performed with GraphPad Prism 8.4.3. P-values are indicated as follows: *$p < 0.05$, **$p < 0.01$.

4. Results

4.1. The CXCR3-B variant is decoupled from G protein but maintains β-arrestin recruitment capacity

To evaluate and compare the functionality and signaling capacity of CXCR3-A and CXCR3-B variants, we first investigated their ability to interact with miniG proteins and β-arrestins in response to their shared ligands CXCL11, CXCL10, and CXCL9 using a Nanoluciferase complementation-based (NanoBiT) assay. CXCR3-A was able to recruit miniGi and miniGq proteins after stimulation with all three chemokines. In stark contrast, CXCR3-B showed a complete loss of miniGi recruitment abilities and miniGq recruitment was almost abolished (Fig. 1A). Both CXCR3-A and CXCR3-B failed to recruit miniGs and miniG12/13 proteins (supplementary Fig 2). Next, we monitored the recruitment of β-arrestins towards both CXCR3 variants upon chemokine stimulation using the above-mentioned NanoBiT assay. CXCL11 or CXCL10 induced a β-arrestin-1 and β-arrestin-2 recruitment to CXCR3-A, with CXCL11 having stronger potency and efficacy compared to CXCL10. Interestingly, although G protein interaction was severely impaired, CXCR3-B efficiently interacted with β-arrestin-1 and -2 upon stimulation with CXCL11 and a weak recruitment for CXCL10 was detected. CXCL9 was only able to induce a slight β-arrestin recruitment towards both CXCR3 variants at the highest concentration tested (Fig. 1B). These results were confirmed using the same assays in U87 cellular background as well as using NanoBRET-based approaches (supplementary Fig 2).

CXCR3-B decoupling from G proteins was further investigated, using a NanoBRET assay, by monitoring the activation of heterotrimeric G proteins and their dissociation from the receptor following chemokine stimulation. CXCR3-A activation by its ligands, triggered the dissociation of Gαi/o and Gαq proteins from the Gβγ dimer, albeit a weaker effect was observed for the latter. In contrast, CXCR3-B displayed a complete loss of Gαi and Gq activation and a severe impairment of Go for CXCL10 and CXCL9. CXCL11 triggered slight activation of Gαi/o protein at high chemokine concentration (Fig 1C). No other G protein subtype activation could be measured for either CXCR3 variant. These results support the data obtained for miniG protein recruitment and further confirm the decoupling of CXCR3-B from G proteins.

To corroborate the impairment of CXCR3-B-driven G protein activation, we investigated the modulation of two downstream secondary messengers: cAMP and calcium. cAMP modulation was monitored using a luciferase-based Glo biosensor. Upon activation with CXCL11, CXCL10, CXCL9, we detected a concentration-dependent decrease in cAMP for CXCR3-A, confirming Gαi/o protein activation. No cAMP modulation was however detected for CXCR3-B (Fig. 1D) nor the untransfected cells after stimulation with CXCL11, CXCL10 or CXCL9 (supplementary Fig 2). The positive control CXCL12 was able to reduce cAMP levels attesting to the functionality of this secondary messenger assay. Intracellular calcium mobilization was investigated to further characterize signaling abilities of the receptors. In this assay, all CXCR3 chemokines induced a calcium flux following CXCR3-A activation. These calcium fluxes could be inhibited using, the CXCR3 antagonist AMG487, confirming the CXCR3-mediated calcium measurements (Fig. 1E). In contrast, CXCR3-B only triggered a weak calcium flux in response to CXCL11 and no response to CXCL10 or CXCL9. Similar results were obtained by monitoring intracellular calcium mobilization with the use of the ratiometric fluorescent indicator Indo-1 (supplementary figure 3). These experiments confirmed CXCR3-B's inability to trigger efficient downstream G protein signaling (Fig. 1E and F), which is in agreement with the impaired Gαi/o activation.

Finally, the ability of the CXCR3-binding chemokines to trigger later downstream signaling events was also examined by monitoring the activation of various transcriptional response elements (RE). Stimulation of CXCR3-A by CXCL11, CXCL10 and CXCL9 showed a significant increase in ERK signaling using a MAPK/ERK-dependent response element (SRE), as opposed to CXCR3-B that showed no modulation of this pathway (Fig. 1G). The specific activation of Gαi or Gαs and subsequent cAMP modulation was monitored by CRE inhibition/activation. A slight decrease in signal for CXCR3-A was observed after stimulation with CXCL11, CXCL10, and CXCL9, while no difference in signal could be seen for CXCR3-B with the exception of CXCL9 that showed a more pronounced decrease. We also examined chemokine-induced activation of Gαq and Gα12/13 protein-specific signaling using NFAT and SRF response elements, respectively, which showed no

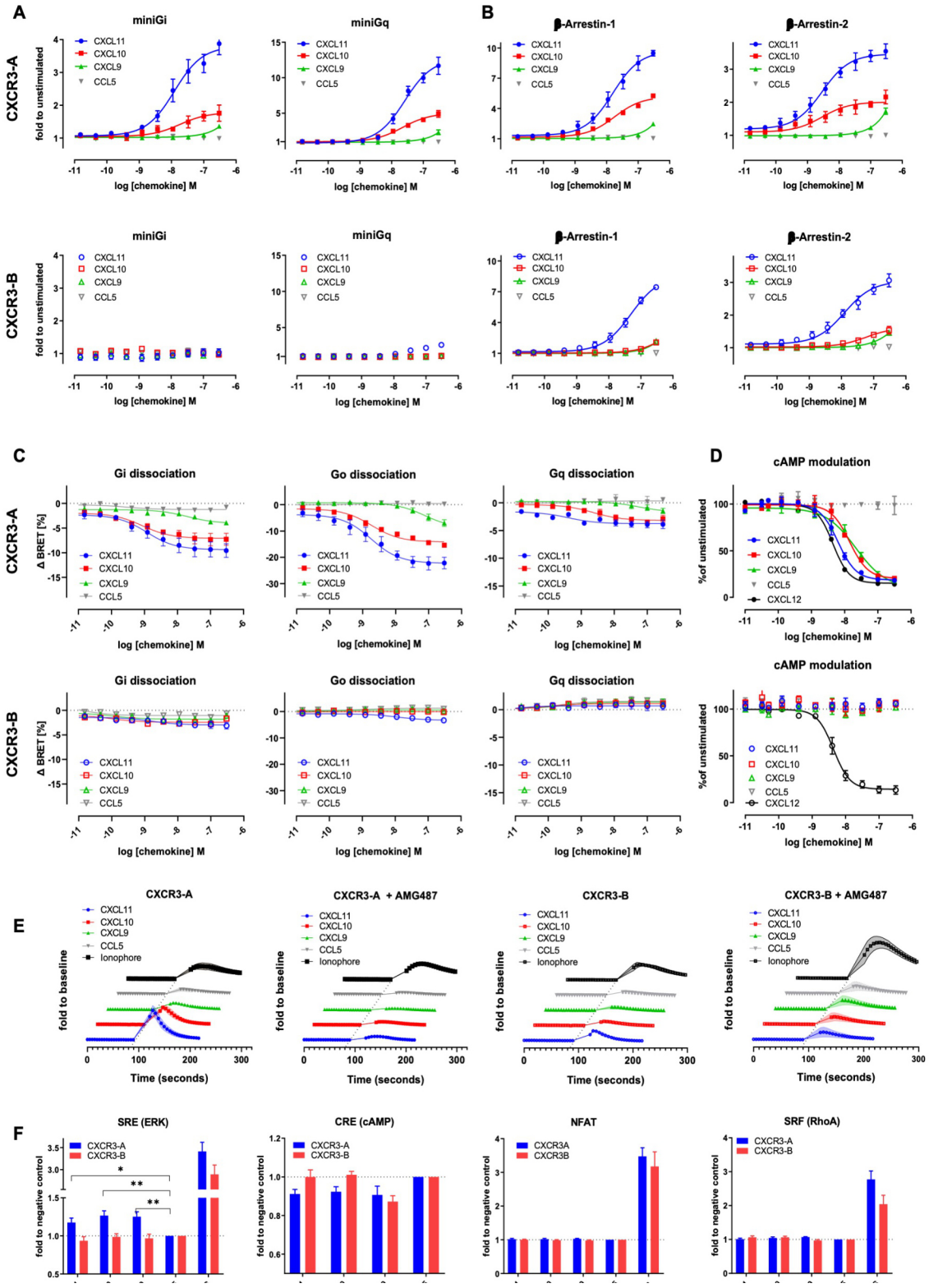


Figure 1 | The CXCR3-B variant does not induce G protein-mediated signaling. (A) miniG proteins or (B) β-Arrestin recruitment towards CXCR3-A and CXCR3-B in response to CXCL11, CXCL10 and CXCL9 monitored by NanoBiT-based assay. CCL5 was used as negative control (C) Heterotrimeric G protein dissociation upon CXCR3-A or CXCR3-B stimulation with CXCL11, CXCL10, CXCL9 and CCL5. Concentration-response relationship for the alpha subunits Gi2, Go and Gq are expressed as ΔBRET. (D) CXCR3-A and CXCR3-B-driven effect on intracellular cAMP levels after stimulation with CXCR3 ligands. (E) Intracellular calcium changes upon activation of CXCR3-A or CXCR3-B monitored by NanoBiT assay. The assay was conducted in absence or presence of CXCR3-antagonist AMG487. (F) CXCR3 variant-mediated downstream signaling events using the Nanoluciferase-based response elements SRE, CRE, NFAT and SRF. CCL5 and CXCL12 were used as

negative and positive control, respectively, for the different assays. Results are expressed as fold RLU to vehicle. All nanoluciferase-based and NanoBRET-based assays were conducted in HEK293T cells. Data points represent mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ by two-way ANOVA with Dunnett post hoc tests.

statistical differences in the signal measured with all CXCR3 chemokines for both variants compared to the negative control CCL5 (Fig. 1G).

Together, these data point to an altered ability of the CXCR3-B variant to couple to and activate G proteins and their downstream signaling events, while preserving the ability to recruit β-arrestins upon chemokine stimulation.

4.2. CXCR3-B has a different cellular localization compared to CXCR3-A

Based on the observations that CXCR3-B is able to efficiently recruit β-arrestins despite the impaired G protein signaling, we examined whether it shows other properties characteristic of atypical chemokine receptors. We first evaluated the basal cellular distribution of both CXCR3 variants and compared it to that of ACKR2 and ACKR3, two receptors of the ACKR family reported to be mostly located in intracellular vesicles under basal conditions [12].

The cellular distribution of CXCR3 variants, was first visualized by confocal fluorescent microscopy using receptors C-terminally fused to the mNeonGreen fluorescent protein and a receptor-specific antibody to detect their presence at the plasma membrane. In line with previous reports [16], CXCR3-A was mainly present at the cell surface as revealed by the strong colocalization of the mNeonGreen and CXCR3-specific antibody. In contrast, CXCR3-B showed a more pronounced intracellular distribution and a reduced fluorescent signal at the plasma membrane, which was reminiscent of the intracellular localization of ACKR2 and ACKR3 (Fig. 2A). The different cellular distribution of the two CXCR3 variants was further confirmed using the HiBiT technology. The data revealed that while CXCR3-A was almost exclusively found at the cell surface, only 45 % of total CXCR3-B was exposed at the cell surface under basal conditions (Fig. 2B).

4.3. CXCR3-B does not cycle in basal conditions but is mobilized to the cell membrane upon chemokine stimulation

Based on the atypical CXCR3-B subcellular distribution reminiscent of ACKRs, we investigated the receptor cycling in the absence and presence of chemokines. Indeed, ACKRs regulate extracellular chemokine availability for classical receptors. Chemokine binding may induce receptor internalization but some ACKRs, like ACKR3, were shown to continuously cycle between the intracellular compartments and the cell surface, independently of ligand stimulation [40, 41].

Basal CXCR3 cycling was thus first evaluated with flow cytometry following extracellular epitope cleavage by proteinase K, by monitoring receptor replenishment at the

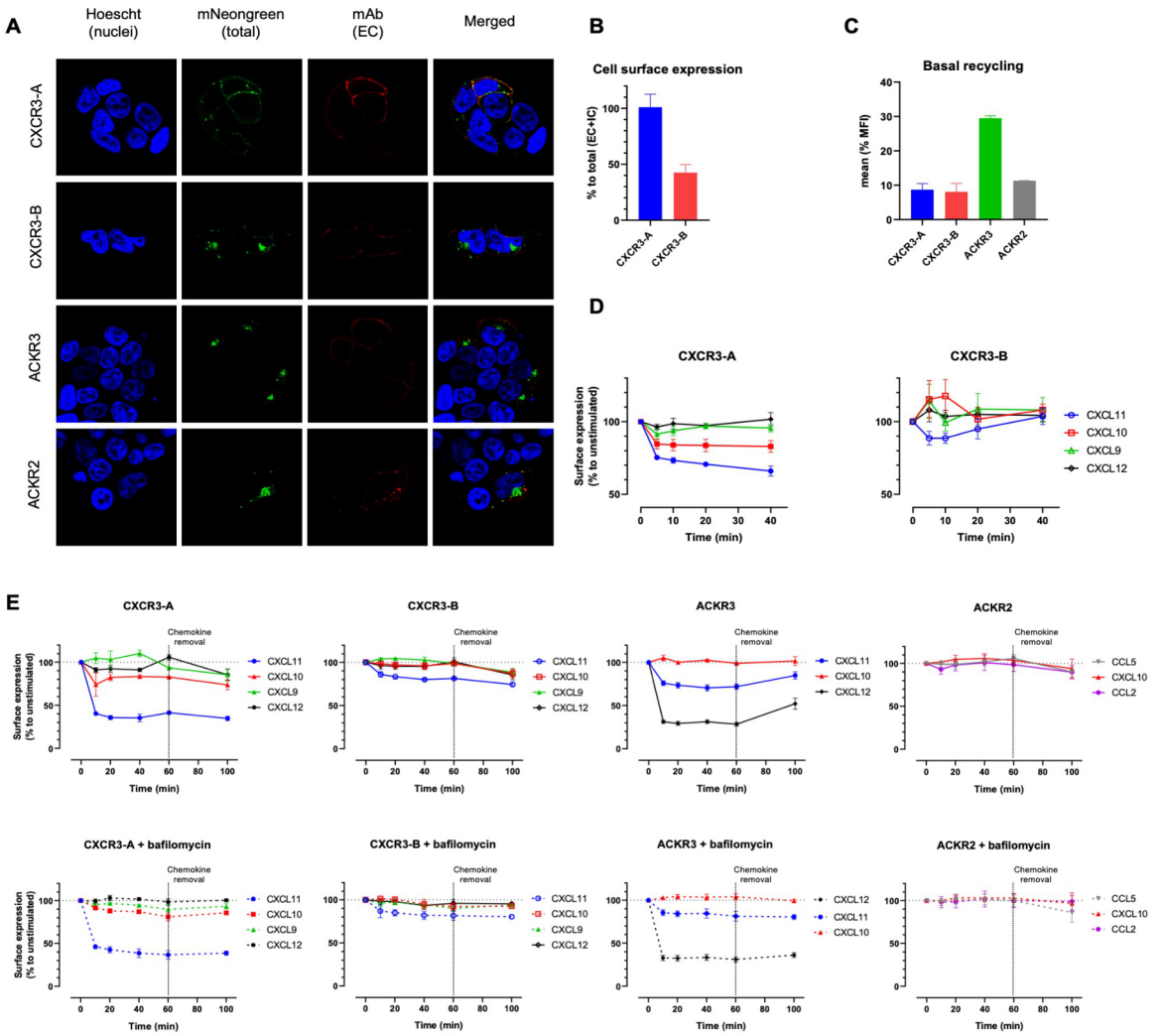


Figure 2 | CXCR3-B shows intracellular localization and does not cycle in basal conditions but is mobilized to the cell membrane upon chemokine stimulation. (A) Receptor cellular distribution of CXCR3-A, CXCR3-B, ACKR2 and ACKR3 fused to mNeongreen fluorescent protein and extracellular staining with 1C6 mAb in basal conditions visualised by confocal microscopy. Pictures are representative of 12 acquired images from three independent experiments. (B) Expression of CXCR3-A and CXCR3-B on the cell surface quantified using HiBiT-mediated nanoluciferase complementation and are expressed as percentage to total (extracellular and intracellular). (C) CXCR3-A, CXCR3-B, ACKR2 and ACKR3 basal cycling after extracellular epitope shaving by proteinase K. Results are expressed as percentage of cell surface expression before proteinase K treatment. (D-E) Cell surface redistribution of CXCR3-A and CXCR3-B after stimulation with chemokines (100 nM) monitored by (D) surface Nanoluciferase complementation (HiBiT) or (E) flow cytometry in the presence or absence of Bafilomycin A1. Results are expressed as percentage with baseline set at 100% for receptor surface expression in basal conditions and represent mean ± SEM of three, independent experiments (N=3).

plasma membrane in the presence of cycloheximide, a de-novo protein synthesis inhibitor. Two distinct trends could be identified for the set of chemokine receptors tested. Both CXCR3 variants and ACKR2 showed an approximately 10% increase of receptor cell surface expression, while ACKR3 demonstrated a more pronounced increase of 30% (Fig. 2C). This suggests that similarly to CXCR3-A and ACKR2, CXCR3-B shows limited cycling from the intracellular compartment to the plasma membrane in the absence of chemokines.

Chemokine-induced internalization and recycling was then assessed for the two CXCR3 variants, first using flow cytometry (Fig. 2E). CXCL11 and CXCL10 induced the

internalization of CXCR3-A after 10-minute stimulation, with CXCL11 reducing the receptor surface expression by 60% and CXCL10 by 20%, while CXCL9 had no impact on receptor internalization (Fig. 2E). In contrast, for CXCR3-B, CXCL11 induced only about 20% internalization, while CXCL10 and CXCL9 had no effect (Fig. 2E). ACKR2 surface expression was not modified by chemokine stimulation, reminiscent of CXCR3-B behaviour, whereas ACKR3 stimulation with CXCL12 and CXCL11 led to the internalization of approximately 70 % and 25 % of the receptor present at the plasma membrane, respectively (Fig. 2C). In addition, receptor surface expression was evaluated 40 minutes after chemokine removal. None of the CXCR3 variants nor ACKR2 recycled back to the cell surface in the presence or absence of the V-ATPase inhibitor Bafilomycin A1, an, suggesting an absence of rapid recycling following ligand stimulations (Fig. 2E) in contrast to ACKR3, for which Bafilomycin A1-sensitive recovery at the plasma membrane could be detected [32, 42].

The limited level of CXCR3-B internalization, compared to CXCR3-A, upon stimulation was further studied using a highly sensitive cell surface detection approach based on the HiBiT peptide and Nanoluciferase complementation technology. Cells expressing N-terminally HiBiT-tagged CXCR3-A or CXCR3-B were stimulated with chemokines and the remaining membrane receptors were quantified at different time points by adding soluble LgBiT protein. A decrease in CXCR3-A receptor level at the cell surface was induced by CXCL10 and CXCL11 and reflected their potencies. In contrast, although an initial reduction of CXCR3-B levels was observed in response to CXCL11, a gradual replenishment of the receptor at the cell surface was then measured. Moreover, an immediate increase in surface CXCR3-B was triggered by CXCL10 and CXCL9, suggesting a rapid transport of a part of the intracellular receptor pool to the plasma membrane as recently described for ACKR2 following ligand stimulation (Fig. 2D) [33].

Altogether, these results confirm that CXCR3-A and CXCR3-B have different cellular distribution patterns under basal and ligand-induced conditions. CXCR3-A exhibits a classical chemokine receptor profile, with a more pronounced cell surface expression and chemokine-induced internalization, while CXCR3-B resides inside the cell in basal conditions and generally shows a slower internalization upon ligand stimulation and a mobilization to the plasma membrane upon stimulation that are reminiscent of the profile observed for ACKR2 [33].

4.4. Both CXCR3 variants mediate efficient uptake of endogenous chemokines

ACKRs play important roles in the immune responses by regulating chemokine availability for classical chemokine receptors [40]. CXCR3-B being unable to induce canonical G protein signaling events in response to ligand binding, while maintaining its

ability to recruit β-arrestin, we next examined whether this receptor was able to internalize CXCR3 ligands.

We first investigated the uptake of all CXCR3 ligands coupled to Alexa Fluor 647 by flow cytometry. CXCL11, CXCL10 and CXCL9 uptake was detected for both variants, albeit with reduced intensities for CXCR3-B (Fig. 3A), which may reflect its lower expression level at the plasma membrane. No uptake was observed with the irrelevant chemokine CCL5 labelled with the same fluorophore. Chemokine targeting to intracellular compartments was confirmed with the use of proteinase K, a non-selective protease allowing to remove remaining cell surface-bound proteins, as illustrated by the reduced fluorescence signal in non-internalizing conditions for both CXCR3-A and CXCR3-B-expressing proteinase K-treated cells. In contrast, this treatment had no impact on the signal detected following chemokine incubation in internalizing conditions for the two CXCR3 variants, strongly pointing to chemokine uptake following receptor activation.

Receptor-dependent internalization of fluorescently labelled chemokines was also analyzed by confocal microscopy. Cells expressing CXCR3-A or CXCR3-B internalized their related chemokines (Fig. 3B) in contrast to non-transfected cells (supplementary Fig 4). Lysotracker, a fluorescent dye for labeling and tracking acidic organelles in living cells, confirmed the intracellular co-localization of the receptor and the internalized chemokines, hinting towards their degradation. Furthermore, a noticeable change in receptor subcellular distribution could be observed for CXCR3-A after stimulation, but not for CXCR3-B, corroborating the different receptor internalization profiles described above.

These data demonstrate that CXCR3-B is able to mediate the uptake of CXCR3 ligands from the extracellular space and to address them to intracellular compartment without triggering efficient G protein signaling.

4.5. The N-terminal extension of CXCR3-B does not modify receptor selectivity and chemokine binding mode

The extracellular N-terminal domain of chemokine receptors plays an important role in chemokine binding and selectivity [43]. The presence of the unique 51 additional residues of CXCR3-B N terminus and its impact on the ability of the receptor to efficiently couple to G protein prompted us to investigate other receptor properties such as selectivity and activation mode.

To this end, we first undertook a β-arrestin-1 recruitment screening of the 43 human chemokines (24 CCLs, 16 CXCLs, 2 XCLs, and 1 CX3CL) and 2 viral chemokines (vCCL1 and vCCL2) towards the CXCR3 variants aiming at evaluating the possible impact of CXCR3-B's N-terminus on its selectivity for chemokine. This systematic assessment did not result in the identification of new endogenous agonist chemokines for any of the two variants (Fig. 4A), although we could confirm the activity of the orphan chemokine CXCL4

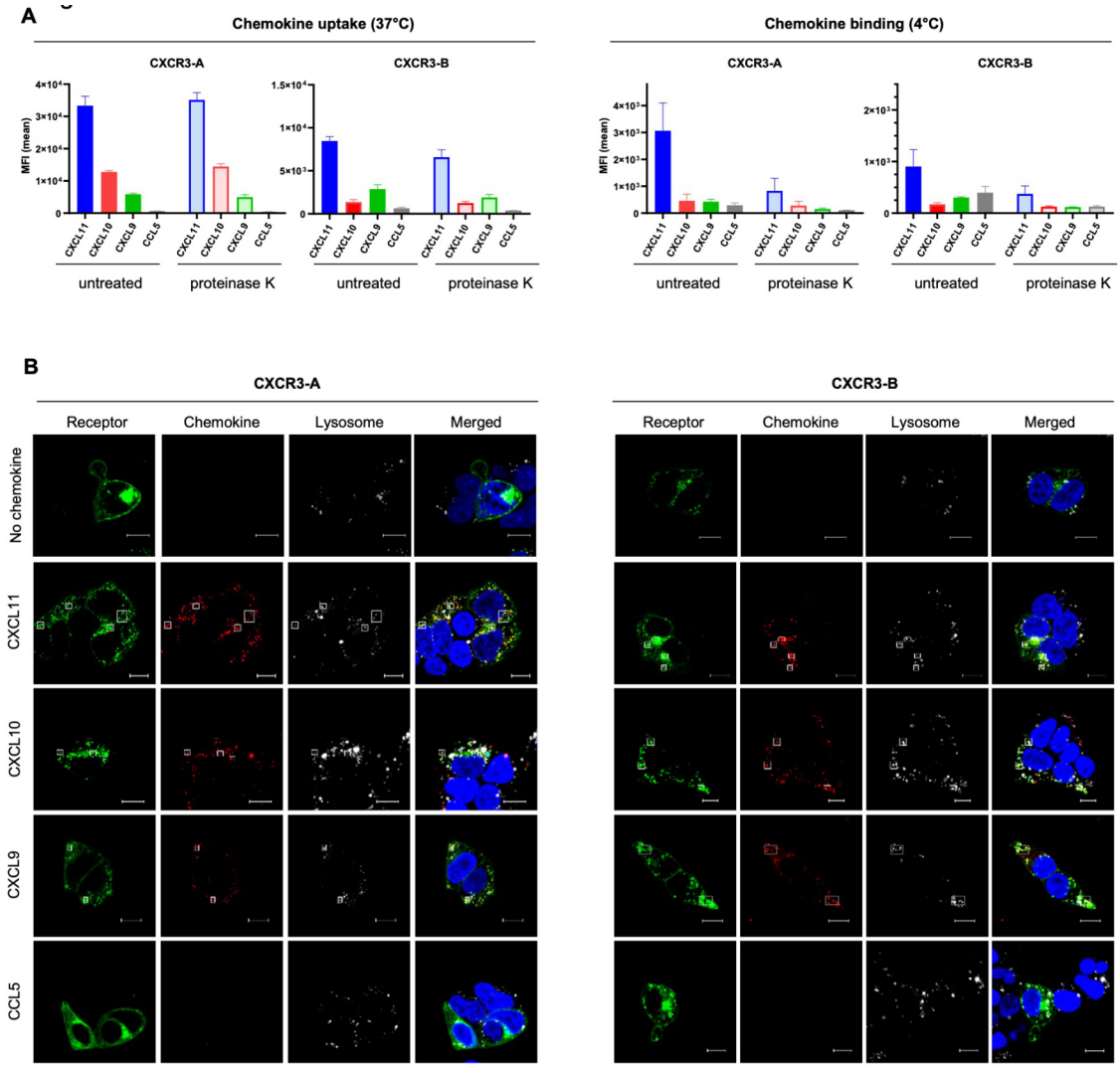


Figure 3 | CXCR3-driven chemokine uptake. (A) Intracellular accumulation and cell surface binding of Alexa Fluor 647-labelled CXCL11, CXCL10, CXCL9 and CCL5 (33 nM) was measured by flow cytometry in CXCR3-A- and CXCR3-B-expressing cells. After 1h incubation at 37°C or 4°C, respectively, cell surface-bound chemokines were removed with proteinase K treatment. Results represent mean ± SEM of three independent experiments (N=3). (B) Uptake of CXCR3 chemokines coupled to Alexa Fluor 647 by CXCR3-A or CXCR3-B visualized by confocal fluorescent microscopy. HEK293T cells transiently expressing CXCR3-A or CXCR3-B fused to mNeongreen (green) were incubated for 2 hours with 100nM CXCR3 ligands or CCL5 (red). Lysosomes and nucleic DNA were stained using LysoTracker™ Red DND-99 (white) and Hoechst 33342 (blue), respectively. Scale bar is 10 µM. Pictures are representative of 12 acquired images from three independent experiments.

proposed in some studies as a CXCR3-B agonist [44]. Surprisingly, we observed that the viral chemokine vCCL2 showed antagonistic properties towards both CXCR3 variants, an interaction that has not been described previously (supplementary Fig 5)[45].

Atypical chemokine receptors may have different recognition determinants and activation modes compared to classical receptors. Notably, ACKR3, which acts as a scavenger for CXCL11, was shown to be insensitive to chemokine N loop substitutions and cleavage by the dipeptidyl peptidase IV (CD26), which, by removing the first two residues turns agonist CXC chemokines into antagonist for a great majority of receptors [46]. Therefore, we investigated the ability of chemokines with N-terminal substitutions and progressive truncations or modified N loops to activate both CXCR3 variants. The removal

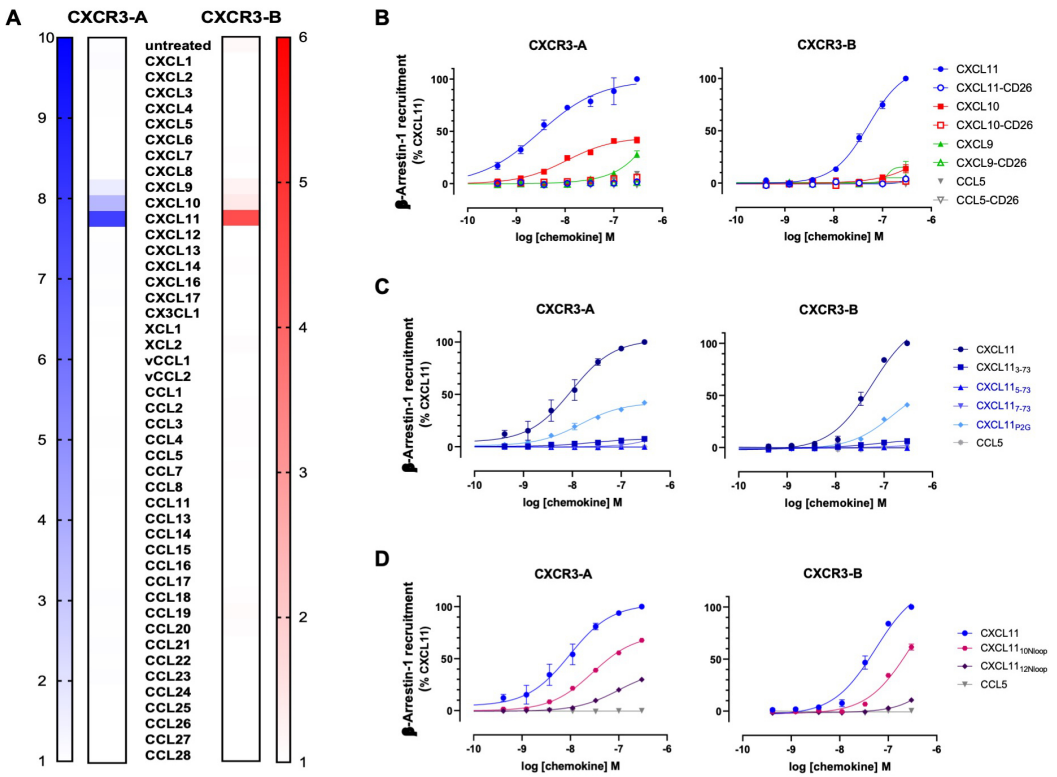


Figure 4 | N-terminal extension of CXCR3-B does not change the receptor specificity and chemokine binding mode. (A) β-arrestin-1 recruitment to CXCR3-A or CXCR3-B in response to all known human and two viral chemokines (100 nM) monitored by NanoBiT-based assay. (B-D) β-arrestin-1 recruitment to CXCR3-A or CXCR3-B by differently processed CXCR3 chemokines monitored by NanoBiT. (B) Recruitment of β-arrestin-1 to CXCR3-A and CXCR3-B induced by CD26-treated CXCR3 chemokines. (C) β-arrestin-1 recruitment to CXCR3-A and CXCR3-B induced by N-terminally truncated and P2G-mutated CXCL11. (D) Recruitment of β-arrestin-1 to CXCR3-A and CXCR3-B in response to CXCL11 Nloop chimeras. All NanoBiT assays were conducted in HEK293T cells. Results are expressed as fold RLU to vehicle and represent mean ± SEM of three independent experiments (N=3).

of the first two residues of CXCL11, CXCL10, and CXCL9 by CD26 exopeptidase abolished the activity of the three chemokines towards both CXCR3 variants (Fig. 4B). These results were confirmed with recombinant CXCL11 lacking the first two residues ($CXCL11_{3-73}$) or bearing further N-terminal truncations ($CXCL11_{5-73}$ and $CXCL11_{7-73}$) (Fig. 4C). Similarly, proline-to-glycine substitution at position 2 ($CXCL11_{P2G}$), known to improve CXCL11 potency towards ACKR3 [29], had a negative impact on the chemokine's ability to activate the two CXCR3 variants. Finally, CXCL11 chimeras with CXCL12 or CXCL10 N loop substitutions ($CXCL11_{12Nloop}$ and $CXCL11_{10Nloop}$), had a reduced potency and efficacy towards both variants, $CXCL11_{12Nloop}$ being the most affected (Fig. 4D).

Taken together, these results demonstrate that the N-terminal extension of the CXCR3-B variants does not change the chemokine selectivity nor recognition determinants of CXCR3-B, suggesting that both variants display similar recognition and activation modes.

4.6. The N-terminal extension of CXCR3 is responsible for its intracellular localization and associated G protein decoupling

Considering the impact of the CXCR3-B N-terminal extension on the receptor localization and coupling to G proteins, we investigated whether the entire extension is required to observe these effects and whether they are specific to CXCR3.

Progressive ten-residue truncations in the N terminus of CXCR3-B were introduced and the membrane expression and ability of the resulting truncated receptors to interact with miniG proteins were evaluated. The progressive N-terminal truncations resulted in a gradual increase of the receptor at the cell surface, the most significant increment being observed with the removal of 30 residues from the extension, reaching a plateau that was nevertheless lower than for the surface expression of CXCR3-A (Fig. 5A). A direct link between surface expression and the recoupling of the receptor to the G proteins was observed (Fig 5B).

Finally, we showed that a similar G protein decoupling effect can be achieved by inserting the CXCR3-B N-terminal extension upstream another classical chemokine receptor, CXCR4. Indeed, the engraftment of the 51-amino-acid extension of CXCR3-B to the N terminus of CXCR4 (CXCR4-B), resulted in its decoupling from Gαi/o protein, while the ability to recruit β-arrestin-1 in response to CXCL12 was conserved (Fig. 5B). The flow cytometry and confocal microscopy analysis of surface expression and cellular distribution also demonstrated that just like for CXCR3 variant, CXCR4-B resides more intracellularly than WT CXCR4 (Fig. 5D and E).

These observations demonstrate that the N-terminal extension of CXCR3-B impacts the receptor's cellular localization and G protein coupling of classical receptor, while it has a limited effect on the receptor ability to recruit β-arrestin and mediate chemokine uptake, giving CXCR3-B, the attributes of an atypical receptor, despite being encoded by the same gene as CXCR3-A.

5. Discussion

CXCR3-B variant is generated following alternative splicing of the cxcr3 gene and presents an extended N terminus compared to CXCR3-A, the classical form of the receptor. Of note, other natural variants of chemokine receptors displaying N- or C-terminal extensions and showing altered biology have also been described for CXCR4, CCR9, CCR2, CX3CR1 and but also in other families of GPCRs [47-50].

CXCR3-B variant is an elusive and enigmatic chemokine receptor for which conflicting functional and signaling results exist [16, 18, 19, 26]. We therefore undertook an in-depth molecular characterization of CXCR3-B and comparison to CXCR3-A to provide signaling and mechanistic insights into the biology and function of CXCR3-B. Our study reveals that CXCR3-B is a β-arrestin-biased receptor that shows many attributes of the atypical chemokine receptor family and could act as a scavenger for the CXCR3 chemokines,

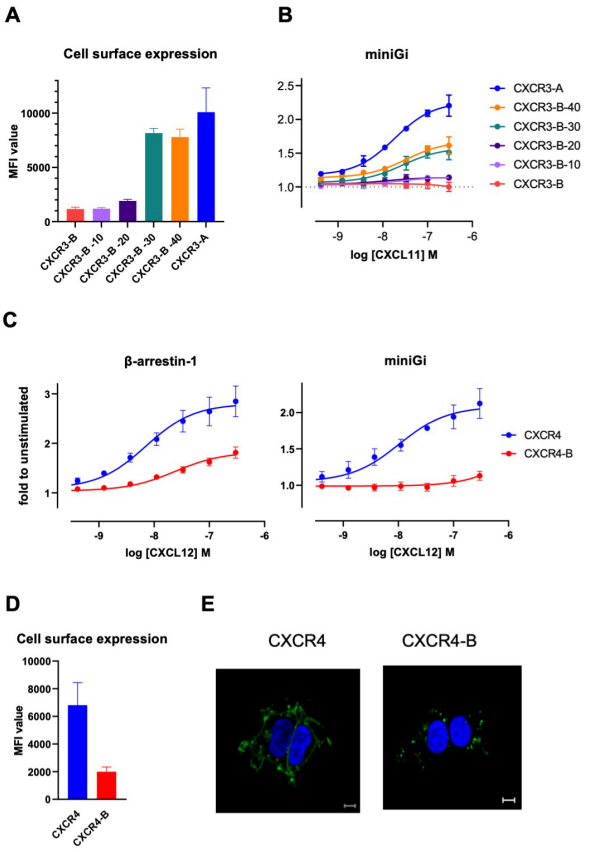


Figure 5 | The N-terminal extension of CXCR3-B is responsible for its intracellular localization and associated G protein decoupling. (A) Evolution of cell surface expression of the different N-terminally truncated CXCR3-B variants. (B) Evolution of concentration-response curves for miniGi protein recruitment to the different N-terminally truncated CXCR3-B variants. (C) β-arrestin-1 or miniGi recruitment to CXCR4 and CXCR4-B upon stimulation with CXCL12. (D) Cell surface expression of CXCR4 and CXCR4-B in HEK293T cells in the absence of chemokine by flow cytometry. (D) CXCR4 and CXCR4-B cellular distribution visualized by confocal fluorescent microscopy in HEK293T cells transiently expressing CXCR4 or CXCR4-B fused to mNeongreen. Results are expressed fold RLU to vehicle and represent mean ± SEM of three independent experiments (N=3).

possibly explaining its opposite biological effects compared to CXCR3-A.

A common characteristic of ACKRs is their inability to trigger downstream G protein-dependent signaling events upon agonist stimulation. Instead, ACKRs recruit β-arrestins to mediate receptor internalization, although recent reports suggest the presence of β-arrestins is not essential for the scavenging function [11, 51]. Our study demonstrated that, similarly to ACKRs, CXCR3-B is unable to efficiently activate G proteins and the related downstream signaling pathways following chemokine stimulation, as opposed to CXCR3-A. However, as observed for several ACKRs, CXCR3-B retained its ability to recruit β-arrestins and to mediate chemokine uptake.

CXCR3-B is mostly localized intracellularly, which may be attributed to its N-terminal extension. The N-terminal domains of chemokine receptors were shown to play an important role in chemokine binding but are generally tolerant to elongation, as illustrated by the many tags or reporters already fused to their N terminus without significant impact on the receptor biology and pharmacology. This appears not to be the case for the 51-residue extension of CXCR3-B, which drastically changes the receptor cellular distribution, limiting its presence at the plasma membrane. Our data also showed that under basal conditions CXCR3-B does not cycle between the plasma membrane and intracellular compartments. In contrast, upon stimulation, CXCR3-B intracellular pool is mobilized to the cell surface. Different directionality of CXCR3-B plasma membrane level modulation after chemokine treatment might arise from their different potential to induce internalization [4, 52]. Indeed, CXCL10 and CXCL9 known to be weak CXCR3 internalizing chemokines led to a net positive increase of CXCR3-B at the plasma membrane after stimulation whereas the strong

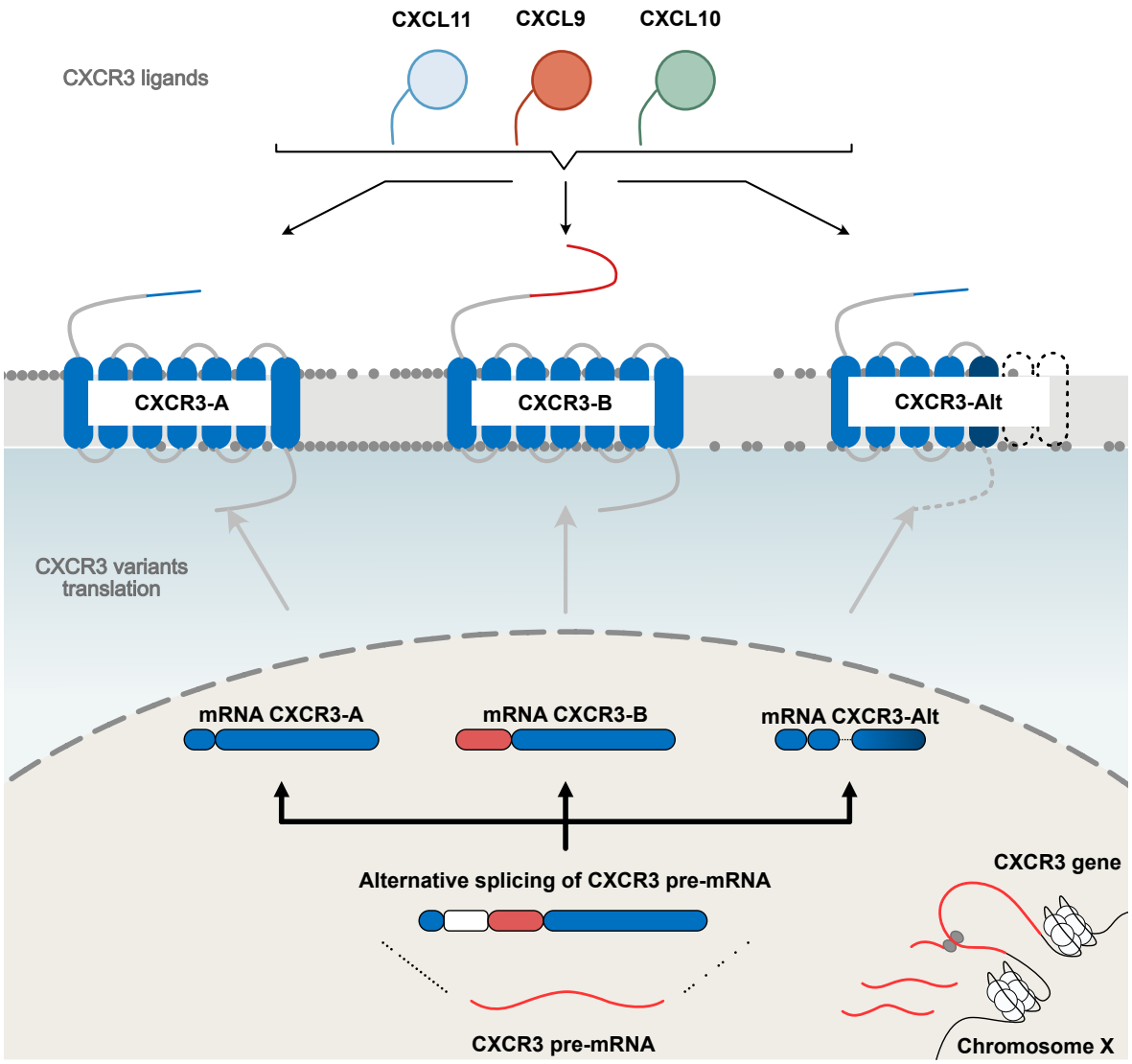
receptor internalizing chemokine such as CXCL11 led to net reduction of the receptor [52, 53]. Uptake experiments using fluorescently labelled chemokines demonstrated that the overall mobilization of the receptor ultimately results in specific intracellular accumulation of all the three CXCR3 chemokines, albeit with different efficacies compared to CXCR3-A. This scavenging ability was confirmed by confocal microscopy showing co-localization of the receptor with the chemokines in acidic degradation compartments, reminiscent of the behavior of well-characterized ACKRs such as ACKR2, ACKR3 or ACKR4. Moreover, the observation that CXCL9 and CXCL10 are efficiently and specifically taken up by both CXCR3 variants, while they show no or reduced ability to induce β-arrestin recruitment towards CXCR3, suggests that β-arrestin-independent mechanisms may mediate the chemokine-induced receptor internalization and trafficking, as recently suggested for other ACKRs [10, 11].

Our results generated using chimeric or truncated chemokines indicate that the two CXCR3 variants share the same chemokine binding mode, suggesting that ligand-receptor interactions are not at the origin of the impaired G protein coupling of CXCR3-B. On the other hand, progressive truncation of residues in the N terminus of CXCR3-B extension restored surface expression and the ability of the receptor to couple to G proteins. This implies that the change in the cellular localization of the receptor is the main driver of this shift of signaling properties, most probably limiting its ability to interact and activate efficiently G proteins, while preserving its ability to actively cycle and internalize chemokines conferring consequently to CXCR3-B attributes of atypical chemokine receptors.

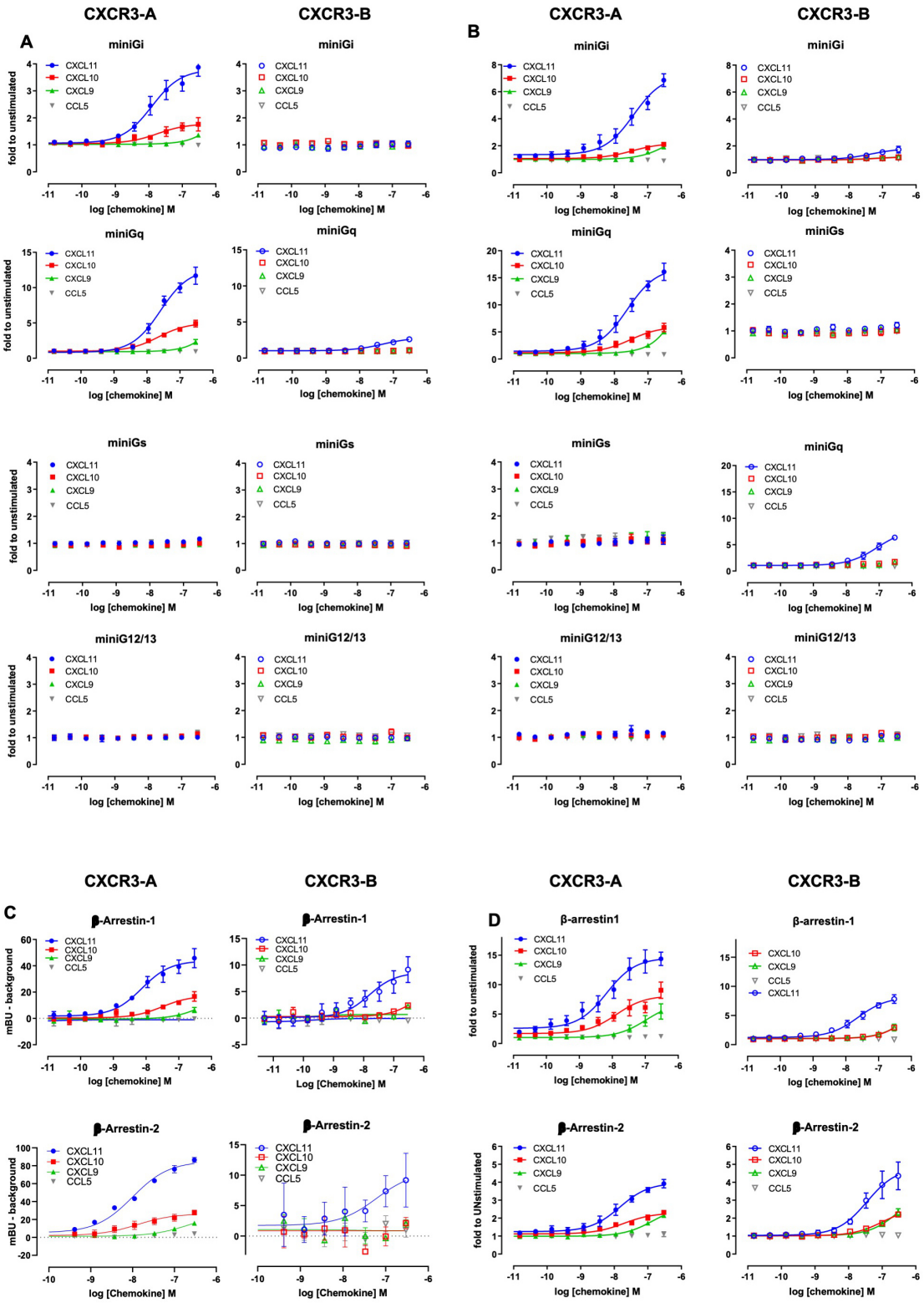
Indeed, the mostly intracellular localization, the absence of G protein signaling and the ability to actively take up chemokines are three major attributes of atypical chemokine receptors. We therefore postulate that CXCR3-B could represent a new kind of atypical chemokine receptor that modulates the bioavailability of CXCR3 chemokines thereby regulating the activation and cellular effects of CXCR3-A. Interestingly, in inflammatory conditions and in the tumor environment, CXCR3-A and CXCR3-B were reported to display opposing effects, which could be explained, in light of the present study, by their distinct function of signaling and scavenging receptors, respectively.

In conclusion, this study provides signalling and mechanistic insights into the differences of the CXCR3 variants that may explain their opposite effects. Our data indicate a strong impairment of G protein signalling for CXCR3-B and corroborate the gain of more atypical properties including intracellular localization and chemokine uptake capacities, which can be attributed to its N-terminal 51-amino acid extension. We therefore propose to consider the possibility to include CXCR3 chemokine receptor variant in the atypical chemokine receptor subfamily. Conclusions of previous reports might now need to be reanalysed in light of the newly suggested functions and additional investigations are

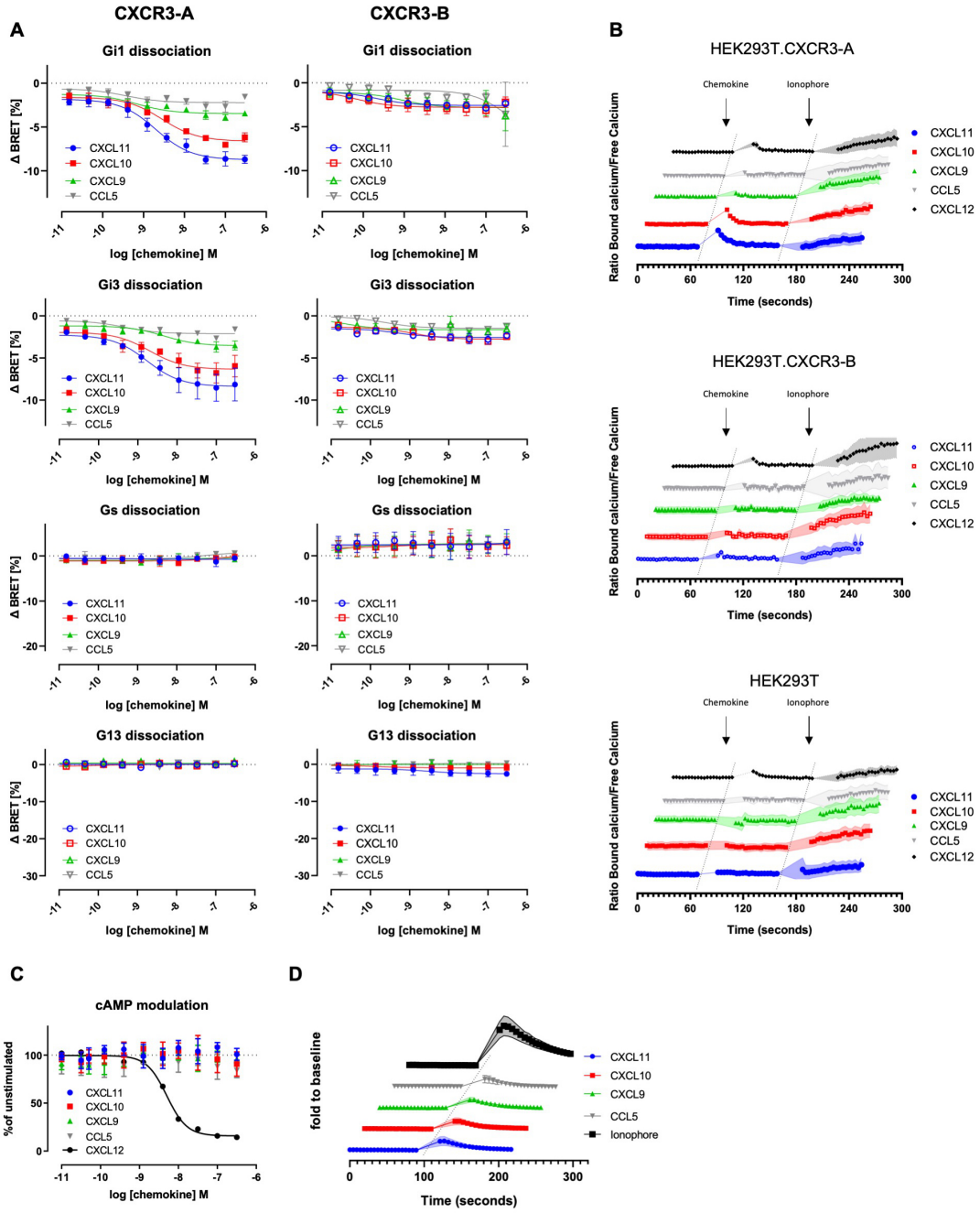
required to get a better understanding of this enigmatic receptor and to be able to develop molecules or antibodies capable to specifically modulate the different CXCR3 variants.



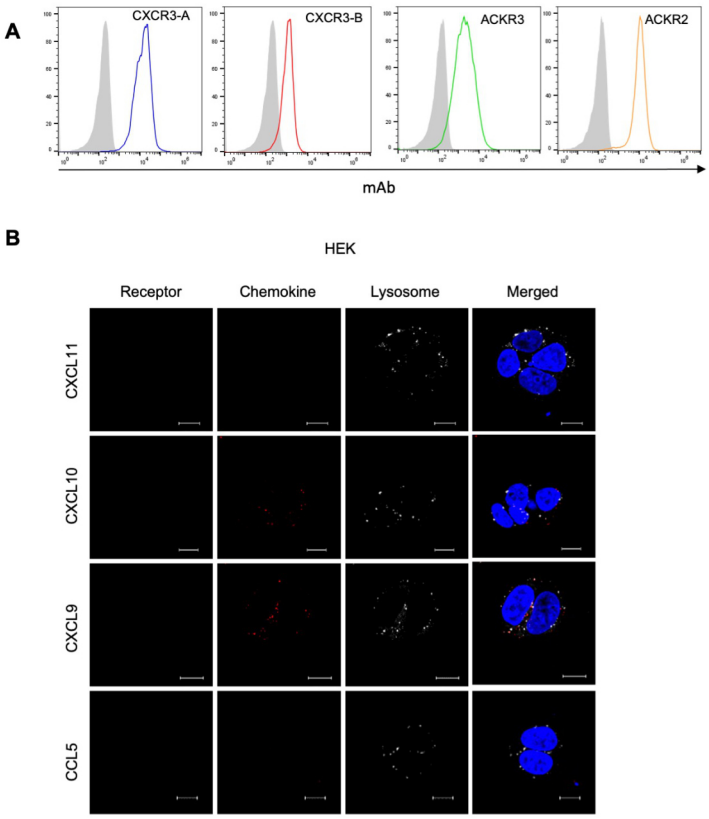
Supplementary figure 1 | Due to alternative splicing of the pre-mRNA of the cxcr3 gene, located on chromosome X, three CXCR3 variants can be generated. The CXCR3-A variant is the product of the splicing of the exon 1 and exon 3 within the CXCR3 gene. The assembly of exon 2 and exon 3 results in the CXCR3-B variant which has an N terminus longer by 51 AA compared with CXCR3-A. The removal of the intron, exon 2 and a 337-bp region within the third exon during RNA splicing results in the CXCR3-Alt variant that comprises the N terminus and the first four transmembrane domains identical to CXCR3-A as well as a possible fifth transmembrane region and a C terminus, which are different from CXCR3-A or CXCR3-B. All CXCR3 variants are able to bind three endogenous ligands CXCL9, CXCL10 and CXCL11, each with a different binding affinity and activation potential.



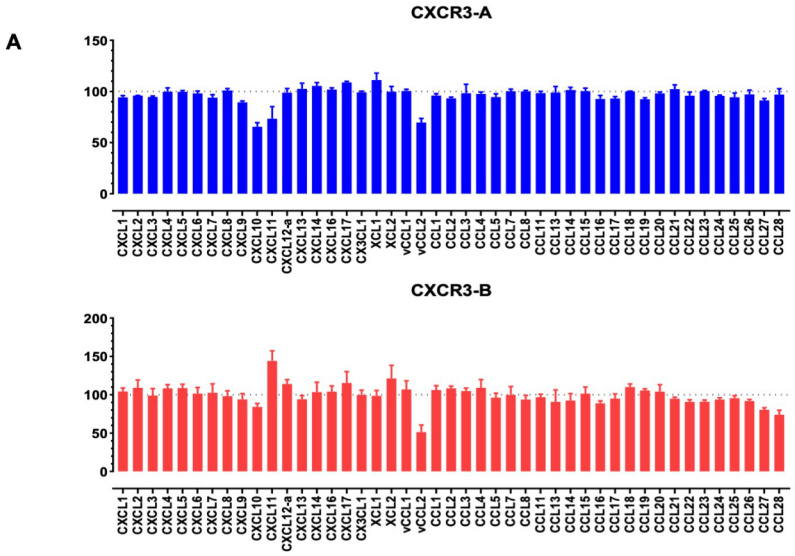
Supplementary figure 2 | (A-B) Recruitment of miniGi, miniGq, miniGs and miniG12/13 protein to CXCR3-A and CXCR3-B after stimulation by CXCL11, CXCL10, CXCL9 and CCL5monitored by NanoBiT-based assay in HEK293T (A) or U87-MG cellular background (B). (C) β-arrestin-1 and β-arrestin-2 recruitment to CXCR3-A or CXCR3-B after activation with CXCR3 ligands monitored by NanoBRET assay. (D) Recruitment of β-arrestin-1 and β-arrestin-2 to CXCR3-A and CXCR3-B after chemokine stimulation in a U87-MG cellular background using NanoBiT complementation assay.



Supplementary figure 3 | (A) Ligand-induced heterotrimeric G protein dissociation profiles for Gi1, Gi3, Gs, G13 monitored in CXCR3-A- or CXCR3-B-expressing HEK293T cells expressed as ΔBRET. (B) Intracellular calcium mobilization in HEK293T cells transiently transfected with vectors encoding CXCR3 variants or empty vector. Calcium fluxes were monitored with the ratiometric fluorescent indicator Indo-1 AM after stimulation with CXCL12, CXCL11, CXCL10, CXCL9 or CCL5. (C-D) Downstream G protein signaling in HEK293T cells after treatment with CXCL12, CXCL11, CXCL10, CXCL9 or CCL5 by intracellular cAMP modulation (C) or NanoBiT-based calcium intracellular calcium release (D). CXCL12 or Ionophore A23187 were used as positive control to confirm assay functionality.



Supplementary figure 4 | (A) Flow cytometry analysis of stable HEK293T-derived cell lines used in receptor recycling studies. Cell surface expression of CXCR3-A, CXCR3-B, ACKR2 or ACKR3, was evaluated with receptor-specific mAb (clone 1C6 for CXCR3 variants, clone 8F11 for ACKR3 and clone 196124 for ACKR2). (B) Confocal images of HEK293T cells after 2 hours incubation with CXCR3-related chemokines or CCL5 (100 nM). Alexa Fluor 647-labelled chemokines are represented in red, lysosomes stained with LysoTracker™ Red DND-99, in white and nuclei stained with Hoechst 33342, in blue. Scale bar is 10 µM. Pictures are representative of 12 acquired images from three independent experiments.



Supplementary figure 5 | (A) Antagonistic activity of all known human and 2 viral chemokines (100nM) towards CXCR3-A or CXCR3-B evaluated by β-arrestin-1 recruitment in HEK293T cells. Antagonistic activity was measured in presence of CXCL11 (20 nM).

References

1. Zlotnik, A. and O. Yoshie, The chemokine superfamily revisited. Immunity, 2012. 36(5): p. 705-16.
2. Bachelerie, F., et al., New nomenclature for atypical chemokine receptors. Nat Immunol, 2014. 15(3): p. 207-8.
3. Bonecchi, R. and G.J. Graham, Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response. Front Immunol, 2016. 7: p. 224.
4. Weber, M., et al., The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. Mol Biol Cell, 2004. 15(5): p. 2492-508.
5. Galliera, E., et al., beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. J Biol Chem, 2004. 279(24): p. 25590-7.
6. Vacchini, A., et al., Control of Cytoskeletal Dynamics by beta-Arrestin1/Myosin Vb Signaling Regulates Endosomal Sorting and Scavenging Activity of the Atypical Chemokine Receptor ACKR2. Vaccines (Basel), 2020. 8(3).
7. Comerford, I., et al., The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. Eur J Immunol, 2006. 36(7): p. 1904-16.
8. McCulloch, C.V., et al., Multiple roles for the C-terminal tail of the chemokine scavenger D6. J Biol Chem, 2008. 283(12): p. 7972-82.
9. Montpas, N., et al., Ligand-specific conformational transitions and intracellular transport are required for atypical chemokine receptor 3-mediated chemokine scavenging. J Biol Chem, 2018. 293(3): p. 893-905.
10. Saaber, F., et al., ACKR3 Regulation of Neuronal Migration Requires ACKR3 Phosphorylation, but Not beta-Arrestin. Cell Rep, 2019. 26(6): p. 1473-1488 e9.
11. Matti, C., et al., ACKR4 Recruits GRK3 Prior to beta-Arrestins but Can Scavenge Chemokines in the Absence of beta-Arrestins. Front Immunol, 2020. 11: p. 720.
12. Vacchini, A., M. Locati, and E.M. Borroni, Overview and potential unifying themes of the atypical chemokine receptor family. J Leukoc Biol, 2016. 99(6): p. 883-92.
13. Sjoberg, E., et al., The diverse and complex roles of atypical chemokine receptors in cancer: From molecular biology to clinical relevance and therapy. Adv Cancer Res, 2020. 145: p. 99-138.
14. Groom, J.R. and A.D. Luster, CXCR3 in T cell function. Exp Cell Res, 2011. 317(5): p. 620-31.
15. Reynders, N., et al., The Distinct Roles of CXCR3 Variants and Their Ligands in the Tumor Microenvironment. Cells, 2019. 8(6).
16. Lasagni, L., et al., An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. J Exp Med, 2003. 197(11): p. 1537-49.
17. Ehlert, J.E., et al., Identification and partial characterization of a variant of human CXCR3 generated by posttranscriptional exon skipping. J Immunol, 2004. 173(10): p. 6234-40.
18. Berchiche, Y.A. and T.P. Sakmar, CXC Chemokine Receptor 3 Alternative Splice Variants Selectively Activate Different Signaling Pathways. Mol Pharmacol, 2016. 90(4): p. 483-95.
19. Smith, J.S., et al., C-X-C Motif Chemokine Receptor 3 Splice Variants Differentially Activate Beta-Arrestins to Regulate Downstream Signaling Pathways. Mol Pharmacol, 2017. 92(2): p. 136-150.
20. Loetscher, M., et al., Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med, 1996. 184(3): p. 963-9.
21. Thompson, B.D., et al., Inhibition of G alpha i2 activation by G alpha i3 in CXCR3-mediated signaling. J Biol Chem, 2007. 282(13): p. 9547-9555.
22. Smit, M.J., et al., CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. Blood, 2003. 102(6): p. 1959-65.
23. Korniejewska, A., et al., Expression and agonist responsiveness of CXCR3 variants in human T lymphocytes. Immunology, 2011. 132(4): p. 503-15.
24. Romagnani, P., et al., Role for interactions between IP-10/Mig and CXCR3 in proliferative glomerulonephritis. J Am Soc Nephrol, 1999. 10(12): p. 2518-26.
25. Bonacchi, A., et al., Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. J Biol Chem, 2001. 276(13): p. 9945-54.
26. Campanella, G.S., R.A. Colvin, and A.D. Luster, CXCL10 can inhibit endothelial cell proliferation independently of CXCR3. PLoS One, 2010. 5(9): p. e12700.
27. Sommer, F., et al., Frontline Science: Antagonism between regular and atypical Cxcr3 receptors regulates macrophage migration during infection and injury in zebrafish. J Leukoc Biol, 2020. 107(2): p. 185-203.
28. Garcia-Lopez, M.A., et al., CXCR3 chemokine receptor distribution in normal and inflamed tissues: expression on activated lymphocytes, endothelial cells, and dendritic cells. Lab Invest, 2001. 81(3): p. 409-18.
29. 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, 2018. 175(9): p. 1419-1438.
30. Veldkamp, C.T., et al., Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. Sci Signal, 2008. 1(37): p. ra4.
31. Takekoshi, T., et al., A locked, dimeric CXCL12 variant effectively inhibits pulmonary metastasis of CXCR4-expressing melanoma cells due to enhanced serum stability. Mol Cancer Ther, 2012. 11(11): p. 2516-25.
32. Meyrath, M., et al., Proadrenomedullin N-Terminal 20 Peptides (PAMPs) Are Agonists of the Chemokine Scavenger Receptor ACKR3/CXCR7. ACS Pharmacol Transl Sci, 2021. 4(2): p. 813-823.
33. Chevigne, A., et al., CXCL10 Is an Agonist of the CC Family Chemokine Scavenger Receptor ACKR2/D6. Cancers (Basel), 2021. 13(5).
34. Wouters, E., et al., Assessment of Biased Agonism among Distinct Synthetic Cannabinoid Receptor Agonist Scaffolds. ACS Pharmacol Transl Sci, 2020. 3(2): p. 285-295.
35. Meyrath, M., et al., The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides. Nat Commun, 2020. 11(1): p. 3033.
36. Dixon, A.S., et al., NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS Chem Biol, 2016. 11(2): p. 400-8.
37. Machleidt, T., et al., NanoBRET--A Novel BRET Platform for the Analysis of Protein-Protein Interactions. ACS Chem Biol, 2015. 10(8): p. 1797-804.
38. Schihada, H., R. Shekhani, and G. Schulte, Quantitative assessment of constitutive G protein-coupled receptor activity with BRET-based G protein biosensors. Sci Signal, 2021. 14(699): p. eabf1653.
39. Gilissen, J., et al., Forskolin-free cAMP assay for Gi-coupled receptors. Biochem Pharmacol, 2015. 98(3): p. 381-91.
40. Nibbs, R.J. and G.J. Graham, Immune regulation by atypical chemokine receptors. Nat Rev Immunol, 2013. 13(11): p. 815-29.
41. Naumann, U., et al., CXCR7 functions as a scavenger for CXCL12 and CXCL11. PLoS One, 2010. 5(2): p. e9175.
42. Canals, M., et al., Ubiquitination of CXCR7 controls receptor trafficking. PLoS One, 2012. 7(3): p. e34192.
43. Szpakowska, M., et al., Function, diversity and therapeutic potential of the N-terminal domain of

human chemokine receptors. Biochem Pharmacol, 2012. 84(10): p. 1366-80.
44. !!! INVALID CITATION !!! [16].
45. Szpakowska, M. and A. Chevigne, vCCL2/vMIP-II, the viral master KEYmokine. J Leukoc Biol, 2016. 99(6): p. 893-900.
46. Ulvmar, M.H., E. Hub, and A. Rot, Atypical chemokine receptors. Exp Cell Res, 2011. 317(5): p. 556-68.
47. Gupta, S.K. and K. Pillarisetti, Cutting edge: CXCR4-Lo: molecular cloning and functional expression of a novel human CXCR4 splice variant. J Immunol, 1999. 163(5): p. 2368-72.
48. Duquenne, C., et al., The two human CXCR4 isoforms display different HIV receptor activities: consequences for the emergence of X4 strains. J Immunol, 2014. 193(8): p. 4188-94.
49. Yu, C.R., et al., CCR9A and CCR9B: two receptors for the chemokine CCL25/TECK/Ck beta-15 that differ in their sensitivities to ligand. J Immunol, 2000. 164(3): p. 1293-305.
50. Garin, A., et al., Two novel fully functional isoforms of CX3CR1 are potent HIV coreceptors. J Immunol, 2003. 171(10): p. 5305-12.
51. Nguyen, H.T., et al., CXCR7: a beta-arrestin-biased receptor that potentiates cell migration and recruits beta-arrestin2 exclusively through Gbetagamma subunits and GRK2. Cell Biosci, 2020. 10(1): p. 134.
52. Rajagopal, S., et al., Biased agonism as a mechanism for differential signaling by chemokine receptors. J Biol Chem, 2013. 288(49): p. 35039-48.
53. Colvin, R.A., et al., Intracellular domains of CXCR3 that mediate CXCL9, CXCL10, and CXCL11 function. J Biol Chem, 2004. 279(29): p. 30219-27.

Chapter 3

Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/ CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists

Martyna Szpakowska, Max Meyrath, **Nathan Reynders**, Manuel Counson, Julien Hanson, Jan Steyaert, Andy Chevigné

Contribution:

Generation of the CXCR3 mutants (C37S-C38S), performing β-arrestin recruitment and flow cytometeric assays, interpretation of the results, and contribution to the writing of the manuscript.

1. Abstract

The atypical chemokine receptor ACKR3/CXCR7 plays crucial roles in numerous physiological processes but also in viral infection and cancer. ACKR3 shows strong propensity for activation and, unlike classical chemokine receptors, can respond to chemokines from both the CXC and CC families as well as to the endogenous peptides BAM22 and adrenomedullin. Moreover, despite belonging to the G protein coupled receptor family, its function appears to be mainly dependent on β-arrestin. ACKR3 has also been shown to continuously cycle between the plasma membrane and the endosomal compartments, suggesting a possible role as a scavenging receptor. So far, the molecular basis accounting for these atypical binding and signalling properties remains elusive. Noteworthy, ACKR3 extracellular domains bear three disulphide bridges. Two of them lie on top of the two main binding subpockets and are conserved among chemokine receptors, and one, specific to ACKR3, forms an intra-N terminus four-residue-loop of so far unknown function. Here, by mutational and functional studies, we examined the impact of the different disulphide bridges for ACKR3 folding, ligand binding and activation. We showed that, in contrast to most classical chemokine receptors, none of the extracellular disulphide bridges was essential for ACKR3 function. However, the disruption of the unique ACKR3 N-terminal loop drastically reduced the binding of CC chemokines whereas it only had a mild impact on CXC chemokine binding. Mutagenesis also uncovered that chemokine and endogenous non-chemokine ligands interact and activate ACKR3 according to distinct binding modes characterized by different transmembrane domain subpocket occupancy and N-terminal loop contribution, with BAM22 mimicking the binding mode of CC chemokine N terminus.

2. Introduction

Chemokine receptors are class A G protein-coupled receptors (GPCRs) present at the surface of various cell types. By interacting with their cognate chemokines, they regulate vital cellular mechanisms, including cell trafficking, development, immune-modulation and adhesion as well as growth and survival [1]. They are also involved in pathological processes such as inflammation, cancer and HIV-1 infection [2,3].

ACKR3, the atypical chemokine receptor 3, formerly known as CXCR7, is one of the most recently deorphanized chemokine receptors [4]. It is expressed in various cells such as B and T lymphocytes, neurons and endothelial cells and plays a crucial role in many processes including cardiovascular and neuronal development as well as in migration and homing of hematopoietic stem/progenitor cells [5–11]. ACKR3 is also present in many cancer cell types and on tumour-associated vasculature and accumulating evidence demonstrates its involvement in metastasis development [12–15]. ACKR3 binds to two endogenous chemokines, CXCL12 and CXCL11, which are also the ligands for CXCR4 and CXCR3, respectively [10,16–19]. In addition, ACKR3 and CXCR4 can interact with vCCL2,

the human herpesvirus 8 (HHV-8)-encoded chemokine, which is an antagonist for a broad spectrum of chemokine receptors, including CXCR4, but acts as agonist towards ACKR3 [19–22]. Unlike CXCR4 and CXCR3, which signal via G protein pathways, ACKR3 activity relies mainly on β-arrestin recruitment, although its ability to trigger signalling may be cell context-dependent [22–25]. In addition ACKR3 is proposed to act as a scavenger or "sink" receptor for CXCL12, CXCL11 and vCCL2, regulating their availability for other chemokine receptors [19,26–29]. Recently, it has also been shown that ACKR3 is a high-affinity receptor for BAM22, a non-chemokine peptide derived from the proenkephalin A family, which plays a role in the modulation of circadian glucocorticoid oscillation [30]. Similarly, based on phenotypic analysis of knock-out mice, ACKR3 was also proposed as a scavenger receptor for adrenomedullin (ADM), a pro-angiogenic peptide involved in the regulation of vascularisation [31].

Over the last few years, the tridimensional structures of several chemokine receptors, including CXCR4, have been resolved in complexes with small molecules or chemokines [32–38]. These structures revealed the typical receptor architecture comprising a flexible extracellular N terminus followed by a bundle of seven hydrophobic membrane-spanning alpha-helices (TM1-TM7) connected by three extracellular (ECL1-3) and three intracellular (ICL1-3) hydrophilic loops. In addition to the conserved disulphide bond linking the top of TM3 (end of ECL1) to the middle of ECL2, present in a large majority of class A GPCRs, all currently available tridimensional structures of chemokine receptors reveal a second disulphide bridge between the N terminus and the top of TM7 (end of ECL3) [39,40]. As a consequence, the last residues of the receptor N terminus form an additional extracellular loop ("ECL4") connecting TM1 and TM7 and closing the receptor into a ring [39,40] (Fig. 1A and B). These structures together with recent mutational and functional studies were also key to refining our current understanding of chemokine-receptor interactions, moving from a simple two-step mechanism to a more continuous one characterized by extensive contacts between the two partners and 1:1 stoichiometry [33,41–44]. These interactions involve the core of the chemokine, including the N-loop region, with the flexible N terminus of the receptor (chemokine recognition site 1, CRS1), a key determinant for selectivity [41,43,45] ensuring optimal chemokine orientation with respect to the top of the ligand-binding pocket and ECL4 (CRS1.5) [33,39]. This enables the insertion of the flexible chemokine N terminus into the receptor transmembrane cavity (CRS2), made of the transmembrane segments and the extracellular loops, stabilizing an active state of the receptor and leading to intracellular signalling [33,43,46–48]. These tridimensional structures also showed that the orientation relative to the receptor varies for the different classes of chemokines and confirmed the presence of two distinguishable binding pockets – the major ligand binding pocket formed by the TM3, 4, 5, 6 proposed to be mainly occupied by the N terminus of CXC chemokines, and the minor pocket delineated by TM1, 2, 3, 7 suggested to accommodate the N terminus of CC chemokines (Fig. 1D) [33,34,48–50].

We have recently shown that ligand binding and activation of ACKR3 is in some measure different compared with CXCR4 and CXCR3 [51]. Indeed, ACKR3 has a strong propensity to activation, possibly linked to its scavenging role, and as opposed to CXCR4 and CXCR3, the most N-terminal residues of the chemokine ligands as well as the N-loop have a minor importance for ACKR3 binding and activation. Moreover, vCCL2 seems to stabilize a different conformation of ACKR3 than do its endogenous CXC chemokine ligands, whereas very little is known on the binding mode of BAM22 and adrenomedullin to ACKR3 [19,30] .

An intriguing particularity of ACKR3, which may contribute to its atypical nature, is the presence of two additional cysteine residues at positions 21 and 26, which have recently been shown to be linked by a disulphide bridge, giving rise to an intra-N terminus four-residue loop (Fig. 1) [32,39,52]. With the exception of CXCR3 that bears two additional successive cysteines at positions 37 and 38, other chemokine receptors do not have analogous cysteine residues. Given the crucial role of the flexible receptor N terminus in chemokine recognition, the intra-N terminus loop in ACKR3 could account for the existence

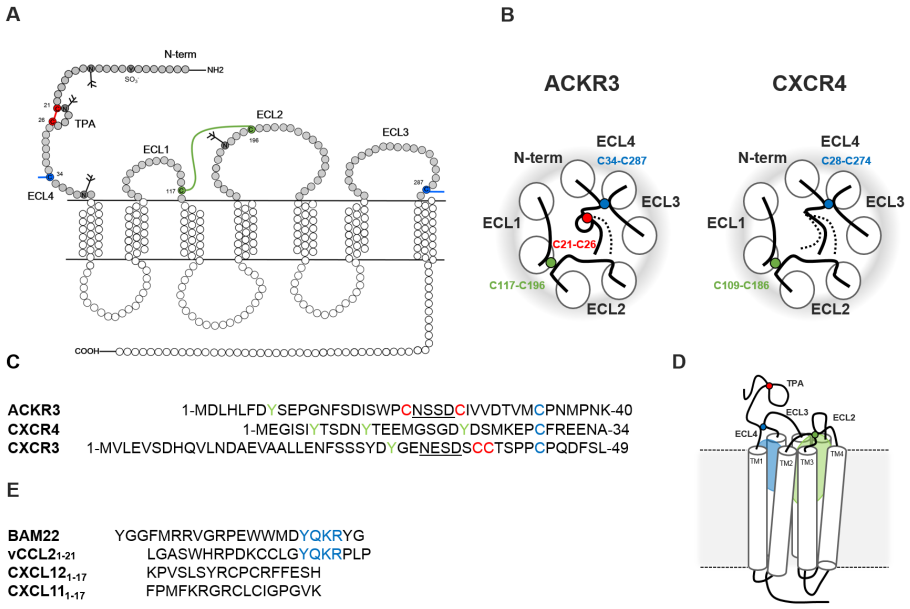


Figure 1 | Localisation and pairing of the extracellular disulphide bridges of ACKR3 and CXCR4. (A) Snake diagram of ACKR3. The putative post-translational modification sites identified in the N terminus and the extracellular loops are shown. Three potential disulphide bridges: within the N terminus (C21-C26), between the top of TM7 and the distal part of the N terminus (C34-C287) and between the top of TM3 and ECL2 (C117-C196) are coloured in red, blue and green, respectively. Putative N-glycosylation sites (Ψ) and a sulfotyrosine (SO3−) are indicated in dark grey. (B) Representations in top-down view of the extracellular disulphides bridges in ACKR3 and CXCR4. The seven transmembrane segments are represented as white circles. The disulphide bridges connecting TM3 to ECL2 (highly conserved in class A GPCRs), the N terminus to TM7 (specific to chemokine receptors), or forming a unique ring within the ACKR3 N terminus are coloured in red, blue and green, respectively. (C) Sequence comparison between the extracellular domains of ACKR3, CXCR4 and CXCR3. The sequences were aligned with respect to the cysteine residues predicted to be involved in the disulphide bridge with the top of TM7 (ECL4). Underlined sequences highlight partial conservation of the ACKR3 TPA-decorating residues within the N terminus of CXCR3. (D) ACKR3 architecture showing the position of the different disulphide bridges on top of the major (green) and minor (blue) ligand binding pockets. (E) Sequence comparison between BAM22 and the N termini of chemokines. The sequences of BAM22 and vCCL21-17 were aligned with respect to the shared YQKR motif (green) at their C terminus. The sequences of chemokines N terminus were aligned with respect to their first cysteine residue (blue).

of either a unique receptor structure or an unusual mode of interaction with its ligands [39,41].

Here, by targeted mutagenesis, we examined the role of the extracellular disulphide bridges in chemokine receptors CXCR4 and ACKR3. Using various binding and functional assays, we investigated the role of the unique ACKR3 intra-N terminus disulphide bridge for receptor-ligand interactions and compared the importance of the ECL4-forming disulphide bridge as well as the one linking TM3 and ECL2 for chemokine binding, receptor integrity and activation. We demonstrated that, in contrast to classical chemokine receptors, the other conserved extracellular disulphide bridges are not essential for ACKR3 surface expression, ligand binding and activation. In addition, we showed that the unique N terminal loop of ACKR3 is important for activation by CC but not CXC chemokines and that BAM22 mimics the binding mode of CC chemokine N terminus.

3. Materials and methods

3.1. Peptides and chemokines

Chemokines CXCL12, CXCL11, vCCL2, CXCL10 and CXCL9 were purchased from PeproTech. Alexa Fluor 647-labelled CXCL12 (CXCL12-AF647) was purchased from Almac. BAM22 and adrenomedullin (ADM) were purchased from Bachem. Peptides derived from the N terminus of chemokines and peptide TPA (21-CNSSDC-26, with C21-C26 cycliza- tion) were purchased from JPT. Peptides CCS (encompassing residues 1-MDLHLFDYSEPGNFSDISWPCNSSDCIVVDTVMSPNMPNKS-40, with a disulphide bridge between cysteines 21 and 26 and a cysteine-to-serine mutation at position 34), SSS (encompassing residues 1-MDLHLFDYSEPGNFSDISWPSNSS-DSIVVDTVMSPNMPNKS-40, in which the three cysteine residues were mutated to serines) and SSS_{scrbld} (1-PPYDVISSMLKDSISNENFVSL-PNGPSDTVHMSWDNFMDS-40, in which all residues were randomly permuted) were purchased from ChinaPeptides. All peptides contain a free amine at the N terminus and an amide group at the C terminus to avoid additional negative charge.

3.2. Generation of U87 cell lines expressing wild-type and mutant ACKR3 and CXCR4

pBABE.puromycin vectors (Addgene) encoding wild type ACKR3, variants bearing double cysteine-to-serine mutations (C21S-C26S, C34S-C287S and C117S-C186S) or glycine-substituted variant (C21-G_4-C26), and wild-type CXCR4 or C28S-C274S or C109S-C186S mutants were transfected into U87 cells [51]. This cellular background was chosen for its absence of endogenous CXCR4, ACKR3 and CXCR3 as previously demonstrated [19]. Cells stably expressing the modified receptors were obtained following puromycin selection and subsequent single-cell sorting using BD FACSAria II cell sorter (BD Biosciences). The presence of the mutations and the surface expression level of the

mutated receptors were verified by genomic DNA sequencing and flow cytometry using antibodies recognising the proximal N terminal part of CXCR4 (clone 4G10, Santa Cruz Biotechnology) or ACKR3 (clones 11G8, R&D Systems and 9C4, MBL Life Science). CXCR3 in transiently transfected U87 cells was detected using the mAb 1C6 (BD Pharmingen).

3.3. Chemokine and peptide binding

U87 cells expressing wild-type or mutant ACKR3 or CXCR4 were distributed into 96-well plates (15×10^4 cells per well) and incubated with Alexa Fluor 647-labelled CXCL12 at concentrations ranging from 35 pM to 115 nM for 90 min on ice or for 45 min at 37 °C, respectively. Non-specific binding of CXCL12-AF647 was evaluated on ACKR3- and CXCR4-negative U87 cells and subtracted. For binding competition studies, U87 cells expressing wild-type or mutant ACKR3 or CXCR4 were incubated with CXCL12-AF647 at concentrations equivalent to twice the EC_{50} values determined for each ACKR3 variant in saturation binding experiments (Table 1) or 11.5 nM for CXCR4, mixed with un-labelled CXCL12, CXCL11, vCCL2, CXCL10, BAM22 or ADM at concentrations ranging from 6 pM to 1 µM. All binding experiments were performed in PBS containing 1% BSA and 0.1% NaN_3 (FACS buffer). Nonspecific chemokine binding was evaluated by the addition of 250-fold excess of unlabeled CXCL12 and the signal obtained was used to define 0% specific binding. The signal obtained for CXCL12-AF647 in the absence of unlabeled chemokines was used to define 100% binding. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

3.4. β-arrestin recruitment

β-arrestin-2 recruitment to wild-type and mutant ACKR3, CXCR4 or CXCR3 induced by chemokines, chemokine N terminus-derived peptides or BAM22 was monitored by NanoLuc complementation assay (NanoBit, Promega) [51,53,54]. 1.2×10^6 U87 cells were plated in 10cm-culture dishes and 48h later transfected with pNBe vectors containing human β-arrestin-2N-terminally fused to LgBiT and receptors C-terminally fused to SmBiT. 48 h post-transfection cells were harvested, incubated 40 min at 37 °C with 200-fold diluted Nano-Glo Live Cell substrate and distributed into white 96-well plates (5×10^4 cells per well). β-arrestin-2 recruitment in response to chemokines, N-terminus derived peptides or BAM22 was evaluated after 10-minute incubation with a Mithras LB940 luminometer (Berthold Technologies). For each receptor and each experiments the maximum signal recorded with a saturating concentration (200 nM) of full agonist (i.e CXCL12 for ACXCR3 and CXCR4) was set as 100%.

3.5. Neutralisation of mAb 9C4 by ACKR3 N-terminal peptides

The mAb 9C4 (3.3 µg/ml) was first incubated for 30 min at room temperature with peptides CCS, SSS (100 nM) or TPA (100 µM) derived from the N terminus of ACKR3.

Peptide SSS_{scrbld} (100 nM) was used as negative control. The antibody-peptide mix was then incubated for 60 min at 4 °C with U87 cells expressing wild-type ACKR3 (15×10^4 cells/well in a 96-well plate). The binding of 9C4 to ACKR3 was revealed with an allophycocyanin-conjugated goat anti-mouse IgG $F(ab')_2$ (Jackson ImmunoResearch) and quantified by mean fluores- cence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

3.6. Immunocytochemistry and fluorescent imaging

For microscopic analysis of CXCR4 and ACKR3 distribution, 5×10^4 U87 cells were plated on sterile coverslips in a 24-well plate and cultured overnight. Cells were transiently transfected with equal amounts of pBABE plasmid encoding wild type or mutated CXCR4 or ACKR3 using X-tremeGENE 9. 48 h later, cells were washed with PBS and fixed for 20 min on ice with 4% (w/v) paraformaldehyde. After one washing step with PBS supplemented with 50 mM NH_4Cl and two washes with PBS, cells were permeabilised with 0.1% Triton X-100 for 20 min at room temperature. Subsequently, cells were blocked with 10% (w/v) normal goat serum for 1 h and incubated overnight at 4 °C with the mAb 4G10 (Santa Cruz Biotechnology) diluted 1:75 for CXCR4 or the mAb 11G8 (R&D Systems) diluted 1:100 for ACKR3 staining in 1% BSA PBS, respectively. After three washing steps, cells were incubated for 1 h at room temperature with a goat anti-mouse Alexa Fluor 647-conjugated secondary antibody (abcam) diluted 1:1200 in PBS complemented with 5% normal goat serum. Cells were washed twice, stained with Hoechst 33,342 dye (Sigma) for 10 min at 4 °C, washed twice and mounted on glass slides with 5µl ProLong Diamond anti-fade mounting medium (Molecular Probes). Images were acquired using a 63 × oil-immersion objective on a Zeiss Axio Observer Z1 microscope equipped with an Apotome.2 and a Colibri LED illumination system. Representative images of two independent experiments are shown.

3.7. Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 7.02) to provide pEC_{50}, pIC_{50}, EC_{50} or IC_{50} values, standard errors of the mean (SEM) and the Hill coefficient. Fitting was performed on data from at least three independent experiments (n = 3). Unpaired t tests were used to analyse the differences in pEC_{50}/pIC_{50} for each ligand using the wild-type receptor as reference. p value of < 0.05 was considered as statistically significant.

4. Results

4.1. Extracellular disulphide bridges are dispensable for ACKR3 surface expression and folding

4.1.1. The conserved disulphide bridges

First, the importance for ACKR3 conformational fidelity of the disulphide bridge between the N terminus and the top of TM7 (C34-C287) as well as the one between TM3 and ECL2 (C117-C196) was evaluated and compared to CXCR4 (Fig. 1A and B). The impact of cysteine pair mutations on the receptor cellular distribution was monitored by flow cytometry and fluorescence microscopy using antibodies detecting linear epitopes located in the receptor N terminus (11G8 for ACKR3 and 4G10 for CXCR4) or recognising more complex epitope spread over different extracellular domains (8F11-M16 for ACKR3 and 12G5 for CXCR4).

In line with previous reports, a higher proportion of the wild-type ACKR3 was present intracellularly compared to the cell surface, while CXCR4 was localised at the plasma membrane (Fig. 2E). Mutation of the cysteine pair engaged in the disulphide bridge between the N terminus and top of TM7 (the ECL4-forming bridge), typical of chemokine receptors, had little effect on ACKR3 (C34S-C287S) and CXCR4 (C28S- C274S) surface expression but strongly impacted CXCR4 architecture, as shown by the inability of the conformational mAb 12G5 to recognise the receptor (Fig.2A and B). In contrast, recognition of the ACKR3.C34S-C287S variant by the conformational mAb 8F11-M16 was equivalent to that of wild-type receptor. The disruption of the TM3-ECL2 bridge (C117S-C196S) only moderately affected the ACKR3 distribution (Fig. 2E), in stark contrast to CXCR4, for which the equivalent mutation (C109S-C186S) abolished the receptor surface expression (Fig. 2B) and resulted in a strong intracellular retention (Fig. 2E). Nonetheless, a 75% reduction of ACKR3 surface expression was observed with the conformational mAb 8F11-M16 compared to the wild-type receptor (Fig. 2A), indicating that the TM3-ECL2 bridge does play a role in ACKR3 structural integrity, although it is not critical for receptor export.

Overall, these results show that the receptor surface expression and architecture in the absence of disulphide bridges was much less affected for ACKR3 than for CXCR4.

4.1.2. The atypical disulphide bridge of ACKR3

Next, the potential role played by the unique four-residue intra-N terminal loop of ACKR3 formed by the disulphide bridge between residues C21 and C26 (TPA for TetraPeptidyl Arch) in receptor expression, localisation and conformation was assessed. No difference in ACKR3 surface expression or folding was observed when each of the four residues within the loop (NSSD) was substituted with a glycine (C21-G4-C26) (Fig. 2A and E). The disruption of the loop-forming cysteine bridge (C21S-C26S) did however have an effect on the receptor cellular distribution, resulting in an apparent 40% increase in surface expression compared with wild-type ACKR3 (Fig. 2A and E). Similar tendency was observed for CXCR3 variant in which the two N-terminal cysteines were mutated to serines (C37S-C38S) (Fig. 2C).

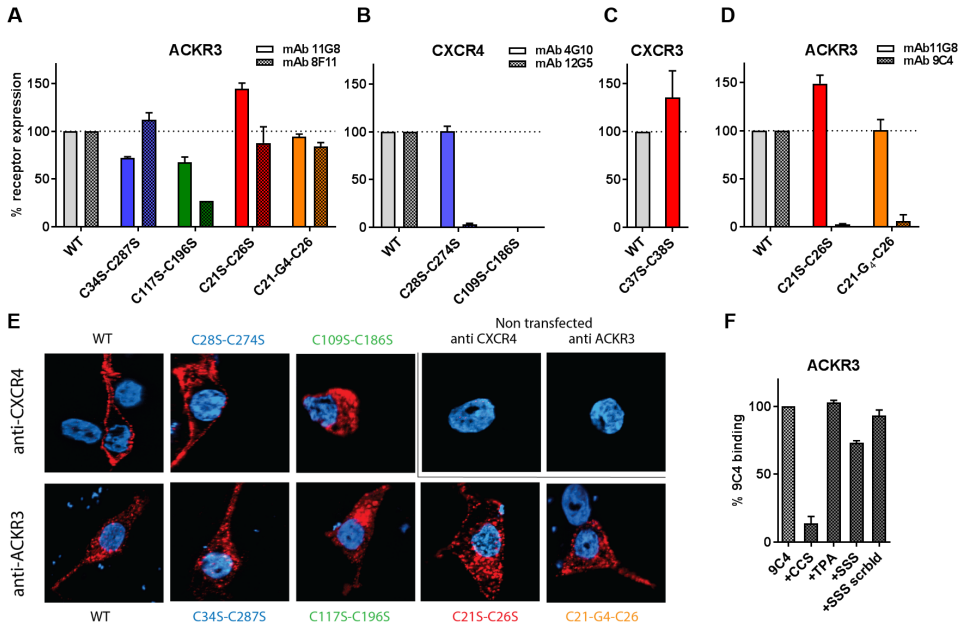


Figure 2 | Surface expression and folding of receptor cysteine variants and 9C4 recognition of ACKR3. (A and B) Receptor surface expression in U87 cells transiently transfected with pBABE vectors encoding wild-type and mutant ACKR3 (A) and CXCR4 (B) revealed with monoclonal antibodies recognising linear N terminus (11G8 and 4G10) or conformational (8F11-M16 and 12G5) epitopes and quantified by flow cytometry. (C) Receptor surface expression in U87 cells transiently transfected with a vector encoding wild-type and C37S-C38S variant of CXCR3 revealed with the mAb 1C6 and quantified by flow cytometry. Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments. (E) Cellular localisation of the wild-type and cysteine variants of CXCR4 (top) and ACKR3 (bottom) monitored by fluorescent microscopy using mAb 4G10 or 11G8 and a secondary Alexa Fluor 647-conjugated antibody (red). The nuclear DNA was stained with Hoechst (blue). A representative picture of at least 10 acquired images from two independent experiments is shown. (D and F) mAb 9C4 recognition of ACKR3. (D) Receptor surface expression in U87 cells transiently transfected with pBABE vectors encoding wild-type and TPA-mutated ACKR3 revealed with mAbs 11G8 and 9C4 recognising the receptor N terminus. (F) 9C4 neutralisation by peptides derived from the N terminus of ACKR3. 9C4 (3.3 µg/ml) binding in the presence of peptides CCS, SSS, SSSscrbld (100 nM) and TPA (100 uM) was monitored in U87.ACKR3 cells by flow cytometry. Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments. ** $p < 0.01$, **** $p < 0.0001$, NS: not significant.

Surprisingly, although the mutants C21S-C26S and C21-G4-C26 were detected at the cell surface using the mAb 11G8, they were not recognised by the mAb 9C4, also raised against the N terminal domain of ACKR3 (Fig. 2D) [55]. In line with this observation, 9C4 binding to the wild-type ACKR3 was reduced by 86% in the presence of the cyclic peptide CCS, derived from the N terminus of ACKR3 and bearing the TPA, whereas the equivalent linear SSS peptide caused only a 25% reduction (Fig. 2F). Peptide SSS_{scbld} had no effect on 9C4 binding to the wild-type receptor. These results show that the four-residue intra-N terminal loop of ACKR3 (TPA) is a crucial contributor of the 9C4 binding, although it is not the sole determinant of its epitope as demonstrated by the inability of the shorter cyclic peptide comprising only the TPA (C21-NSSD-C26) to neutralise the antibody binding to the wild-type receptor, even at a concentration as high as 100 µM. In addition, these data reveal that the TPA is well exposed and accessible to the extracellular ligands and makes mAb 9C4 a useful probe to evaluate its presence on the receptor in different cellular contexts.

4.2. CXC and CC chemokines have different binding and activation modes on ACKR3

The binding of Alexa Fluor 647-labelled CXCL12 to cysteine-mutated ACKR3 and CXCR4 was first evaluated and compared in U87 cell lines stably expressing wild-type or mutated receptor.

In contrast to CXCR4, all cysteine mutants of ACKR3 retained their ability to bind CXCL12-AF647, although the maximal binding and potency were affected to different extents. The mutation of the TM3-ECL2 cysteine bridge (C117S-C196S) had the most pronounced effect, resulting in a marked reduction of potency because of which no EC_{50} nor maximum binding could be determined (Fig. 3B). The mutation of the ECL4-forming cysteine bridge linking the N terminus and TM7 of ACKR3 had less effect, but still led to a 60%-decrease of the maximum CXCL12-AF647 binding and a significant 4-fold increase in EC_{50} as compared to the wild-type receptor (Fig. 3B, Table 1). Interestingly, the analogous mutant of CXCR4 (C28S-C274S) was no longer capable of binding CXCL12 (Fig. 3A and Table 1), further confirming the stronger tolerance of ACKR3 to alterations in TM-linking disulphide bridges. The C21-G_4-C26 mutant showed an EC_{50} value equivalent to that of wild-type ACKR3, while the C21S-C26S mutation led to an approximately two-fold increase in EC_{50} (Fig.3B, Table 1) indicating that it is the constraint brought by the intra-N terminus loop rather than its dec- orating residues that plays a role in CXCL12 binding.

The effect of cysteine mutations on the ACKR3 interactions with its other chemokine ligands was then evaluated in binding competition studies with CXCL12-AF647. CXCL11 binding to mutant ACKR3.C34S-C287S was reduced by approximately two-fold compared to the wild-type receptor, while vCCL2 binding was improved by nearly 4-fold (Fig. 3C and D, Table 1). Both CXCL11 and vCCL2 were less potent in displacing CXCL12-AF647 from ACKR3.C21S-C26S where the binding of the viral chemokine was the most impacted (2.4- and 14.3-fold increase in IC50 values respectively). Binding of CXCL11 and vCCL2 to the mutant C21-G4-C26 was affected to a similar extent (3-fold increase in IC50 values), despite the absence of effect of the mutation on CXCL12-AF647 binding. These results suggest that the presence of the TPA has an impact on the binding of all chemokines with a significantly higher effect on vCCL2 binding while the residues of the TPA themselves appear to be involved in interactions with vCCL2 and CXCL11 but not CXCL12.

Table 1. Binding properties of full-length chemokines and BAM22 towards ACKR3.

ACKR3	Binding												
	CXCL12-AF647				CXCL11			vCCL2			BAM22		
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)*	pIC_{50} ± SEM	IC_{50} (nM)	Fold WT	pIC_{50} ± SEM	IC_{50} (nM)	Fold WT	pIC_{50} ± SEM	IC_{50} (nM)	Fold WT
WT	8.60 ± 0.04	2.5	1.0	100	8.79 ± 0.07	1.6	1.0	7.36 ± 0.05	43.4	1.0	7.49 ± 0.03	32.2	1.0
C34S-C287S	7.99 ± 0.18	10.2	4.1	40 ± 7	8.58 ± 0.07	2.7	1.7	7.95 ± 0.08	11.2	0.3	7.77 ± 0.11	17.1	0.5
C117S-C196S	< 6.3	>500	> 200	ND	ND	ND	-	ND	ND	-	ND	ND	-
C21S-C26S	8.25 ± 0.02	5.6	2.2	96 ± 1	8.41 ± 0.06	3.9	2.4	6.21 ± 0.15	622.5	14.3	7.40 ± 0.06	39.4	1.2
C21-G_4-C26	8.61 ± 0.06	2.5	1.0	106 ± 4	8.32 ± 0.05	4.8	3.0	6.90 ± 0.03	124.6	2.9	7.46 ± 0.05	34.4	1.1

CXCL12-AF647 binding to ACKR3 mutants was normalised to receptor cell surface expression based on staining with mAb 8F11-M16 considering WT receptor as 100%.

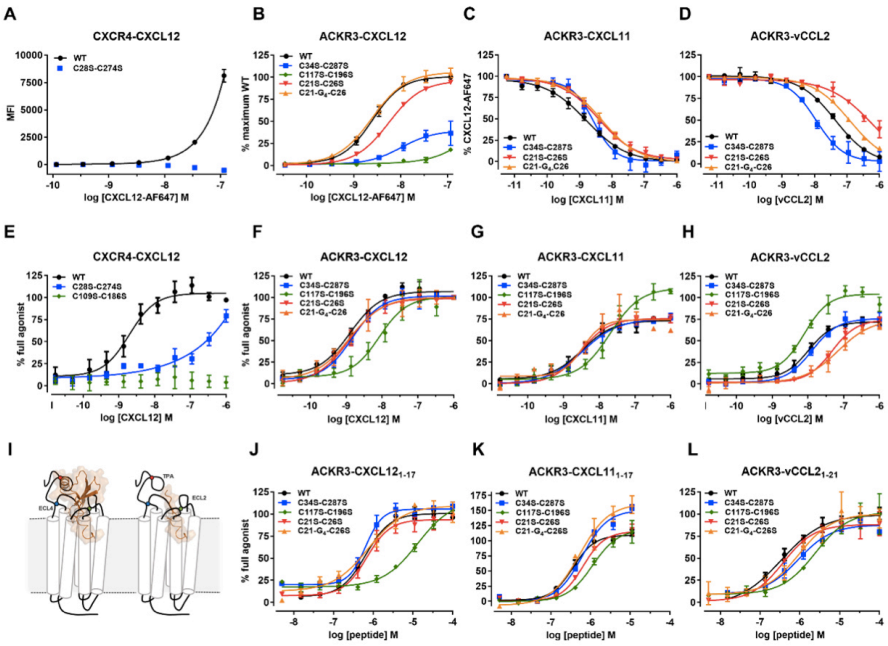


Figure 3 | Binding and activation of cysteine-mutated receptors by chemokine ligands. (A-B) Binding of Alexa Fluor 647-labelled CXCL12 to wild-type and cysteine mutants of CXCR4 (A) and ACKR3 (B) monitored by flow cytometry. CXCL12-AF647 binding to ACKR3 was normalised to receptor cell surface expression based on staining with mAb 8F11-M16. (C-D) Binding of CXCL11 (C) and vCCL2 (D) to wild-type and mutated ACKR3 assessed by binding competition with CXCL12-AF647 and analysed by flow cytometry. Binding of CXCL12-AF647 in the absence of unlabelled chemokines was considered as 100%, whereas binding in the presence of a 250-fold excess of unlabelled CXCL12 was used to define 0%. (I) Schematic representation of the interaction between ACKR3 and a full-length chemokine (left) or a peptide derived from its N terminus (right). (E-L) β-arrestin-2 recruitment to CXCR4 (E) and ACKR3 (F-L) variants induced by CXCL12 (E and F), CXCL11 (G) vCCL2 (H), CXCL121-17 (J), CXCL111-17 (K) and vCCL21-21 (L). Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments.

The effect of disulphide bridge disruption on ACKR3 activation was then monitored in a β-arrestin-2 recruitment assay. Overall two clearly contrasting trends could be observed in how disrupting the disulphide bridges affected the ability of chemokines to activate ACKR3, one — for the two CXC chemokines, CXCL12 and CXCL11, second — for the CC chemokine vCCL2. Firstly, β-arrestin-2 recruitment induced by CXCL12 and CXCL11 was only negatively impacted by the mutation disrupting the TM3-ECL2 disulphide bridge located on top of the major ligand- binding pocket (6.5- and 6.5-fold increase in EC_{50} values, respectively) (Fig. 3F and G, Table 2). This mutation however had no effect on vCCL2 potency to induce β-arrestin-2 recruitment (Fig. 3H, Table 2). Interestingly, whereas the TM3-ECL2 mutation resulted in a non-functional CXCR4, in case of ACKR3 it allowed CXCL11 and vCCL2 to achieve efficacies equivalent to CXCL12, as opposed to their partial response (75%) observed with the wild-type receptor and all the other cysteine mutants. The mutation of ECL4 (C34S-C287S) did not affect β-arrestin- 2 recruitment to ACKR3 for any of the ligands (Fig. 3F–H), but had a strongly negative effect on CXCL12-induced recruitment to the equivalent mutant (C28S-C274S) of CXCR4 (Fig. 3E). β-arrestin-2 recruitment to the TPA-modified ACKR3 variants in response to CXCL12 and CXCL11 was comparable to the wild-type receptor, whereas it was considerably impaired in response to vCCL2 (4.6- and 6.6-fold reduction for the C21S-C26S and C21-G4-C26 mutants, respectively), showing the importance of the TPA in the vCCL2-induced ACKR3 activation.

Table 2. β-arrestin-2 recruitment induced by full-length chemokines and BAM22 to ACKR3.

ACKR3	β-arrestin-2 recruitment															
	CXCL12				CXCL11				vCCL2				BAM22			
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)
WT	8.89 ± 0.07	1.27	1.0	100	8.48 ± 0.11	3.35	1.0	73 ± 4	7.99 ± 0.08	10.35	1.0	72 ± 3	7.70 ± 0.10	20	1.0	93 ± 5
C34S-C287S	8.83 ± 0.07	1.44	1.1	100	8.47 ± 0.10	3.41	1.0	75 ± 3	7.88 ± 0.07	13.08	1.3	76 ± 3	7.48 ± 0.10	33	1.7	95 ± 6
C117S-C196S	8.07 ± 0.13	8.45	6.5	100	7.70 ± 0.11	20.01	6.0	111 ± 7	8.10 ± 0.08	7.96	0.8	104 ± 4	>6.40	>400	>20	ND
C21S-C26S	8.93 ± 0.09	1.17	0.9	100	8.51 ± 0.05	3.07	0.9	75 ± 2	7.33 ± 0.05	47.31	4.6	75 ± 3	7.31 ± 0.09	49	2.5	83 ± 6
C21-G_R-C26	8.90 ± 0.16	1.25	1.0	100	8.47 ± 0.15	3.42	1.0	74 ± 5	7.16 ± 0.19	68.79	6.6	75 ± 7	7.24 ± 0.11	57	2.9	89 ± 7

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n =3).

By comparison, mutagenesis of cysteine residues at position 37 and 38 of the CXCR3 N terminus had no significant impact on the receptor activation by CXCL11, CXCL10 but decreased the potency of CXCL9 by more than 2 fold (Table 4).

Overall, these data reveal that whereas all cysteine-mutated ACKR3 variants were able to bind and efficiently respond to chemokine ligands, the disruption of the disulphide bridges in CXCR4, without exception, led to severe impairment of the receptor functionality. Furthermore, the differences in the impact of cysteine pair mutations for the CXC chemokines on one side and vCCL2 on the other clearly point to distinct binding and activation modes for ACKR3, either largely relying on the TM3-ECL2 disulphide bridge or on the N-terminal TPA (Fig. 5).

To gain further insight into the determinants involved in the recognition and activation of ACKR3, peptides derived from the N-terminal region of CXCL12, CXCL11 and vCCL2 were tested for their ability to induce β-arrestin-2 recruitment to the wild-type and mutated receptors. Similarly to what has previously been shown for the wild-type ACKR3 [51], CXCL12-, CXCL11- and vCCL2-derived peptides covering the flexible N terminus, the cysteine motif and the N loop ($CXCL12_{1-17}$, $CXCL11_{1-17}$ and $vCCL2_{1-21}$) were able to trigger β-arrestin-2 recruitment to the cysteine-mutated receptors, although differences were observed between the peptides. The mutation of the cysteine bridge between TM3 and ECL2, covering the major binding pocket, strongly reduced the activity of $CXCL12_{1-17}$ and $vCCL2_{1-21}$ (EC_{50} increase by>20 and 6.5 fold, respectively) and to a lesser extent that of $CXCL11_{1-17}$ (3 fold), suggesting that small peptides directly targeting the transmembrane pocket require this structure for efficient receptor activation. In contrast, the disruption of the disulphide bridge between the N terminus and TM7 stabilising the minor binding pocket had no effect on $CXCL12_{1-17}$ activity but slightly decreased that of $CXCL11_{1-17}$ and $vCCL2_{1-21}$ (1.7 and 2.3 fold respectively) (Fig. 3, Table 3).

Finally, the mutation of cysteine residues forming the TPA (C21S- C26S) had no effect on the activity of $CXCL12_{1-17}$ and slightly impacted that of $CXCL11_{1-17}$ (1.8 fold). Surprisingly however, this mutation had little impact on $vCCL2_{1-21}$, in contrast to what was observed for full- length vCCL2, indicating that the core of the viral chemokine is the main contributor in the interactions with TPA.

4.3. The endogenous peptide BAM22 reveals a vCCL2-like binding and activation mode towards ACKR3

Table 3. β-arrestin-2 recruitment induced by peptides derived from chemokine N-terminal regions to ACKR3.

ACKR3	β-arrestin-2 recruitment											
	$CXCL12_{1-17}$				$CXCL11_{1-17}$				$vCCL2_{1-21}$			
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)
WT	6.22 ± 0.04	610	1.0	101 ± 2	6.45 ± 0.04	360	1.0	110 ± 3	6.45 ± 0.09	360	1.0	99 ± 4
C34S-C287S	6.23 ± 0.7	600	1.0	106 ± 3	6.21 ± 0.05	620	1.7	151 ± 6	6.10 ± 0.09	790	2.2	89 ± 4
C117S-C196	4.85 ± 0.18	14270	23.4	104 ± 4	5.95 ± 0.09	1120	3.1	113 ± 10	5.63 ± 0.16	2340	6.5	104 ± 9
C21S-C26S	6.18 ± 0.09	660	1.1	94 ± 4	6.19 ± 0.04	650	1.8	115 ± 4	6.43 ± 0.10	370	1.0	88 ± 4
C21-G_4-C26	6.20 ± 0.12	630	1.0	106 ± 10	6.27 ± 0.10	540	1.5	160 ± 12	6.04 ± 0.14	920	2.6	100 ± 7

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n =3).

Table 4. β-arrestin-2 recruitment induced by full-length chemokines to CXCR3.

CXCR3	β-arrestin-2 recruitment											
	CXCL11				CXCL10				CXCL9			
	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)	pEC_{50} ± SEM	EC_{50} (nM)	Fold WT	Max (%)
WT	8.03 ± 0.19	9.4	1.0	100	7.60 ± 1.17	25.7	1.0	45 ± 5	7.03 ± 0.2	92.9	1.0	46 ± 7
C37S-C38S	8.68 ± 0.10	8.7	0.9	100	7.41 ± 0.18	38.5	1.5	65 ± 3	6.60 ± 0.15	226.4	2.5	54 ± 4

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n =3).

Among the different non-chemokine ligands described to bind to ACKR3, only BAM22, a 22-amino acid peptide showing sequence similarities with chemokine N termini (Fig. 1E), was able to compete with the binding of labelled CXCL12 to wild-type ACKR3 (IC_{50} = 32.2 nM, pIC_{50} = 7.49 ± 0.03). No displacement was observed with adrenomedullin (ADM) in the concentration range tested (Fig. 4A, Table 1).

BAM22 was then tested towards the cysteine mutants to provide the first information about its binding and activation modes. Reminiscent of what was observed for vCCL2, the mutation C34S-C287S disrupting ECL4, reduced the IC_{50} of BAM22 binding by nearly two-fold compared to the wild-type receptor (Fig.4B). However, in contrast to vCCL2, mutations of the TPA had no effect on BAM22 binding to ACKR3. BAM22 was also able to induce β-arrestin-2 recruitment towards all ACKR3 mutants (Fig. 4C). The strongest impairment was observed with the C117-C196 mutant, just like for the CXC chemokines, all small chemokine N-terminal peptides but not for vCCL2. While the mutation in ECL4 had a modest effect on ACKR3 functionality, the two mutations affecting the TPA significantly impacted the potency of BAM22 to induce β-arrestin-2 recruitment (2.5 fold for C21S-C26S and 2.9 fold for C21-G4-C26, respectively), comparable to what was observed for vCCL2 and its N-terminal peptides. These data suggest that the endogenous peptide BAM22 has a vCCL2-like ACKR3 binding and activation modes, consistent with its sequence homology with the N-terminal region of vCCL2 (Fig. 1E).

5. Discussion

ACKR3 is involved in different vital physiological but also pathological processes and has emerged as a highly relevant drug target, especially for cancer therapy. Although many efforts have been undertaken over the last years, no small molecule antagonist of ACKR3 has been identified so far. ACKR3 shows several distinctive functional characteristics some of which have been suggested to be linked to its chemokine scavenging function. The most remarkable ones are its lack of G protein coupling, its continuous cycling, predominantly

intracellular localization, responsiveness to both CXC and CC chemokines as well as to endogenous non-chemokine peptides, and its high propensity for activation. In addition to these functional particularities, an uncommon structural feature of ACKR3 is the disulphide bridge within its N terminus, which creates a small loop whose function and impact on the receptor biology remain unknown [32,41,52]. In this study, by disrupting this additional disulphide bridge, as well as the two conserved disulphide bridges, we uncovered new functional properties of ACKR3 and demonstrated that chemokine and endogenous non-chemokine ligands interact and activate ACKR3 following different binding modes characterized by distinct transmembrane domain subpocket occupancy and intra-N terminus loop contribution.

The roles of the conserved disulphide bridges for chemokine receptor functions have previously been shown to reflect unique traits of a single receptor rather than being shared between receptors, even within the same subfamily [40]. Their impact was also shown to vary depending on the ligand, thus offering an interesting means to explore different binding and/or activation modes for a specific receptor. Indeed, the two conserved disulphide bridges are positioned at opposing critical regions of the receptor extracellular surface and shape the entrance of the ligand-binding subpockets (Fig. 1D).

Our data showed that ACKR3 folding and export is largely independent of the presence of its extracellular disulphide bridges. Only the disruption of the TM3-ECL2 disulphide reduced the presence of the receptor at the plasma membrane and moderately impacted the structure of the receptor. These results markedly contrast with the observations for TM3-ECL2 disulphide mutants in this and previous studies for CXCR4 [56] or CCR6 [57], which were both fully retained within the cell. However, maintenance of folding and export were reported for CXCR2 and CCR1 [40,58].

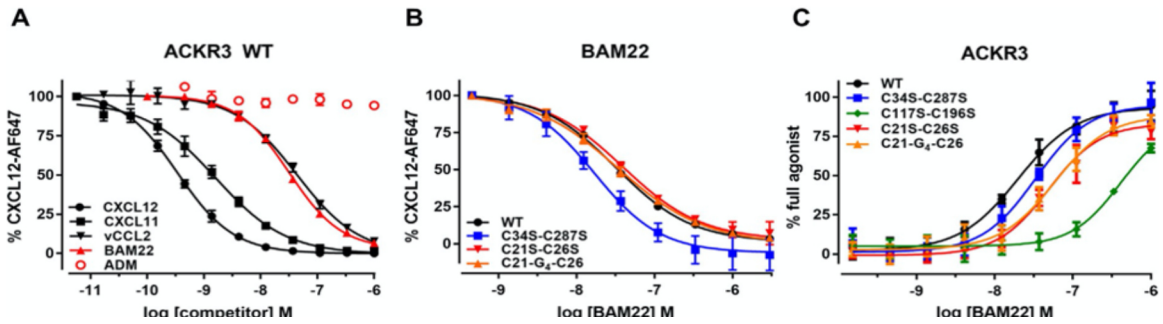


Figure 4 | Binding and activation of non-chemokine ligands to the wild-type and cysteine-mutated ACKR3. (A) Binding of CXCL12, CXCL11, vCCL2, BAM22 and adrenomedullin (ADM) to wild-type ACKR3 assessed by binding competition with CXCL12-AF647 and analysed by flow cytometry. (B) Binding of BAM22 to WT and mutated ACKR3 assessed by binding competition with CXCL12-AF647 and analysed by flow cytometry. (C) β-arrestin-2 recruitment to wild-type ACKR3 and cysteine-mutated variants induced by BAM22. Values represent the mean ± standard error of the mean (SEM) of at least three independent experiments.

The substitution of the two N-terminal cysteine residues engaged in the formation of the intra-N terminus loop (TPA) resulted in a slight increase of surface expression levels compared to the wild-type receptor. Similar results were observed for the CXCR3 variant bearing the C37S-C38S mutation, suggesting that this apparent higher surface expression in N-terminal cysteine mutants may either reflect a facilitated transfer through the cell secretory pathway or simply a better accessibility of the epitope to the probe. A recent mutagenesis study of ACKR3 focusing on its N-terminal features reported corroborating results and found that the disruption of the N terminus-TM7 or the TPA-forming disulphide bridges did not significantly affect ACKR3 surface expression [52].

The maintenance of cell surface expression for all ACKR3 mutants allowed us to further probe the role of the disulphide bridges in ligand binding and receptor activation, providing new insights into the importance of the different extracellular regions and subpockets of ACKR3. The absence of the disulphide bridge linking TM3 to ECL2, lying on top of the major subpocket, reduced the ability of ACKR3 to bind CXCL12 and respond to CXC chemokines, but not the CC chemokine. Noteworthy, the disruption of this disulphide turned CXCL11 and vCCL2 from partial to full agonists, suggesting that this structural constraint regulates the maximal responsiveness of the receptor to certain chemokines. Conversely, the disruption of the disulphide bridge linking the N-terminal domain to TM7, surrounding the minor ligand binding pocket, enhanced the apparent binding of vCCL2, which was likely linked to the weaker interaction of the fluorescent tracer and may point to differential ACKR3 binding pocket occupancy by CXC and CC chemokines. Strikingly, the disruption of or substitution within the N-terminal loop (TPA) markedly impaired the recognition and activation by CC and to a lesser extent by CXC chemokines. Altogether these results provide strong evidence for a different binding modes for CXC versus CC chemokines to ACKR3 characterised by different roles of the N-terminal arch and different ligand subpocket occupancies. Our data suggest that CXC chemokines mainly occupy the major subpocket, whereas vCCL2 interacts with structural determinants located in the vicinity of the minor subpocket, including the TPA. These observations are supported by structural data and comparative models of the binding mode of CXCL12 and vCCL2 to CXCR4, which shares 29% identity with ACKR3, that indicate that although the tip of the N-termini of the two chemokines reach similar depths in the transmembrane region, they occupy the extracellular surface and the receptor subpockets differently. While vCCL2 lies mainly above TM1 and TM7 with its N terminus spanning the minor binding pocket, CXCL12 shows a rotation of approximately 80° relative to vCCL2, consequently positioning it more on top of the major binding pocket (TM5-TM6) [33,59].

In this study, no interaction between adrenomedullin and ACKR3 was detected in the concentration range for which all other ligands were highly potent. This observation may be due the requirement of additional partners for adrenomedullin binding to occur, which are absent in the U87 cells. The binding and activation by BAM22, showed an apparent mixed

CXC/CC profile for the effect of disulphide bridge disruptions. BAM22 interactions was negatively impacted by the disruption of the disulphide bridge TM3-ECL2 similarly to what was observed for CXC chemokines, suggesting that it also activates the receptor by occupying mainly the major binding pocket (Fig. 5). However, its binding and activity were also significantly affected by the disruption or the substitution of the TPA indicating that its C terminus is most likely positioned more on top of the minor ligand binding pocket reminiscent of vCCL2 binding mode. These binding similarities between vCCL2 and BAM22 may be partially explained by the sequence identity between the N-loop of vCCL2 and the C terminus of BAM22, both regions bearing the YQKR motif. We recently demonstrated that the N-loop of vCCL2, but not that of CXCL12 and CXCL11, is important for ACKR3 binding and activation further supporting the observation that this stretch could interact with the TPA by for instance forming electrostatic interactions with negatively charged residues (D25) located within the TPA [51]. Sequence alignment revealed that D25 occupies a position similar to that of the sulfotyrosine sY21 in the CXCR4 N terminus, a residue known to be crucial for CXCR4-chemokine interactions [42]. Furthermore, the shortening of CRS1 resulting from the formation of the TPA would also bring the potential sY8 of ACKR3 at a position equivalent to that of sY7 in CXCR4. Moreover, although it is highly speculative, the oxidation state of the two TPA-forming cysteines, by influencing the strength of ACKR3 interactions with internalised ligands, may regulate their handling or release during the trafficking through the different intracellular compartments. Finally, it cannot be excluded that this arch could also support the binding of other yet unknown ligands or interacting partners.

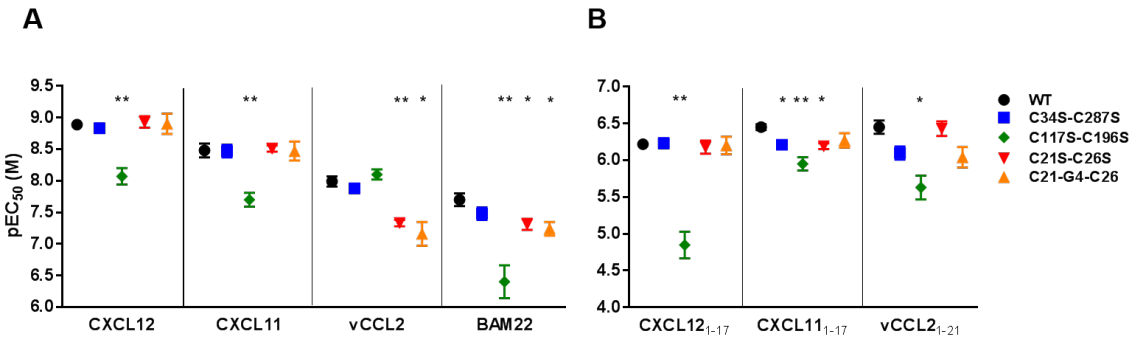


Figure 5 | Overview of the effects of cysteine mutation on ACKR3 activity. ACKR3 activation in response to full-length chemokines and BAM22 (A) and chemokine N-terminal peptides (B) monitored in β-arrestin recruitment assay. pEC50 values represent the mean ± standard error of the mean (SEM) of at least three independent experiments. Unpaired t tests were used to compare the differences in pEC50/pIC50 for each mutant using the WT receptor as reference. * $p < 0.05$, ** $p < 0.01$.

References

1. D. Rossi, A. Zlotnik, The biology of chemokines and their receptors, Ann. Rev. Immunol. 18 (2000) 217–242.
2. P. Romagnani, L.Lasagni, F.Annunziato, M.Serio, S.Romagnani, CXC chemokines: the regulatory link between inflammation and angiogenesis, Trends Immunol. 25 (4) (2004) 201–209.
3. F. Balkwill, Cancer and the chemokine network, Nat. Rev. Cancer 4 (7) (2004) 540–550.
4. F. Bachelerie, A. Ben-Baruch, A.M. Burkhardt, C. Combadiere, J.M. Farber, G.J. Graham, R. Horuk, A.H. Sparre-Ulrich, M. Locati, A.D. Luster, A. Mantovani, K. Matsushima, P.M. Murphy, R. Nibbs, H. Nomiyama, C.A. Power, A.E. Proudfoot, M.M. Rosenkilde, A. Rot, S. Sozzani, M. Thelen, O. Yoshie, A. Zlotnik, International union of basic and clinical pharmacology. [corrected]. LXXXIX. Update on the ex- tended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors, Pharmacol. Rev. 66 (1) (2014) 1–79.
5. S. Shimizu, M. Brown, R. Sengupta, M.E. Penfold, O. Meucci, CXCR7 protein ex- pression in human adult brain and differentiated neurons, PLoS ONE 6 (5) (2011) e20680.
6. M.L.Humpert, M.Tzouros, S.Thelen, A.Bignon, A.Levoye, F.Arenzana-Seisdedos, K. Balabanian, F. Bachelerie, H. Langen, M. Thelen, Complementary methods pro- vide evidence for the expression of CXCR7 on human B cells, Proteomics 12 (12) (2012) 1938–1948.
7. Y. Wang, G. Li, A. Stanco, J.E. Long, D. Crawford, G.B. Potter, S.J. Pleasure, T. Behrens, J.L. Rubenstein, CXCR4 and CXCR7 have distinct functions in regulating interneuron migration, Neuron 69 (1) (2011) 61–76.
8. H. Gerrits, D.S. van Ingen Schenau, N.E. Bakker, A.J. van Disseldorp, A. Strik, L.S. Hermens, T.B. Koenen, M.A. Krajnc-Franken, J.A. Gossen, Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice, Genesis 46 (5) (2008) 235–245.
9. F. Sierro, C. Biben, L. Martinez-Munoz, M. Mellado, R.M. Ransohoff, M. Li, B. Woehl, H. Leung, J. Groom, M. Batten, R.P. Harvey, A.C. Martinez, C.R. Mackay, F. Mackay, Disrupted cardiac development but normal hematopoiesis in mice de- ficient in the second CXCL12/SDF-1 receptor, CXCR7, Proc. Natl. Acad. Sci. U.S.A. 104 (37) (2007) 14759–14764.
10. J.M. Burns, B.C. Summers, Y. Wang, A. Melikian, R. Berahovich, Z. Miao, M.E. Penfold, M.J. Sunshine, D.R. Littman, C.J. Kuo, K. Wei, B.E. McMaster, K. Wright, M.C. Howard, T.J. Schall, A novel chemokine receptor for SDF-1 and I- TAC involved in cell survival, cell adhesion, and tumor development, J. Exp. Med. 203 (9) (2006) 2201–2213.
11. L. Sanchez-Martin, P. Sanchez-Mateos, C. Cabanas, CXCR7 impact on CXCL12 biology and disease, Trends Mol. Med. 19 (1) (2013) 12–22.
12. K. Grymula, M. Tarnowski, M. Wysoczynski, J. Drukala, F.G. Barr, J. Ratajczak, M. Kucia, M.Z. Ratajczak, Overlapping and distinct role of CXCR7-SDF-1/ITAC and CXCR4-SDF-1 axes in regulating metastatic behavior of human rhabdomyo- sarcomas, Int. J. Cancer (Journal International du Cancer) 127 (11) (2010) 2554–2568.
13. K.E. Luker, S.A. Lewin, L.A. Mihalko, B.T. Schmidt, J.S. Winkler, N.L. Coggins, D.G. Thomas, G.D. Luker, Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells, Oncogene 31 (45) (2012) 4750–4758.
14. X. Sun, G. Cheng, M. Hao, J. Zheng, X. Zhou, J. Zhang, R.S. Taichman, K.J. Pienta, J. Wang, CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression, Cancer Metastasis Rev. 29 (4) (2010) 709–722.
15. R. Wurth, A. Bajetto, J.K. Harrison, F. Barbieri, T. Florio, CXCL12 modulation of CXCR4 and CXCR7 activity in human glioblastoma stem-like cells and regulation of the tumor microenvironment, Front. Cell. Neurosci. 8 (2014) 144.
16. K.E. Cole, C.A. Strick, T.J. Paradis, K.T. Ogborne, M. Loetscher, R.P. Gladue, W. Lin, J.G. Boyd, B. Moser, D.E. Wood, B.G. Sahagan, K. Neote, Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent ac- tivity on activated T cells through selective high affinity binding to CXCR3, J. Exp. Med. 187 (12) (1998) 2009–2021.
17. M. Loetscher, B. Gerber, P. Loetscher, S.A. Jones, L. Piali, I. Clark-Lewis, M. Baggiolini, B. Moser, Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes, J. Exp. Med. 184 (3) (1996) 963–969.
18. K. Balabanian, B. Lagane, S. Infantino, K.Y. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, F. Bachelerie, The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes, J. Biol. Chem. 280 (42) (2005) 35760–35766.
19. M. Szpakowska, N. Dupuis, A. Baragli, M. Counson, J. Hanson, J. Piette, A. Chevigne, Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7, Biochem. Pharmacol. (2016).
20. M. Szpakowska, A. Chevigne, vCCL2/vMIP-II, the viral master KEYmokine, J. Leukocyte Biol. (2015).
21. T.N. Kledal, M.M. Rosenkilde, F. Coulin, G. Simmons, A.H. Johnsen, S. Alouani, C.A. Power, H.R. Luttichau, J. Gerstoft, P.R. Clapham, I. Clark-Lewis, T.N. Wells, T.W. Schwartz, A broad-spectrum chemokine antagonist encoded by Kaposi's sar- coma-associated herpesvirus, Science 277 (5332) (1997) 1656–1659.
22. R.K. Ganju, S.A. Brubaker, J. Meyer, P. Dutt, Y. Yang, S. Qin, W. Newman, J.E. Groopman, The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways, J. Biol. Chem. 273 (36) (1998) 23169–23175.
23. I. Kalatskaya, Y.A. Berchiche, S. Gravel, B.J. Limberg, J.S. Rosenbaum, N. Heveker, AMD3100 is a CXCR7 ligand with allosteric agonist properties, Mol. Pharmacol. 75 (5) (2009) 1240–1247.
24. S. Rajagopal, J. Kim, S. Ahn, S. Craig, C.M. Lam, N.P. Gerard, C. Gerard, R.J. Lefkowitz, Beta-arrestin- but not G protein-mediated signaling by the "decoy" re-ceptor CXCR7, Proc Natl Acad Sci U.S.A. 107(2) 628–32.
25. Y. Sun, Z. Cheng, L. Ma, G. Pei, Beta-arrestin2 is critically involved in CXCR4- mediated chemotaxis,

and this is mediated by its enhancement of p38 MAPK acti- vation, J. Biol. Chem. 277 (51) (2002) 49212–49219.
26. U. Naumann, E. Cameroni, M. Pruenster, H. Mahabaleshwar, E. Raz, H.G. Zerwes, A. Rot, M. Thelen, CXCR7 functions as a scavenger for CXCL12 and CXCL11, PLoS ONE 5 (2) (2010) e9175.
27. K.E. Luker, J.M. Steele, L.A. Mihalko, P. Ray, G.D. Luker, Constitutive and che- mokine-dependent internalization and recycling of CXCR7 in breast cancer cells to degrade chemokine ligands, Oncogene 29 (32) (2010) 4599–4610.
28. R.D. Berahovich, B.A. Zabel, S. Lewen, M.J. Walters, K. Ebsworth, Y. Wang, J.C. Jaen, T.J. Schall, Endothelial expression of CXCR7 and the regulation of sys- temic CXCL12 levels, Immunology 141 (1) (2014) 111–122.
29. B. Boldajipour, H. Mahabaleshwar, E. Kardash, M. Reichman-Fried, H. Blaser, S. Minina, D. Wilson, Q. Xu, E. Raz, Control of chemokine-guided cell migration by ligand sequestration, Cell 132 (3) (2008) 463–473.
30. Y. Ikeda, H. Kumagai, A. Skach, M. Sato, M. Yanagisawa, Modulation of circadian glucocorticoid oscillation via adrenal opioid-CXCR7 signaling alters emotional be- havior, Cell 155 (6) (2013) 1323–1336.
31. K.R. Klein, N.O. Karpinich, S.T. Espenschied, H.H. Willcockson, W.P. Dunworth, S.L. Hoopes, E.J. Kushner, V.L. Bautch, K.M. Caron, Decoy receptor CXCR7 mod- ulates adrenomedullin-mediated cardiac and lymphatic vascular development, Dev. Cell 30 (5) (2014) 528–540.
32. M. Gustavsson, L. Wang, N. van Gils, B.S. Stephens, P. Zhang, T.J. Schall, S. Yang, R. Abagyan, M.R. Chance, I. Kufareva, T.M. Handel, Structural basis of ligand in- teraction with atypical chemokine receptor 3, Nat. Commun. 8 (2017) 14135.
33. L. Qin, I. Kufareva, L.G. Holden, C. Wang, Y. Zheng, C. Zhao, G. Fenalti, H. Wu, G.W. Han, V. Cherezov, R. Abagyan, R.C. Stevens, T.M. Handel, Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral che- mokine, Science 347 (6226) (2015) 1117–1122.
34. B. Wu, E.Y. Chien, C.D. Mol, G. Fenalti, W. Liu, V. Katritch, R. Abagyan, A. Brooun, P. Wells, F.C. Bi, D.J. Hamel, P. Kuhn, T.M. Handel, V. Cherezov, R.C. Stevens, Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists, Science 330 (6007) (2010) 1066–1071.
35. Q. Tan, Y. Zhu, J. Li, Z. Chen, G.W. Han, I. Kufareva, T. Li, L. Ma, G. Fenalti, J. Li, W. Zhang, X. Xie, H. Yang, H. Jiang, V. Cherezov, H. Liu, R.C. Stevens, Q. Zhao, B. Wu, Structure of the CCR5 chemokine receptor-HIV entry inhibitor maraviroc complex, Science 341 (6152) (2013) 1387–1390.
36. J.S. Burg, J.R. Ingram, A.J. Venkatakrishnan, K.M. Jude, A. Dukkipati, E.N. Feinberg, A. Angelini, D. Waghray, R.O. Dror, H.L. Ploegh, K.C. Garcia, Structural biology Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor, Science 347 (6226) (2015) 1113–1117.
37. Y. Zheng, L. Qin, N.V. Zacarias, H. de Vries, G.W. Han, M. Gustavsson, M. Dabros, C. Zhao, R.J. Cherney, P. Carter, D. Stamos, R. Abagyan, V. Cherezov, R.C. Stevens, A.P. IJ, L.H. Heitman, A. Tebben, I. Kufareva, T.M. Handel, Structure of CC che- mokine receptor 2 with orthosteric and allosteric antagonists, Nature 540 (7633) (2016) 458–461.
38. C. Oswald, M. Rappas, J. Kean, A.S. Dore, J.C. Errey, K. Bennett, F. Deflorian, J.A. Christopher, A. Jazayeri, J.S. Mason, M. Congreve, R.M. Cooke, F.H. Marshall, Intracellular allosteric antagonism of the CCR9 receptor, Nature 540 (7633) (2016) 462–465.
39. M. Szpakowska, D. Perez Bercoff, A. Chevigne, Closing the ring: a fourth extra- cellular loop in chemokine receptors, Sci. Signaling 7 (341) (2014) 21.
40. P.C. Rummel, S. Thiele, L.S. Hansen, T.P. Petersen, A.H. Sparre-Ulrich, T. Ulven, M.M. Rosenkilde, Extracellular disulfide bridges serve different purposes in two homologous chemokine receptors, CCR1 and CCR5, Mol. Pharmacol. 84 (3) (2013) 335–345.
41. M. Szpakowska, V. Fievez, K. Arumugan, N. van Nuland, J.C. Schmit, A. Chevigne, Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors, Biochem. Pharmacol. 84 (10) (2012) 1366–1380.
42. I. Kufareva, B.S. Stephens, L.G. Holden, L. Qin, C. Zhao, T. Kawamura, R. Abagyan, T.M. Handel, Stoichiometry and geometry of the CXC chemokine receptor 4 com- plex with CXC ligand 12: molecular modeling and experimental validation, Proc. Natl. Acad. Sci. U.S.A. 111 (50) (2014) E5363–E5372.
43. C.T. Veldkamp, C. Seibert, F.C. Peterson, N.B. De la Cruz, J.C. Haugner 3rd, H. Basnet, T.P. Sakmar, B.F. Volkman, Structural basis of CXCR4 sulfotyrosine re- cognition by the chemokine SDF-1/CXCL12, Sci. Signaling 1 (37) (2008) 4.
44. M.P. Crump, E. Elisseeva, J. Gong, I. Clark-Lewis, B.D. Sykes, Structure/function of human herpesvirus-8 MIP-II (1–71) and the antagonist N-terminal segment (1–10), FEBS Lett. 489 (2–3) (2001) 171–175.
45. A.B. Kleist, A.E. Getschman, J.J. Ziarek, A.M. Nevins, P.A. Gauthier, A. Chevigne, M. Szpakowska, B.F. Volkman, New paradigms in chemokine receptor signal transduction: moving beyond the two-site model, Biochem. Pharmacol. (2016).
46. A. Chevigne, V. Fievez, J.C. Schmit, S. Deroo, Engineering and screening the N- terminus of chemokines for drug discovery, Biochem. Pharmacol. 82 (10) (2011) 1438–1456.
47. M.P. Crump, J.H. Gong, P. Loetscher, K. Rajarathnam, A. Amara, F. Arenzana- Seisdedos, J.L. Virelizier, M. Baggiolini, B.D. Sykes, I. Clark-Lewis, Solution struc- ture 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 (23) (1997) 6996–7007.
48. A. Chevigne, V. Fievez, M. Szpakowska, A. Fischer, M. Counson, J.M. Plesseria, J.C. Schmit, S. Deroo, Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1 infection, Biochim. Biophys. Acta 1843 (5) (2014) 1031–1041.
49. J.S. Surgand, J. Rodrigo, E. Kellenberger, D. Rognan, A chemogenomic analysis of the transmembrane binding cavity of human G-protein-coupled receptors, Proteins 62 (2) (2006) 509–538.
50. D.J. Scholten, M. Canals, D. Maussang, L. Roumen, M.J. Smit, M. Wijtmans, C. de Graaf, H.F. Vischer, R. Leurs, Pharmacological modulation of chemokine r e c e p t o r function, Br. J. Pharmacol. 165 (6) (2012) 1617–1643.
51. M. Szpakowska, A. Nevins, M. Meyrath, D. Rhainds, T. D'huys, F. Guité-Vinet, N. Dupuis, P.-A. Gauthier, M.

Counson, A. Kleist, G. St-Onge, J. Hanson, D. Schols, B. Volkman, N. Heveker, A. Chevigné, Different contribution of chemokine N- terminal features attest a different ligand binding mode and a bias towards acti- vation of the atypical chemokine receptor ACKR3/CXCR7 compared to CXCR4 and CXCR3, Br. J. Pharmacol. (2017).
52. B. Benredjem, M. Girard, D. Rhainds, G. St-Onge, N. Heveker, Mutational analysis of atypical chemokine receptor 3 (ACKR3/CXCR7) interaction with its chemokine li- gands CXCL11 and CXCL12, J. Biol. Chem. 292 (1) (2017) 31–42.
53. N. Dupuis, C. Laschet, D. Franssen, M. Szpakowska, J. Gilissen, P. Geubelle, A. Soni, A.S. Parent, B. Pirotte, A. Chevigne, J.C. Twizere, J. Hanson, Activation of the or- phan G protein-coupled receptor GPR27 by surrogate ligands promotes beta-ar- restin 2 recruitment, Mol. Pharmacol. 91 (6) (2017) 595–608.
54. A.S. Dixon, M.K. Schwinn, M.P. Hall, K. Zimmerman, P. Otto, T.H. Lubben, B.L. Butler, B.F. Binkowski, T. Machleidt, T.A. Kirkland, M.G. Wood, C.T. Eggers, L.P. Encell, K.V. Wood, NanoLuc complementation reporter optimized for accurate measurement of protein interactions in Cells, ACS Chem. Biol. 11 (2) (2016) 400–408.
55. S. Infantino, B. Moepps, M. Thelen, Expression and regulation of the orphan re- ceptor RDC1 and its putative ligand in human dendritic and B cells, J. Immunol. 176 (4) (2006) 2197–2207.
56. D.J. Chabot, P.F. Zhang, G.V. Quinnan, C.C. Broder, Mutagenesis of CXCR4 iden- tifies important domains for human immunodeficiency virus type 1 X4 isolate en- velope-mediated membrane fusion and virus entry and reveals cryptic coreceptor activity for R5 isolates, J. Virol. 73 (8) (1999) 6598–6609.
57. L.S. Ai, F. Liao, Mutating the four extracellular cysteines in the chemokine receptor CCR6 reveals their differing roles in receptor trafficking, ligand binding, and sig- naling, Biochemistry 41 (26) (2002) 8332–8341.
58. C. Limatola, S. Di Bartolomeo, M. Catalano, F. Trettel, S. Fucile, L. Castellani, F. Eusebi, Cysteine residues are critical for chemokine receptor CXCR2 functional properties, Exp. Cell Res. 307 (1) (2005) 65–75.
59. J.J. Ziarek, A.B. Kleist, N. London, B. Raveh, N. Montpas, J. Bonneterre, G. St-Onge, C.J. DiCosmo-Ponticello, C.A. Koplinski, I. Roy, B. Stephens, S. Thelen, C.T. Veldkamp, F.D. Coffman, M.C. Cohen, M.B. Dwinell, M. Thelen, F.C. Peterson, N. Heveker, B.F. Volkman, Structural basis for chemokine recognition by a G pro- tein-coupled receptor and implications for receptor activation, Sci. Signaling 10 (471) (2017).

Chapter 4

Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22

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*Contributed equally

Contribution:

Performing β-arrestin recruitment assays, analysis and interpretation of the results, and contribution to writing and revising the manuscript.

1. Abstract

Atypical chemokine receptors (ACKRs) have emerged as important regulators or scavengers of homeostatic and inflammatory chemokines. Among these atypical receptors, ACKR4 is reported to bind the homeostatic chemokines CCL19, CCL21, CCL25 and CXCL13. In a recent study by Matti *et al.*, the authors show that ACKR4 is also a receptor for CCL20, previously established to bind to CCR6 only. They provide convincing evidence that, just as for its other chemokine ligands, ACKR4 rapidly internalizes CCL20 both in vitro and in vivo. Independently of this discovery, we undertook a screening program aiming at reassessing the activity of the 43 human chemokines toward ACKR4 using a highly sensitive β-arrestin recruitment assay. This systematic analysis confirmed CCL20 as a new agonist ligand for ACKR4 in addition to CCL19, CCL21, and CCL25. Furthermore, CCL22, which plays an important role in both homeostasis and inflammatory responses, and is known as a ligand for CCR4 and ACKR2 was found to also act as a potent partial agonist of ACKR4. In contrast, agonist activity of CXCL13 toward ACKR4 was disproved. This independent wide-range systematic study confirms the pairing of CCL20 with ACKR4 newly discovered by Matti and co-authors, and further refines the spectrum of chemokines activating ACKR4.

2. Brief conclusive report

Atypical chemokine receptors (ACKRs) form a subfamily of 4 chemokine receptors unable to trigger G protein-dependent signaling or to directly induce cell migration in response to chemokines [1]. ACKRs play however a crucial role in chemokine biology by capturing, scavenging, or transporting chemokines, thereby regulating their availability and signaling through classical chemokine receptors [2,3]. Among ACKRs, ACKR4, formerly known as CCX-CKR, CCR11, or CCRL1, which is expressed on keratinocytes, astrocytes, lymphatic endothelial cells and on thymic epithelial cells, is an important regulator of homeostatic chemokines [4–6].

Human ACKR4 was deorphanized in 2000 based on competition studies with radiolabeled CCL19 [7]. It was initially proposed to bind CCL19, CCL21, CCL25, and CXCL13, which are the ligands for CCR7, CCR9, and CXCR5, respectively [7,8]. Of note, CXCL13 interaction with the mouse ACKR4 could not be confirmed [5] and the human CXCL13-ACKR4 pairing inferred from binding competition studies [7] was later reevaluated and the observations reattributed to cooperative GAG binding rather than direct receptor interactions [9].

By scavenging its chemokine ligands, ACKR4 was shown to regulate mainly the trafficking and positioning of T-cells and dendritic cells (DCs) [6,10]. ACKR4 is best known for its role in shaping the gradient of CCL19 and CCL21 for CCR7-expressing DCs in the subcapsular sinuses of lymph nodes during the initiation phase of the adaptive immune

response [11,12]. ACKR4 is also involved in anti-tumor immunity and modulates epithelial-mesenchymal transition and metastasis [13–15]; and studies with ACKR4-deficient mice in an EAE model also demonstrated the receptor implication in autoimmune diseases notably by accelerated disease onset an more severe symptomes attributable to increased Th17 response [4,16]. Of note, 2 ACKR4-defincient mouse strains (ACKR4$^{-/-}$ and ACKR4GFP/GFP) are available and display distinct phenotypes. While ACKR4$^{-/-}$ mice show a strong accumulation of plasma blasts in mesenteric lymph node and spleen as well as increased B cell proliferation after in vitro activation [17], B cells from ACKR4GFP/GFP mice exhibit a phenotype comparable to wild-type cells [18], suggesting that the results from the above-mentioned ACKR4-deficient mouse studies should be interpreted cautiously.

In a recent study, Matti and co-authors reported CCL20, previously established to bind to CCR6 only, as a novel chemokine ligand for ACKR4 [19]. The authors had predicted the existence of this interaction based on CCL20 sequence and expression similarities with CCL19 and CCL21. They showed that CCL20 induces β-arrestin-1 and β-arrestin-2 recruitment to ACKR4, and is rapidly internalized and scavenged by ACKR4-expressing cells, both in vitro and in vivo. They proposed that by scavenging CCL20, ACKR4 regulates its availability for the classical signaling receptor CCR6 and thereby plays a role in the positioning of CCR6-positive leukocytes within secondary lymphoid tissues to initiate effective humoral and memory immune responses [19].

Independently of this discovery, we undertook a systematic screening program aiming at reassessing the agonist activity of the 43 human chemokines (24 CCLs, 16 CXCLs, 2 XCLs, and 1 CX3CL) and 2 viral chemokines (vCCL1 and vCCL2) toward ACKR4, by monitoring β-arrestin recruitment to the receptor using a highly sensitive Nanoluciferase complementation-based assay (NanoBiT) (Fig. 1A) [20–22]. In agreement with the initial description by Matti and co-authors, our systematic analysis also identified CCL20 as an ACKR4 ligand capable of inducing β-arrestin-1 and β-arrestin-2 recruitment to the receptor and behaving as a partial agonist (80% efficacy) with a potency (EC50 = 42 nM) somewhat lower than potencies observed for CCL19 (EC50 = 3 nM), CCL21 (EC50 = 5 nM), and CCL25 (EC50 = 24 nM) (Fig. 1B). Besides CCL19, CCL20, CCL21, and CCL25, our systematic reassessment of chemokine-receptor interactions revealed that CCL22, known to bind to CCR4 and ACKR2, is also a ligand for ACKR4. CCL22 was slightly less potent (EC50 = 128 nM) than the other ligands in inducing β-arrestin-1 recruitment to ACKR4 and acted as a partial agonist, displaying about 60% of the maximum efficacy observed with CCL19. Interestingly, the CCL22 variant lacking the first 2 N-terminal residues (CCL22 3-69) and hence mimicking the dipeptidyl peptidase 4 (DPP4 or CD26)-cleaved chemokine, retained significant activity toward ACKR4, which contrasts with its absence of activity toward ACKR2 [23] but is reminiscent of the agonist effect of processed CXC chemokines toward ACKR3 [20,24] (Fig. 1B).

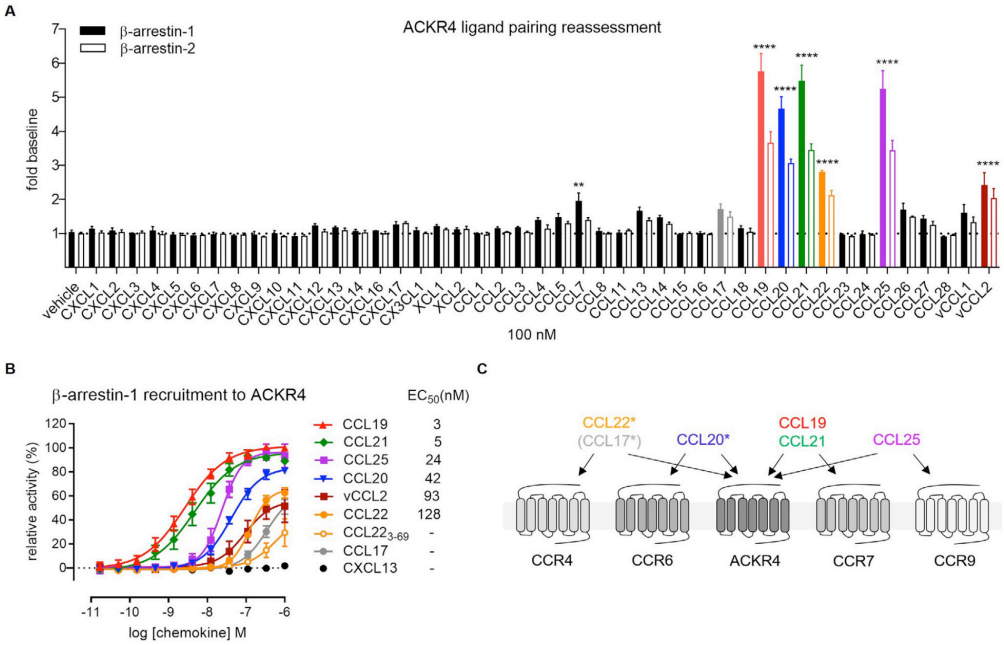

Figure 1 | Systematic reassessment of human ACKR4 activation by chemokines using a highly sensitive β-arrestin recruitment assay based on NanoBiT technology. (A) β-arrestin-1 and β-arrestin-2 recruitment to ACKR4 in response to all known human and 2 viral chemokines (100 nM). Experiments were conducted inU87 cells as previously described20. for statistical analysis, 1-wayANOVAwith Dunnett'smultiple comparison test with vehicle as reference was performed. **P<0.01 (β-arrestin-1 only), ****P<0.0001 (in both β-arrestin-1 and β-arrestin-2 recruitment assays). (B) β-arrestin-1 recruitment to ACKR4 by the previously reported and newly identified chemokines CCL17, CCL19, CCL20, CCL21, CCL22, CCL25, CXCL13, and vCCL2, showing the concentration-response relationship. EC50 values are indicated (nM). (A and B) Data are represented as mean ± SEM of at least 3 independent experiments. (C) Schematic representation of the interactions between ACKR4 and CC receptors CCR4, CCR6, CCR7, and CCR9, and their shared endogenous chemokines. The newly identified ligands are indicated with an asterisk

CCL22 was not the only CCR4-related chemokine acting on ACKR4. CCL17, the second ligand of CCR4, showed detectable activity toward ACKR4 but statistical significance was not reached for this pairing. A similar low activity was also observed for several other human CC chemokines including CCL7, CCL13, CCL14, CCL26, and CCL27, which are ligands for CCR1, CCR2, CCR3, and CCR10. Moreover, the viral broad-spectrum antagonist chemokine vCCL2/vMIP-II encoded by the Kaposi sarcoma-associated herpesvirus HHV-8 [25], known to bind to ACKR4 [7], and to activate ACKR3 [26], also behaved as a partial agonist of ACKR4 (EC50 = 93 nM) inducing 50% of the maximum efficacy observed with CCL19. In contrast, CXCL13, for which discordant observations have been reported regarding its interaction with ACKR4 [7,9], did not induce β-arrestin recruitment to ACKR4 in our assay, narrowing down ACKR4 specificity to CC chemokines only. In a similar screening conducted with all 45 chemokines (100 nM) in antagonist mode, that is, in the presence of CCL19 at a concentration equivalent to its EC50 (3 nM), no chemokine other than the above-mentioned agonists was able to modulate CCL19-induced β-arrestin recruitment to ACKR4 (data not shown).

A phylogenetic analysis based on amino acid sequences shows that CCL22 clusters together with all other ACKR4 ligands, including CCL20, further supporting its pairing with ACKR4. Moreover, CCL22 is constitutively expressed in lymphoid tissues, the intestine, lung, and skin, where ACKR4 is also expressed [4,10]. CCL22 acts as a potent agonist for

CCR4, primarily expressed on Th2 cells [27,28] but it has also been described to be involved in self-tolerance and mediate DC-Treg interaction. Indeed, deletion of CCR4 or CCL22 in mice showed a disruption of T-cell immunity, which caused an accumulation of autoreactive T-cells and, as a result, autoimmune diseases [29,30]. Therefore, together with the reported acceleration of CD4+ T-cell skewing toward Th17 in ACKR4-deficient mice [4], one could postulate the involvement of ACKR4 in the CCR4/CCL22-axis to mediate effective T-cell immunity. The relevance of this newly identified interaction remains nevertheless to be established, especially considering the crosstalk of CCL22 with CCR4 and ACKR2. It also needs to be determined whether the interaction between CCL22 and ACKR4 holds true for the murine homologues. Altogether, this systematic reassessment of chemokine-receptor interactions confirmed the pairing between CCL20 and ACKR4 in a different cellular background and using a different readout [31], reinforcing the initial description by Matti and co-authors [19]. In addition, it identified another ligand for ACKR4, CCL22. These novel pairings add a level of complexity to ACKR4 interactions within the chemokine-receptor network and extend its regulatory functions to the CCL20/CCR6 and CCL22/CCR4 axes (Fig. 1C). Nevertheless, complementary pharmacological experiments, including binding and scavenging or internalization assays, remain to be performed to validate these results. The importance of these novel interacting partners for ACKR4 in pathophysiological context also remains to be elucidated. Finally, our study focused exclusively on chemokine-induced β-arrestin recruitment and thus chemokines with different mode of action toward ACKR4 may have been overlooked [32]. Indeed, although ACKR4-driven chemokine scavenging is generally considered as dependent on β-arrestins, recent studies indicated that they are not essential for this activity, suggesting that further investigations are necessary to fully understand this still enigmatic receptor [8,33].

References

1. Bachelerie F, Graham GJ, Locati M, et al. New nomenclature for atypical chemokine receptors. Nat Immunol. 2014;15:207-208.
2. Graham GJ, Locati M, Mantovani A, Rot A, Thelen M. The biochemistry and biology of the atypical chemokine receptors. Immunol Lett. 2012;145:30-38.
3. Nibbs RJ, Graham GJ. Immuneregulation by atypical chemokine receptors. Nat Rev Immunol. 2013;13:815-829.
4. Comerford I, Nibbs RJ, Litchfield W, et al. The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in circulation and tissues and suppresses Th17 responses. Blood. 2010;116 4130-4140.
5. Townson JR, Nibbs RJ. Characterization of mouse CCX-CKR, a receptor for the lymphocyte-attracting chemokines TECK/mCCL25, SLC/mCCL21 and MIP-3beta/mCCL19: comparison to human CCXCKR. Eur J Immunol. 2002;32:1230-1241.
6. Heinzel K, Benz C, Bleul CC. A silent chemokine receptor regulates steady-state leukocyte homing in vivo. Proc Natl Acad Sci U S A. 2007;104:8421-8426.
7. Gosling J, Dairaghi DJ, Wang Y, et al. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cellactive chemokines including ELC, SLC, and TECK. J Immunol. 2000;164:2851-2856.
8. Comerford I, Milasta S,Morrow V, Milligan G, Nibbs R. The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. Eur J Immunol. 2006;36:1904-1916.
9. Verkaar F, van Offenbeek J, van der Lee MMC, et al. Chemokine cooperativity is caused by competitive glycosaminoglycan binding. J Immunol. 2014;192:3908-3914.
10. Lucas B, White AJ, Ulvmar MH, et al. CCRL1/ACKR4 is expressed in key thymic microenvironments but is dispensable for T lymphopoiesis at steady state in adult mice. Eur J Immunol. 2015;45:574-583.
11. Ulvmar MH, Werth K, Braun A, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. Nat Immunol. 2014;15:623-630.
12. Bryce SA, Wilson RA, Tiplady EM, et al. ACKR4 on stromal cells scavenges CCL19 to enable CCR7-dependent trafficking of APCs from Inflamed skin to lymph nodes. J Immunol. 2016;196:3341-3353.
13. Sjoberg E,Meyrath M, Chevigne A, Ostman A, Augsten M, Szpakowska M. The diverse and complex roles of atypical chemokine receptors in cancer: from molecular biology to clinical relevance and therapy. Adv Cancer Res. 2020;145:99-138.
14. Harata-Lee Y, Turvey ME, Brazzatti JA, et al. The atypical chemokine receptorCCX-CKRregulatesmetastasis ofmammary carcinoma via an effect on EMT. Immunol Cell Biol. 2014;92:815-824.
15. Whyte CE, Osman M, Kara EE, et al. ACKR4 restrains antitumor immunity by regulating CCL21. J Exp Med. 2020;217(6):e20190634.
16. Bunting MD, Comerford I, Seach N, et al. CCX-CKR deficiency alters thymic stroma impairing thymocyte development and promoting autoimmunity. Blood. 2013;121:118-128.
17. Kara EE, Bastow CR, McKenzie DR, et al. Atypical chemokine receptor 4 shapes activated B cell fate. J Exp Med. 2018;215:801-813.
18. Eckert N, Werth K, Willenzon S, Tan L, Forster R. B cell hyperactivation in an Ackr4-deficient mouse strain is not caused by lack of ACKR4 expression. J Leukoc Biol. 2019;16.
19. Matti C, D'Uonnolo G, Artinger M, et al. CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4. J Leukoc Biol. 2020. In press.
20. Szpakowska M, Nevins AM, Meyrath 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. 2018;175:1419-1438.
21. Dixon AS, Schwinn MK, Hall MP, et al. NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. ACS Chem Biol. 2016;11:400-408.
22. Szpakowska M, Meyrath M, Reynders N, et al. Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists. Biochem Pharmacol. 2018;153:299-309.
23. Bonecchi R, Locati M, Galliera E, et al. Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. J Immunol. 2004;172:4972-4976.
24. Janssens R, Mortier A, Boff D, et al. 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;132:92-101.
25. Szpakowska M, Chevigne A. (2016) vCCL2/vMIP-II, the viral master KEYmokine. J Leukoc Biol;99:893-900.
26. Szpakowska M, Dupuis N, Baragli A, et al. Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7. Biochem Pharmacol. 2016;114:14-21.
27. Imai T, Chantry D, Raport CJ, et al. Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. J Biol Chem. 1998;273:1764-1768.
28. Imai T, Nagira M, Takagi S, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol. 1999;11:81-88.
29. Rapp M, WintergerstMWM,KunzWG, et al. CCL22 controls immunity by promoting regulatory T cell communication with dendritic cells in lymph nodes. J Exp Med. 2019;216:1170-1181.
30. Hu Z, Lancaster JN, Sasiponganan C, Ehrlich LI. CCR4 promotes medullary entry and thymocyte-dendritic cell interactions required for central tolerance. J Exp Med. 2015;212:1947-1965.
31. Laschet C,Dupuis N, Hanson J. The G protein-coupled receptors deorphanization landscape. Biochem Pharmacol. 2018;153:62-74.
32. Kleist AB,Getschman AE, Ziarek JJ, et al.New paradigms in chemokine receptor signal transduction: moving beyond the two-site model. Biochem Pharmacol. 2016;114:53-68.
33. Matti C, Salnikov A, Artinger M, et al. ACKR4 recruits GRK3 prior to □-Arrestins but can scavenge chemokines in the absence of □-arrestins. Front Immunol. 2020;11:720.

Chapter 5

CXCL10 Is an Agonist of the CC Family Chemokine Scavenger Receptor ACKR2/D6

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Contribution:

Performing β-arrestin recruitment to CXCR3 assays, analysis and interpretation of the results, and contribution to revising the manuscript.

1. Simple Summary

The atypical chemokine receptor ACKR2 plays an important role in the tumour microenvironment. It has long been considered as a scavenger of inflammatory chemokines exclusively from the CC family. In this study, we identified the CXC chemokine CXCL10 as a new strong agonist ligand for ACKR2. CXCL10 is known to drive the infiltration of immune cells into the tumour bed and was previously reported to bind to CXCR3 only. We demonstrated that ACKR2 acts as a scavenger reducing the availability of CXCL10 for CXCR3. Our study sheds new light onthe complexity of the chemokine network and the potential role of CXCL10 regulation by ACKR2 in tumour immunology.

2. Abstract

Atypical chemokine receptors (ACKRs) are important regulators of chemokine functions. Among them, the atypical chemokine receptor ACKR2 (also known as D6) has long been considered as a scavenger of inflammatory chemokines exclusively from the CC family. In this study, by using highly sensitive arrestin recruitment assays based on NanoBiT and NanoBRET technologies, we identified the inflammatory CXC chemokine CXCL10 as a new strong agonist ligand for ACKR2. CXCL10 is known to play an important role in the infiltration of immune cells into the tumour bed and was previously reported to bind to CXCR3 only. We demonstrated that ACKR2 is able to internalize and reduce the availability of CXCL10 in the extracellular space. Moreover, we found that, in contrast to CC chemokines, CXCL10 activity towards ACKR2 was drastically reduced by the dipeptidyl peptidase 4 (DPP4 or CD26) N-terminal processing, pointing to a different receptor binding pocket occupancy by CC and CXC chemokines. Overall, our study sheds new light on the complexity of the chemokine network and the potential role of CXCL10 regulation by ACKR2 in many physiological and pathological processes, including tumour immunology. Our data also testify that systematic reassessment of chemokine-receptor pairing is critically needed as important interactions may remain unexplored.

3. Introduction

Chemokines are small (8–14 kDa) soluble cytokines that guide directional cell migration and orchestrate many important processes, including leukocyte recruitment during immunosurveillance. They are also involved in numerous inflammatory diseases and the development and spread of many cancers. Based on the presence of specific cysteine motifs in their N termini, chemokines are divided into four classes: CC, CXC, XC and CX3C. Their receptors belong to the G protein-coupled receptor (GPCR) family and are accordingly classified as CCR, CXCR, XCR and CX3CR, depending on the chemokine class they bind. Over the past years, a subfamily of four chemokine receptors has emerged as important regulators of chemokine functions. These receptors are termed atypical chemokine receptors (ACKR1-4) due to their inability to trigger a G protein-dependent

signalling or directly induce cell migration in response to chemokine binding [1,2]. Nevertheless, ACKRs do play an important role within the chemokine-receptor network by shaping the gradient of chemokines, thereby regulating their effect on cells expressing their respective classical chemokine receptors. Most ACKRs have the ability to constitutively cycle between the cell membrane and the intracellular compartments, internalizing and directing for degradation the chemokines that they bind [1,3-5]. Although this activity was previously considered to mainly rely on -arrestins, recent studies showed that alternative mechanisms can drive chemokine scavenging by ACKRs [6-11].

ACKR2 (formerly D6 or CCBP2) has been long reported to bind inflammatory chemokines exclusively from the CC family. ACKR2 main ligands include CCL2-8, CCL11-13, CCL17 and CCL22, which are agonists of the classical receptors CCR1-5 [12–15]. By scavenging this large spectrum of inflammatory chemokines, ACKR2 drives the resolution phase of inflammation and prevents exacerbated immune responses [16–21]. ACKR2 is expressed on lymphatic endothelial cells, epithelial cells, trophoblasts in placenta and some subsets of leukocytes, including alveolar macrophages and innate-like B cells [22–24]. Owing to its anti-inflammatory effect, ACKR2-deficient mice show an increased number of circulating inflammatory monocytes [25] and neutrophils [26,27], as well as defects in lymphatic vessel density and function [28]. ACKR2 was also shown as an important regulator of chemokines in inflammatory and autoimmune diseases, notably in psoriasis [18,29–31]. A scavenging-independent activity of ACKR2 has also been reported in apoptotic neutrophils, where ACKR2 was proposed to present chemokines to macrophages and promote inflammation resolution by shifting their phenotype [32,33].

Importantly, ACKR2 plays diverse and complex roles in tumour biology from initiation to metastasis [27,34,35]. ACKR2-deficient mice were shown to be more prone to tumour development but display increased tumour natural killer (NK) cell infiltration and circulating neutrophils, while opposing effects were reported regarding ACKR2 involvement in tumour dissemination [27,34,36]. Besides CC inflammatory chemokines, several CXC chemokines play important roles in inflammatory responses and are also found as part of tumour-associated inflammatory signatures [37,38]. In particular, the interferon gamma-induced chemokine CXCL10, also known as IP-10, reported to sustain tumour growth via autocrine loops [39] and to drive T lymphocytes and NK cells through activation of CXCR3 [37,40,41], is often upregulated in the same manner or simultaneously with CC inflammatory chemokines [42].

In this study, by applying highly sensitive assays monitoring-arrestin recruitment, we identified CXCL10, previously known to exclusively bind to CXCR3, as a high-affinity agonist for ACKR2. This finding expands the panel of ACKR2 ligands to the CXC chemokine family and at the same time highlights the need for a systematic reassessment of chemokine-receptor pairing, as important interactions may remain unexplored.

4. Material and methods

4.1. Cells and Proteins

HEK-ACKR2 cell line stably expressing human or mouse ACKR2 were established by transfection of HEK293T cells (ATCC, Manassas, VA, USA) with pIRES-puro vector (Addgene, Watertown, MA, USA) encoding the human or mouse ACKR2 and subsequent puromycin selection (5 µg/mL). Receptor surface expression was verified by flow cytometry using hACKR2-specific mAb (clone 196124, R&D Systems, Minneapolis, MI, USA) or polyclonal mACKR2-specific antibody (ab1656, Abcam, Cambridge, UK). The absence of CXCR3 at the cell surface was confirmed using mAb clone 1C6 and the corresponding isotype control (BioLegend, San Diego, CA, USA). The B16.F10 and U87.MG cell lines were purchased from ATCC. Unlabelled chemokines were purchased from PeproTech. CXCL10 was labelled with Cy5 using the Amersham QuickStain Protein Labeling Kit (GE Healthcare Life Sciences, Marlborough, MA, USA). Alexa Fluor 647-labelled CCL2 (CCL2-AF647) was purchased from Almac (Craigavon, UK).

4.2. Chemokine Processing by Dipeptidyl Peptidase 4

CCL5, CCL2, CXCL10, CXCL11 and CXCL12 chemokines (9 µM) were incubated with recombinant dipeptidyl peptidase 4 (CD26) (200 U) in Tris/HCl 50 mM pH 7.5 + 1 mM EDTA for 1 h at 37°C in the presence or absence of the sitagliptin (10 M) (Sigma Aldrich, St. Louis, MO, USA). The efficiency of processing was verified by MALDI-TOF analysis using a RapifleX, Bruker Daltonics instrument (Billerica, MA, USA) in positive ion mode and in reflectron mode.

4.3. Chemokine-Induced b-Arrestin Recruitment

Chemokine-induced β-arrestin recruitment to receptors was monitored by NanoLuc complementation assay (NanoBiT) [43–45] or by NanoBRET using mNeonGreen as acceptor molecule.

NanoBiT: HEK293T or U87.MG cells were co-transfected with pNBe vectors encoding chemokine receptors C-terminally fused to SmBiT and human β-arrestin-1/2 N-terminally fused to LgBiT. Twenty-four hours after transfection cells were harvested, incubated 25 min at 37°C with Nano-Glo Live Cell substrate (1:200) and upon addition of chemokines at the indicated concentrations, β-arrestin recruitment was evaluated with a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany). Each point corresponds to average values acquired for 20 min, represented as percentage of maximum full agonist response.

NanoBRET: HEK293T cells were co-transfected with pNeonGreen and pNLF vectors encoding ACKR2 C-terminally fused to mNeonGreen and -arrestin-1 N-terminally fused to Nanoluciferase. Twenty-four hours after transfection cells were harvested and upon

simultaneous addition of Nano-Glo Live Cell substrate (1:200) and chemokines, BRET signal was measured with a Mithras LB940 luminometer (Berthold Technologies) using a 460/70 BP filter for Nanoluciferase and a 515/40 BP filter for mNeonGreen signal.

4.4. Chemokine Binding

HEK293T and HEK-ACKR2 cells were incubated with CXCL10-Cy5 at indicated concentrations for 45 min at 37°C, then washed twice with FACS buffer (PBS, 1% BSA, 0.1% NaN3). Dead cells were excluded using Zombie Green viability dye (BioLegend). ACKR2-negative HEK293T cells were used to evaluate non-specific binding of CXCL10-Cy5. For binding competition with unlabelled chemokines (50 nM or 10 nM), the signal obtained for CXCL10-Cy5 (100 ng/mL) or CCL2-AF647 (30 ng/mL) in the absence of unlabelled chemokines was used to define 100% binding. Ligand binding was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

4.5. Chemokine-Induced Receptor Mobilisation to the Plasma Membrane

Ligand-induced receptor mobilisation to the plasma membrane was monitored by NanoBRET. A total of 5 x 10^6 HEK293T cells were seeded in 10 cm dishes and co-transfected with plasmids encoding ACKR2 C-terminally tagged with Nanoluciferase and mNeonGreen C-terminally tagged with the plasma membrane targeting polybasic sequence and prenylation signal sequence from K-RAS splice variant b [46]. Twenty-four hours after transfection, cells were distributed into black 96-well plates (1 x 10^5 cells per well) and treated with chemokines (100 nM). After 45 min incubation at 37°C, coelenterazine H (10 µM) was added, and donor emission (460 nm) and acceptor emission (535 nm) were immediately measured on a GloMax plate reader (Promega, Madison, WI, USA).

4.6. Chemokine-Induced Receptor-Arrestin Delivery to Endosomes

Ligand-induced receptor-arrestin delivery to early endosomes was monitored by NanoBRET. In brief, 5 x 10^6 HEK293T cells were seeded in 10 cm dishes and co-transfected with plasmids encoding ACKR2, β-arrestin-2 N-terminally tagged with Nanoluciferase and FYVE domain of endofin interacting with phosphatidylinositol 3-phosphate (PI3P) in early endosomes [46,47], N-terminally tagged with mNeonGreen. Twenty-four hours after transfection, cells were distributed into black 96-well plates (1 x 10^5 cells per well) and treated with full-length or processed chemokines. After 2 h incubation at 37°C, coelenterazine H (10 µM) was added, and donor emission (460 nm) and acceptor emission (535 nm) were immediately measured on a GloMax plate reader (Promega).

4.7. Chemokine Scavenging

Chemokine depletion from the extracellular space was quantified by ELISA. HEK293T and HEK-ACKR2 cells were incubated 8 h at 37°C with chemokines at 0.3 and 30 nM. Chemokine scavenging by ACKR2 was evaluated by quantifying the concentration ofchemokines remaining in the supernatant using commercially available ELISA kits (CXCL10 R&D Systems, CCL5 BioLegend and CXCL11 Peprotech, Rocky Hill, NJ, USA) and was expressed as the percentage of input chemokine concentrations.

4.8. Chemokine Internalization

Chemokine internalization using labelled CXCL10 or CCL2 was visualized by imaging flow cytometry as previously described [7]. HEK.293T or HEK-ACKR2 cells were incubated 15 min at 37°C in the presence or absence of unlabelled chemokines (200 nM) after which Cy5-labelled CXCL10 (100 nM) or AF647-labelled CCL2 (100 ng/mL) was added for 45 min at 37°C. Cells were washed twice with FACS buffer. Dead cells were excluded using Zombie Green viability dye (BioLegend). Images of 1 x 10^4 in-focus living single cells were acquired with an ImageStream MKII imaging flow cytometer (Amnis Luminex, Austin, TX, USA) using 60x magnification. Samples were analysed using Ideas6.2 software. The number of spots per cell was determined using a mask-based software wizard.

For confocal microscopy, 4 x 104 HEK-ACKR2 cells/well were seeded on poly-L-Lysine coated 8-well chamber slides (µ-Slide 8 well, Ibidi, Fitchburg, WI, USA). After 36 h, cells were incubated 2 h at 37°C with 100 nM Cy5-labelled chemokines (CXCL10, CXCL11 or CCL2) and co-incubated one additional hour with 750 nM LysoTracker™ Red DND-99 (ThermoFisher, Schwerte, Germany). Cells were then washed twice with PBS, fixed with 3.5 % (w/v) paraformaldehyde for 20 min at room temperature and washed again twice with PBS. Nuclear staining was performed with Hoechst 33342 dye (1 µg/mL) for 20 min at room temperature, and cells were washed 3 times with PBS. Images were acquired on a Zeiss LSM880 confocal microscope using a 63 oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss, Jena, Germany). Representative cells from 12 image acquisitions of three independent experiments are shown.

4.9. Inhibition of Chemokine Uptake by Anti-mACKR2 Antibodies

HEK-mACKR2 or B16-F10 cells were incubated 45 min at 37°C with Cy5-labelled mCXCL10 (100 nM) in the presence or absence of the polyclonal goat anti-mACKR2 antibody (50 µg/mL) (ab1656, Abcam) or goat IgG control antibody (ab37373, Abcam) and the secondary donkey anti-goat-AF647 antibody (Jackson ImmunoResearch,West Grove, PA, USA). Dead cells were excluded using Zombie Green viability dye (BioLegend). Ligand uptake was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences). Inhibition of mCXCL10 scavenging by anti-mACKR2 was expressed as the percentage relative to conditions where the antibody was absent.

4.10. Data and Statistical Analysis

Concentration–response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 8.0.1) to provide EC50 values and standard errors of the mean. All curves were fitted to data points generated from the mean of at least three independent experiments. All statistical tests, i.e., t-tests, ordinary one-way ANOVA and post hoc analysis, were performed with GraphPad Prism 8.0.1. p-values are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5. Results and Discussion

The pairing of ACKR2 with CC chemokines dates back to when many chemokines, especially the CXC chemokines, had not yet been known or available [12,15,48]. Recent identification of CCL20 and CCL22 as ligands for ACKR4 [49,50] demonstrates that some pairings within the complex chemokine–receptor interaction network may have been overlooked. Several reports point to increased CXC chemokine levels in ACKR2-deficient mice [51,52], and an indirect crosstalk between the orphan CXCL14 and ACKR2 has recently been described [53]. These observations prompted us to re-evaluate the ability of ACKR2 to scavenge chemokines also from the CXC family.

First, we assessed the activity of the 16 human CXC chemokines (100 nM) towards ACKR2 by monitoring their ability to induce β-arrestin-1 recruitment using Nanoluciferase complementation-based assay (NanoBiT). Our screening revealed that at least three CXC chemokines, namely CXCL2, CXCL10 and CXCL12, are capable of inducing β-arrestin-1 recruitment to ACKR2. However, only CXCL10 reached statistical significance in this assay (Figure 1A).

To evaluate the functional relevance of the interactions between these chemokines and ACKR2, especially in light of a possible scavenging function, we next performed an in-depth analysis of intracellular events and monitored the fate of the chemokines and receptor following their interactions.

CXCL2 and CXCL12 consistently showed reduced potency and efficacy in β-arrestin recruitment towards ACKR2 compared to CXCL10 or to the activity they display towards their already known receptors [45,54–56] (Figure 1B,E,H,I). Given this limited activity, they were not further investigated. CXCL10, however, showed a strong potency towards ACKR2 (EC50 = 8.2 nM, pEC50 = 8.08 ± 0.14) and induced approximately half of the maximal response compared to the full agonist CCL5 (Figure 1B). This partial agonist behaviour of CXCL10 was reminiscent of the activity towards its long-established signaling receptor CXCR3 relative to the full agonist CXCL11 (Figure 1C,F,G) [57,58]. The potency of CXCL10 towards ACKR2 appears approximately 3 times stronger than towards CXCR3 (EC50= 24.9 nM, pEC50 = 7.60 0.12), consistent with a potential scavenging role of ACKR2. In NanoBRET, the potency of CXCL10 towards ACKR2 (EC50 = 5.1 nM, pEC50 = 8.29 ± 0.11) was close to that of CCL2 and approximately 20-fold stronger than towards CXCR3. The

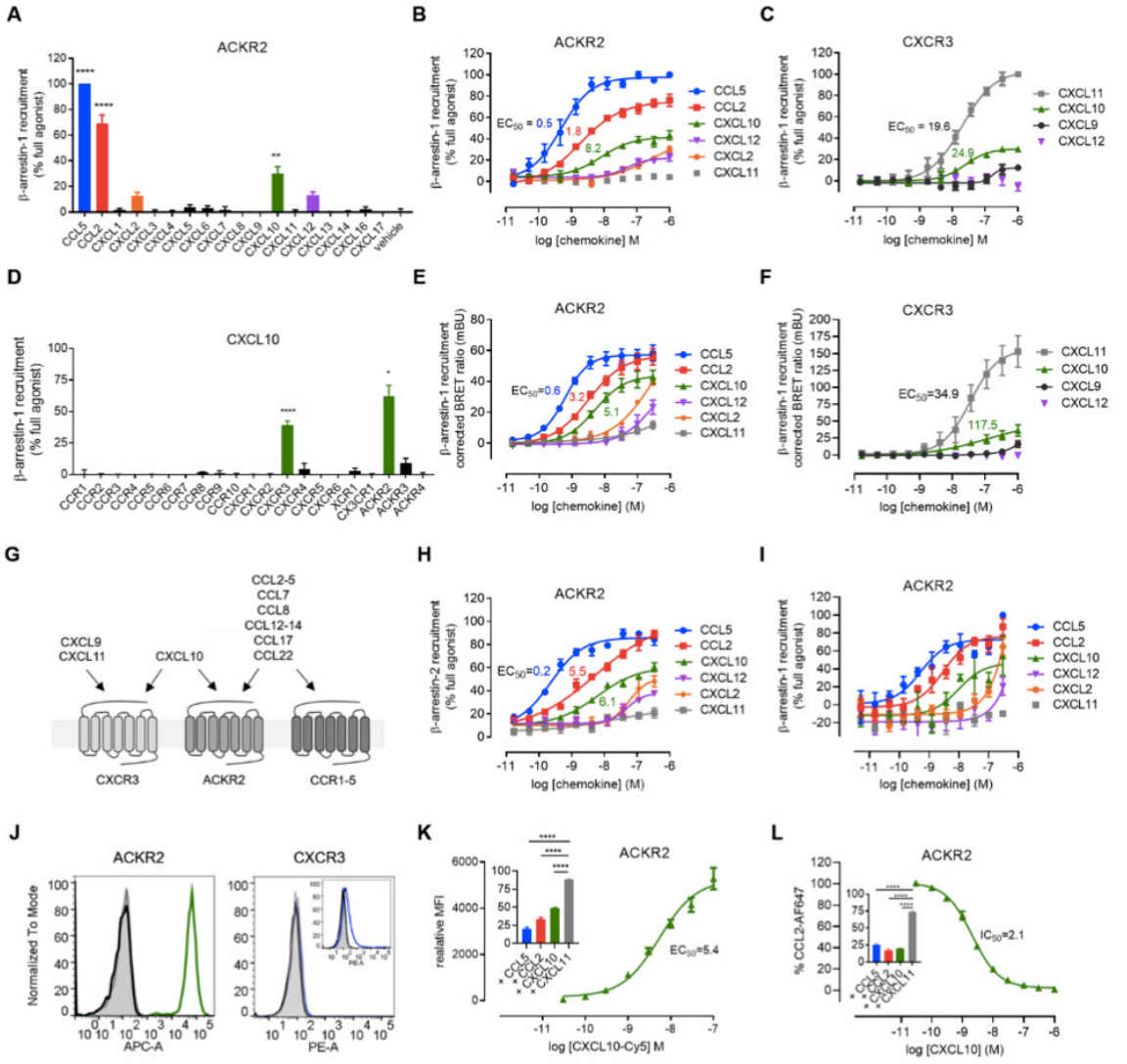

Figure 1 | ACKR2 activation by CXCL10. (A) β-arrestin-1 recruitment to ACKR2 in response to all known human CXC chemokines (100 nM) monitored by NanoBiT-based assay. CCL2 and CCL5 were used as positive control chemokines. (B) β-arrestin-1 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 and CXCL12 monitored by NanoBiT, showing the concentration–response relationship. CXCL11 was used as negative control. (C) β-arrestin-1 recruitment to CXCR3 induced by its cognate ligands CXCL9, CXCL10 and CXCL11 monitored by NanoBiT. CXCL12 was used as negative control. (D) β-arrestin-1 recruitment to all known chemokine receptors in response to CXCL10 (100 nM). (E,F) β-arrestin-1 recruitment to ACKR2 (E) and CXCR3 (F) monitored by NanoBRET. (G) Schematic representation of chemokine–receptor interactions between ACKR2, CXCR3 and the CC receptors CCR1, CCR2, CCR3, CCR4 and CCR5, including the newly identified pairing between CXCL10 and ACKR2. (H) β-arrestin-2 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 and CXCL12 monitored by NanoBiT. (I) β-arrestin-1 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 and CXCL12 monitored by NanoBiT in U87.MG cells. (J) Flow cytometry analysis of cells used in the binding studies, left panel: ACKR2 surface expression in HEK-ACKR2 (green histogram) and the parental HEK293T cell line (greyfilled histogram) evaluated using the ACKR2-specific mAb (clone 196124) or the corresponding isotype control (black histogram); right panel: CXCR3 surface expression in HEK-ACKR2 evaluated using the CXCR3-specific mAb (clone 1C6) (blue histogram) and the corresponding isotype control (black histogram). Unstained cells are represented as grey filled histogram. (inset) Positive control surface expression staining for CXCR3 in HEK293T cells transiently transfected with a CXCR3-encoding vector, using CXCR3-specific mAb (clone 1C6) (blue histogram) and the corresponding isotype control (black histogram). (K) Binding of Cy5-labelled CXCL10 to HEK-ACKR2 cells. (inset) Binding competition (100 ng/mL CXCL10-Cy5) with unlabelled chemokines (50 nM). (L) Binding competition of unlabelled CXCL10 with Alexa Fluor 647-labelled CCL2 (30 ng/mL) on HEK-ACKR2 cells. (inset) Binding competition with unlabelled chemokines (10 nM). EC50 and IC50 values for concentration–response curves (B–L) are indicated (nM). All NanoBiT and NanoBRET assays were conducted in HEK293T cells except for (I) for which U87.MG cells were used. Data points represent mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by one-way ANOVA with Dunnett (A,D) and Bonferroni (K,L) post hoc tests.

efficacy of CXCL10 in this assay reached approximately 70% of the maximal signal measured with CCL5 (Figure 1E,F). Similar observations were made for the recruitment of β-arrestin-2 (Figure 1H) and were further confirmed in a different cellular background (Figure 1I). Moreover, the screening of CXCL10 on 23 chemokine receptors showed that CXCR3 and ACKR2 are the only human receptors activated by CXCL10 (Figure 1D). Fluorescently labelled CXCL10 also strongly and specifically bound to HEK293T cells expressing ACKR2 (IC50 = 5.4, pIC50 = 8.27 0.09) (Figure 1J,K) and was only displaced by ACKR2-related chemokines CCL5, CCL2 and by CXCL10 itself (Figure 1K inset). Inversely, binding competition studies showed that CXCL10 was able to fully displace fluorescently labelled CCL2 from the receptor with an IC50 of 2.1 nM (pIC50 = 8.68 ± 0.03) (Figure 1L).

The ability of ACKR2 to mediate CXCL10 scavenging and control its extracellular concentration was then analysed. CXCL10 stimulation resulted in rapid mobilization of intracellular ACKR2 to the plasma membrane reminiscent of the activity of CC chemokines [59,60] (Figure 2A). The CXCL10-induced receptor mobilisation was followed by its delivery to the endosomes with an EC50 of 6.0 nM (pEC50 = 8.22 ± 0.06) (Figure 2B,C). Imaging flow cytometry also revealed specific and efficient uptake of labelled CXCL10 by ACKR2-expressing cells. A notably higher number of distinguishable intracellular vesiclelike structures and mean fluorescent intensity were observed compared to HEK293T cells or HEK-ACKR2 cells pre-treated with CCL5 (Figure 2D,F). Confocal microscopy further confirmed CXCL10 uptake and in addition showed its distribution within acidic intracellular vesicles (Figure 2E). Moreover, the uptake of CXCL10 by ACKR2 was more efficient compared to that by CXCR3, consistent with the stronger potency of CXCL10 towards ACKR2 and the possible scavenging function (Figure 2G). As an additional selectivity control, CXCL10—just like CCL5 and CCL2—was able to compete with the uptake of fluorescently labelled CCL2 by ACKR2-expressing cells in imaging flow cytometry (Figure 2H). Importantly, the ACKR2-driven intracellular accumulation of CXCL10 was also associated with a reduction of its availability in the extracellular space as demonstrated by ELISA quantification. The efficiency of ACKR2-driven CXCL10 scavenging was similar at high (30 nM) and low (0.3 nM) chemokine concentrations (Figure 2I) and was comparable to the depletion of CCL5, while no reduction was observed for CXCL11. The interaction between CXCL10 and ACKR2 was also observed with the murine counterparts, as illustrated by the uptake of labelled murine CXCL10 (mCXCL10) by HEK-mACKR2 cells or the mouse melanoma cell line B16.F10, which was partially inhibited by mACKR2-specific polyclonal antibody but not the isotype control (Figure 2J).

Similar to many other CC and CXC chemokines, CXCL10 was shown to be subject to post-translational modification by proteolytic enzymes [61]. In particular, N-terminal cleavage by the dipeptidyl peptidase 4 (DPP4 or CD26) was demonstrated to turn CXCL10 from CXCR3 agonist to antagonist [62]. Based on recent reports demonstrating that, in

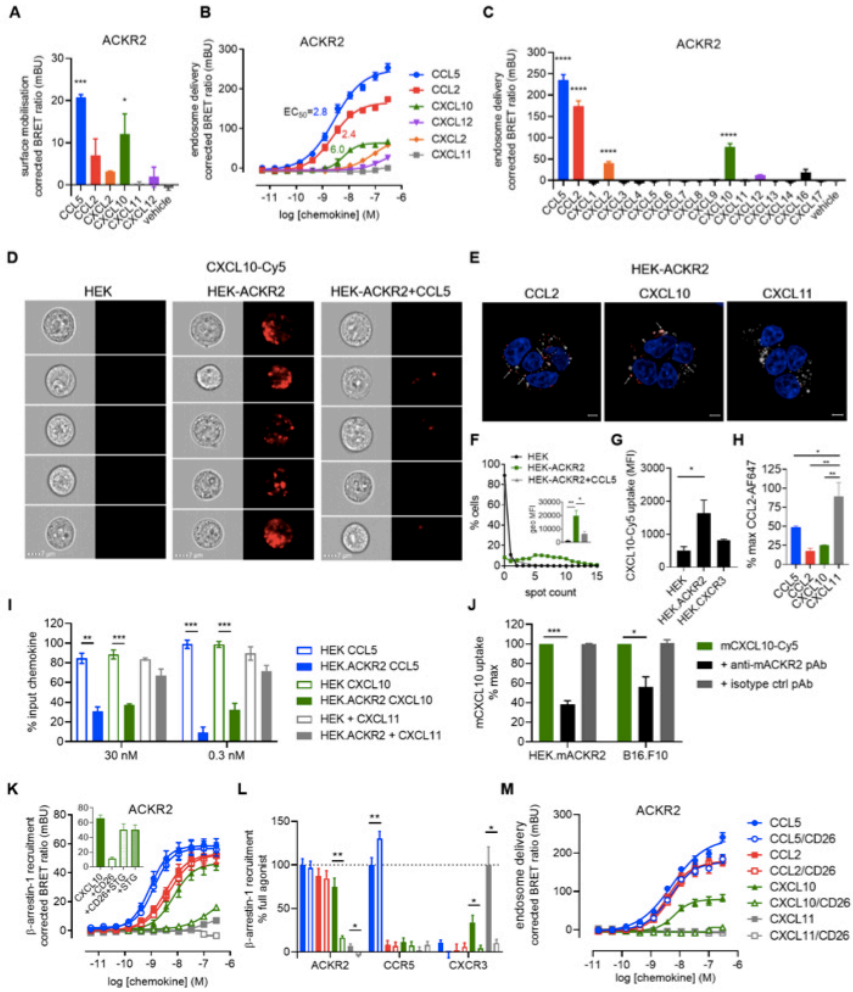

Figure 2 | CXCL10 scavenging by ACKR2. (A) ACKR2 mobilisation to the plasma membrane in response to chemokines (100 nM) monitored by NanoBRET-based assay. (B,C) β-arrestin-1/ACKR2 complex delivery to the early endosomes in response to the CXC chemokines CXCL2, CXCL10 and CXCL12 (B) or the 16 human CXC chemokines (100 nM) (C) monitored by NanoBRET-based assay. CCL2 and CCL5 were used a positive control chemokines. (D–F) Uptake of fluorescently labelled CXCL10 by ACKR2-expressing cells visualized by imaging flow cytometry (D,F) and confocal microscopy (E). (D) HEK, HEK-ACKR2 or HEK-ACKR2 cells pre-treated with CCL5 at saturating concentration (200 nM) were stimulated for 45 min at 37°C with 100 nM (Cy5)-labelled CXCL10 (CXCL10-Cy5, red channel). Five representative cells for each condition are shown (10,000 events recorded). Scale bar: 7 µm. (F) Percentage of cells from (D) with a given number of distinguishable vesicle-like structures (spots), as well as the geometrical mean fluorescence intensity (MFI) for the red channel were determined (inset). Data shown are representative of three independent experiments and for inset, mean ± SEM of three independent experiments. (E) Cellular localization of Cy5-labelled chemokine (red) following HEK-ACKR2 stimulation (100 nM) for 2 h monitored by fluorescent confocal microscopy. Lysosomes and nucleic DNA were stained using LysoTracker™ Red DND-99 (white) and Hoechst 33342 (blue), respectively. Pictures are representative of 12 acquired images from three independent experiments. Scale bar: 5 m. Arrows highlight colocalization of Lysotracker and chemokine-Cy5 signal. (G) Uptake of Cy5-labelled chemokine (100 nM) by HEK cells transfected or not with equal amounts of ACKR2 or CXCR3 vectors analysed by imaging flow cytometry as described in (D). (H) Binding competition between Alexa Fluor 647-labelled CCL2 (100 ng/mL) and unlabelled chemokines (100 nM) in HEK-ACKR2 analysed by imaging flow cytometry. (I) ACKR2-mediated depletion of extracellular CXCL10 monitored by ELISA. Chemokines in the supernatant of HEK293T cells expressing or not ACKR2 were quantified after 8 h stimulation, and expressed as percentage of the input concentrations (30 nM and 0.3 nM). CCL5 and CXCL11 were used as positive and negative controls, respectively. Data points represent mean ± SEM of three independent experiments. (J) Inhibition of mACKR2-mediated mCXCL10 uptake by neutralizing antibodies. Cy5-labelled mouse CXCL10 (mCXCL10-Cy5) (100 nM) was incubated with HEK-mACKR2 or B16.F10 in the presence of mACKR2-specific polyclonal antibody (Ab1656) or corresponding isotype control (Ab37373) for 45 min at 37°C and analysed by flow cytometry. (K–M) Impact of chemokine N-terminal processing by dipeptidyl peptidase 4 (DPP4/CD26) on the activation of ACKR2 and related receptors CXCR3 and CCR5 and ACKR2 delivery to the endosomes. (K,L) β-arrestin-1 recruitment to ACKR2 by processed chemokines monitored by NanoBRET. (L) Comparison of the impact of N-terminal processing on the ability of CXC and CC chemokines (100 nM) to induce β-arrestin-1 recruitment to ACKR2, CXCR3 and CCR5. (Inset) Comparison of ACKR2 activity induced by unprocessed CXCL10 or CXCL10 treated with CD26 in the presence or absence of its specific inhibitor, sitagliptin (STG) (10 M) or with STG alone, demonstrating no interference between CD26 and the

ACKR2-CXCL10 interaction. (M) β-arrestin-1/ACKR2 complex delivery to the early endosomes in response to processed chemokines monitored by NanoBRET. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA with Dunnet (A,C) and Bonferroni (H) post hoc tests or repeated measures one-way ANOVA with Bonferroni post hoc test (J) and two-tailed unpaired Student's t-test (I).

contrast to CXCR3, ACKR3 is responsive to DPP4-inactivated CXCL11 [45], the impact of the CXCL10 N-terminal processing on ACKR2 activation was evaluated and compared to CXCR3. We observed that, in contrast to CC chemokines, truncation of CXCL10 drastically reduced its ability to induce β-arrestin-1 recruitment to ACKR2 (Figure 2K,L) and subsequent receptor targeting to the early endosomes (Figure 2M), indicating that CXCL10 N-terminal residues are critical for its activity towards ACKR2 [60,63]. The uptake of CD26-processed CXCL10 by ACKR2-positive cells was also highly reduced and, similar to the full-length chemokine, competed out by non-truncated CXCL10 or ACKR2-related CC chemokines (data not shown). These results, in addition to partial agonist behavior of CXCL10, point to distinct ACKR2 interaction and activation modes compared to CC chemokines. This may be attributed to notable differences in the N terminus orientation and occupation of the receptor binding pockets of CXC and CC chemokines [64].

6. Conclusions

In conclusion, our study shows that CXCL10 is a novel ACKR2 ligand. CXCL10 is one of the most important inflammatory CXC chemokines and is involved in many physiological and pathological processes such as angiogenesis, chronic inflammation, immune dysfunction, tumour development and dissemination [65,66], in which ACKR2 has also been shown to play critical roles [35]. Together with CCL5, CXCL10 is a key player in driving NK cells and CD8+ T cells into the tumour bed [37,38,40,41]. This novel pairing consequently adds an unforeseen level of complexity to ACKR2 functions and a new level of CXCL10 regulation and could thus encourage re-examination of previous studies taking into account CXCL10–ACKR2 interactions (Figure 1G) [27,51,52,65,67].

The ability to bind and respond to both CXC and CC chemokines has already been reported for ACKR1 [68], ACKR3 [69] and ACKR4 [70], although this property has recently been challenged for the latter. Here, we identified an agonist CXC ligand for ACKR2, which until now has been recognised for binding inflammatory CC chemokines only. Therefore, such cross-family spectrum of chemokine ligands, uncommon among the classical chemokine receptors, seems to represent an additional functional property of ACKRs [2] besides their inability to trigger G protein signalling. Overall, this study highlights that a systematic reassessment of chemokine–receptor pairings for both long-established and recently deorphanized receptors may be necessary, as important interactions may have been overlooked.

References

1. Graham, G.J.; Locati, M.; Mantovani, A.; Rot, A.; Thelen, M. The biochemistry and biology of the atypical chemokine receptors. Immunol. Lett. 2012, 145, 30–38.
2. Bachelerie, F.; Graham, G.J.; Locati, M.; Mantovani, A.; Murphy, P.M.; Nibbs, R.; Rot, A.; Sozzani, S.; Thelen, M. New nomenclature for atypical chemokine receptors. Nat. Immunol. 2014, 15, 207–208.
3. Weber, M.; Blair, E.; Simpson, C.V.; O'Hara, M.; Blackburn, P.E.; Rot, A.; Graham, G.J.; Nibbs, R.J. The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. Mol. Biol. Cell 2004, 15, 2492–2508.
4. Galliera, E.; Jala, V.R.; Trent, J.O.; Bonecchi, R.; Signorelli, P.; Lefkowitz, R.J.; Mantovani, A.; Locati, M.; Haribabu, B. beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. J. Biol. Chem. 2004, 279, 25590–25597.
5. Vacchini, A.; Cancellieri, C.; Milanesi, S.; Badanai, S.; Savino, B.; Bifari, F.; Locati, M.; Bonecchi, R.; Borroni, E.M. Control of Cytoskeletal Dynamics by beta-Arrestin1/Myosin Vb Signaling Regulates Endosomal Sorting and Scavenging Activity of the Atypical Chemokine Receptor ACKR2. Vaccines 2020, 8, 542.
6. Comerford, I.; Milasta, S.; Morrow, V.; Milligan, G.; Nibbs, R. The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. Eur. J. Immunol. 2006, 36, 1904–1916.
7. Meyrath, M.; Szpakowska, M.; Zeiner, J.; Massotte, L.; Merz, M.P.; Benkel, T.; Simon, K.; Ohnmacht, J.; Turner, J.D.; Kruger, R.; et al. The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides. Nat. Commun. 2020, 11, 1–16.
8. McCulloch, C.V.; Morrow, V.; Milasta, S.; Comerford, I.; Milligan, G.; Graham, G.J.; Isaacs, N.W.; Nibbs, R.J. Multiple roles for the C-terminal tail of the chemokine scavenger D6. J. Biol. Chem. 2008, 283, 7972–7982.
9. Montpas, N.; St-Onge, G.; Nama, N.; Rhainds, D.; Benredjem, B.; Girard, M.; Hickson, G.; Pons, V.; Heveker, N. Ligand-specific conformational transitions and intracellular transport are required for atypical chemokine receptor 3-mediated chemokine scavenging. J. Biol. Chem. 2018, 293, 893–905.
10. Saaber, F.; Schutz, D.; Miess, E.; Abe, P.; Desikan, S.; Ashok Kumar, P.; Balk, S.; Huang, K.; Beaulieu, J.M.; Schulz, S.; et al. ACKR3 Regulation of Neuronal Migration Requires ACKR3 Phosphorylation, but Not beta-Arrestin. Cell Rep. 2019, 26, 1473–1488.e1479.
11. Matti, C.; Salnikov, A.; Artinger, M.; D'Agostino, G.; Kindinger, I.; Uguccioni, M.; Thelen, M.; Legler, D.F. ACKR4 Recruits GRK3 Prior to beta-Arrestins but Can Scavenge Chemokines in the Absence of beta-Arrestins. Front. Immunol. 2020, 11, 720.
12. Nibbs, R.J.; Wylie, S.M.; Pragnell, I.B.; Graham, G.J. Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein-1alpha receptors, CCR-1, CCR-3, and CCR-5. J. Biol. Chem. 1997, 272, 12495–12504.
13. Fra, A.M.; Locati, M.; Otero, K.; Sironi, M.; Signorelli, P.; Massardi, M.L.; Gobbi, M.; Vecchi, A.; Sozzani, S.; Mantovani, A. Cutting edge: Scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. J. Immunol. 2003, 170, 2279–2282.
14. Locati, M.; Torre, Y.M.; Galliera, E.; Bonecchi, R.; Bodduluri, H.; Vago, G.; Vecchi, A.; Mantovani, A. Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. Cytokine Growth Factor Rev. 2005, 16, 679–686.
15. Bonini, J.A.; Martin, S.K.; Dralyuk, F.; Roe, M.W.; Philipson, L.H.; Steiner, D.F. Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. DNA Cell Biol. 1997, 16, 1249–1256.
16. Jamieson, T.; Cook, D.N.; Nibbs, R.J.; Rot, A.; Nixon, C.; McLean, P.; Alcami, A.; Lira, S.A.; Wiekowski, M.; Graham, G.J. The chemokine receptor D6 limits the inflammatory response in vivo. Nat. Immunol. 2005, 6, 403–411.
17. Lee, K.M.; McKimmie, C.S.; Gilchrist, D.S.; Pallas, K.J.; Nibbs, R.J.; Garside, P.; McDonald, V.; Jenkins, C.; Ransohoff, R.; Liu, L.; et al. D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. Blood 2011, 118, 6220–6229.
18. Singh, M.D.; King, V.; Baldwin, H.; Burden, D.; Thorrat, A.; Holmes, S.; McInnes, I.B.; Nicoll, R.; Shams, K.; Pallas, K.; et al. Elevated expression of the chemokine-scavenging receptor D6 is associated with impaired lesion development in psoriasis. Am. J. Pathol. 2012, 181, 1158–1164.
19. Bonecchi, R.; Graham, G.J. Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response. Front.Immunol. 2016, 7, 224.
20. Lee, K.M.;Wilson, G.J.; Pingen, M.; Fukuoka, A.; Hansell, C.A.H.; Bartolini, R.; Medina-Ruiz, L.; Graham, G.J. Placental chemokine compartmentalisation: A novel mammalian molecular control mechanism. PLoS Biol. 2019, 17, e3000287.
21. Martinez de la Torre, Y.; Locati, M.; Buracchi, C.; Dupor, J.; Cook, D.N.; Bonecchi, R.; Nebuloni, M.; Rukavina, D.; Vago, L.; Vecchi, A.; et al. Increased inflammation in mice deficient for the chemokine decoy receptor D6. Eur. J. Immunol. 2005, 35, 1342–1346.
22. Teoh, P.J.; Menzies, F.M.; Hansell, C.A.; Clarke, M.; Waddell, C.; Burton, G.J.; Nelson, S.M.; Nibbs, R.J. Atypical chemokine receptor ACKR2 mediates chemokine scavenging by primary human trophoblasts and can regulate fetal growth, placental structure, and neonatal mortality in mice. J. Immunol. 2014, 193, 5218–5228.
23. Hansell, C.A.; Schiering, C.; Kinstrie, R.; Ford, L.; Bordon, Y.; McInnes, I.B.; Goodyear, C.S.; Nibbs, R.J. Universal expression and dual function of the atypical chemokine receptor D6 on innate-like B cells in mice. Blood 2011, 117, 5413–5424.
24. McKimmie, C.S.; Singh, M.D.; Hewit, K.; Lopez-Franco, O.; Le Brocq, M.; Rose-John, S.; Lee, K.M.; Baker, A.H.; Wheat, R.; Blackbourn, D.J.; et al. An analysis of the function and expression of D6 on lymphatic endothelial cells. Blood 2013, 121, 3768–3777.
25. Savino, B.; Castor, M.G.; Caronni, N.; Sarukhan, A.; Anselmo, A.; Buracchi, C.; Benvenuti, F.; Pinho, V.; Teixeira, M.M.; Mantovani, A.; et al. Control of murine Ly6C(high) monocyte traffic and immunosuppressive activities by atypical chemokine receptor D6. Blood 2012, 119, 5250–5260.
26. Castanheira, F.; Borges, V.; Sonego, F.; Kanashiro, A.; Donate, P.B.; Melo, P.H.; Pallas, K.; Russo, R.C.; Amaral, F.A.; Teixeira, M.M.; et al. The Atypical Chemokine Receptor ACKR2 is Protective Against Sepsis. Shock 2018, 49, 682–689.
27. Massara, M.; Bonavita, O.; Savino, B.; Caronni, N.; Mollica Poeta, V.; Sironi, M.; Setten, E.; Recordati, C.; Crisafulli, L.; Ficara, F.; et al. ACKR2 in hematopoietic precursors as a checkpoint of neutrophil release and anti-metastatic activity. Nat. Commun. 2018, 9, 1–11.
28. Lee, K.M.; Danuser, R.; Stein, J.V.; Graham, D.; Nibbs, R.J.; Graham, G.J. The chemokine receptors ACKR2 and CCR2 reciprocally regulate lymphatic vessel density. Embo J. 2014, 33, 2564–2580.
29. Nibbs, R.J.; Graham, G.J. Immune regulation by atypical chemokine receptors. Nat. Rev. Immunol. 2013, 13, 815–829.
30. Shams, K.;Wilson, G.J.; Singh, M.; van den Bogaard, E.H.; Le Brocq, M.L.; Holmes, S.; Schalkwijk, J.; Burden, A.D.; McKimmie, C.S.; Graham, G.J. Spread of Psoriasiform Inflammation to Remote Tissues Is Restricted by the Atypical Chemokine Receptor ACKR2. J. Invest. Derm. 2017, 137, 85–94.
31. Liu, L.; Graham, G.J.; Damodaran, A.; Hu, T.; Lira, S.A.; Sasse, M.; Canasto-Chibuque, C.; Cook, D.N.; Ransohoff, R.M. Cutting edge: The silent chemokine receptor D6 is required for generating T cell responses

that mediate experimental autoimmune encephalomyelitis. J. Immunol. 2006, 177, 17–21.
32. Pashover-Schallinger, E.; Aswad, M.; Schif-Zuck, S.; Shapiro, H.; Singer, P.; Ariel, A. The atypical chemokine receptor D6 controls macrophage efferocytosis and cytokine secretion during the resolution of inflammation. FASEB J. 2012, 26, 3891–3900.
33. Aswad, M.; Assi, S.; Schif-Zuck, S.; Ariel, A. CCL5 Promotes Resolution-Phase Macrophage Reprogramming in Concert with the Atypical Chemokine Receptor D6 and Apoptotic Polymorphonuclear Cells. J. Immunol. 2017, 199, 1393–1404.
34. Hansell, C.A.H.; Fraser, A.R.; Hayes, A.J.; Pingen, M.; Burt, C.L.; Lee, K.M.; Medina-Ruiz, L.; Brownlie, D.; Macleod, M.K.L.; Burgoyne, P.; et al. The Atypical Chemokine Receptor Ackr2 Constrains NK Cell Migratory Activity and Promotes Metastasis. J. Immunol. 2018, 201, 2510–2519.
35. Sjoberg, E.; Meyrath, M.; Chevigne, A.; Ostman, A.; Augsten, M.; Szpakowska, M. The diverse and complex roles of atypical chemokine receptors in cancer: From molecular biology to clinical relevance and therapy. Adv. Cancer Res. 2020, 145, 99–138.
36. Nibbs, R.J.; Gilchrist, D.S.; King, V.; Ferra, A.; Forrow, S.; Hunter, K.D.; Graham, G.J. The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. J. Clin. Investig. 2007, 117, 1884–1892.
37. Noman, M.Z.; Parpal, S.; Van Moer, K.; Xiao, M.; Yu, Y.; Viklund, J.; De Milito, A.; Hasmim, M.; Andersson, M.; Amaravadi, R.K. et al. Inhibition of Vps34 reprograms cold into hot inflamed tumors and improves anti-PD-1/PD-L1 immunotherapy. Sci. Adv. 2020, 6, eaax7881.
38. Nagarsheth, N.; Wicha, M.S.; Zou, W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nat. Rev. Immunol. 2017, 17, 559–572.
39. Maru, S.V.; Holloway, K.A.; Flynn, G.; Lancashire, C.L.; Loughlin, A.J.; Male, D.K.; Romero, I.A. Chemokine production and chemokine receptor expression by human glioma cells: Role of CXCL10 in tumour cell proliferation. J. Neuroimmunol. 2008, 199, 35–45.
40. Zumwalt, T.J.; Arnold, M.; Goel, A.; Boland, C.R. Active secretion of CXCL10 and CCL5 from colorectal cancer microenvironments associates with GranzymeB+ CD8+ T-cell infiltration. Oncotarget 2015, 6, 2981–2991.
41. Mikucki, M.E.; Fisher, D.T.; Matsuzaki, J.; Skitzki, J.J.; Gaulin, N.B.; Muhitch, J.B.; Ku, A.W.; Frelinger, J.G.; Odunsi, K.; Gajewski, T.F.; et al. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. Nat. Commun. 2015, 6, 1–14.
42. Russo, R.C.; Savino, B.; Mirolo, M.; Buracchi, C.; Germano, G.; Anselmo, A.; Zammataro, L.; Pasqualini, F.; Mantovani, A.; Locati, M.; et al. The atypical chemokine receptor ACKR2 drives pulmonary fibrosis by tuning influx of CCR2(+) and CCR5(+) IFNgamma-producing gammadeltaT cells in mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 2018, 314, L1010–L1025.
43. Dixon, A.S.; Schwinn, M.K.; Hall, M.P.; Zimmerman, K.; Otto, P.; Lubben, T.H.; Butler, B.L.; Binkowski, B.F.; Machleidt, T.; Kirkland, T.A.; et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS Chem. Biol. 2016, 11, 400–408.
44. Szpakowska, M.; Meyrath, M.; Reynders, N.; Counson, M.; Hanson, J.; Steyaert, J.; Chevigne, A. Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists. Biochem. Pharm. 2018, 153, 299–309.
45. Szpakowska, M.; Nevins, A.M.; Meyrath, M.; Rhainds, D.; D'Huys, T.; Guite-Vinet, F.; Dupuis, N.; Gauthier, P.A.; Counson, M.; Kleist, A.; 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. Pharm. 2018, 175, 1419–1438.
46. Namkung, Y.; Le Gouill, C.; Lukashova, V.; Kobayashi, H.; Hogue, M.; Khoury, E.; Song, M.; Bouvier, M.; Laporte, S.A. Monitoring G protein-coupled receptor and beta-arrestin trafficking in live cells using enhanced bystander BRET. Nat. Commun. 2016, 7, 1–12.
47. Schink, K.O.; Raiborg, C.; Stenmark, H. Phosphatidylinositol 3-phosphate, a lipid that regulates membrane dynamics, protein sorting and cell signalling. Bioessays 2013, 35, 900–912.
48. Nibbs, R.J.; Wylie, S.M.; Yang, J.; Landau, N.R.; Graham, G.J. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. J. Biol. Chem. 1997, 272, 32078–32083.
49. Meyrath, M.; Reynders, N.; Uchanski, T.; Chevigne, A.; Szpakowska, M. Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22. J. Leukoc. Biol. 2020, 109, 373–376.
50. Matti, C.; D'Uonnolo, G.; Artinger, M.; Melgrati, S.; Salnikov, A.; Thelen, S.; Purvanov, V.; Strobel, T.D.; Spannagel, L.; Thelen, M.; et al. CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4. J. Leukoc. Biol. 2020, 107, 1137–1154.
51. Lux,M.; Blaut, A.; Eltrich, N.; Bideak, A.; Muller, M.B.; Hoppe, J.M.; Grone, H.J.; Locati, M.; Vielhauer, V. The Atypical Chemokine Receptor 2 Limits Progressive Fibrosis after Acute Ischemic Kidney Injury. Am. J. Pathol. 2019, 189, 231–247.
52. Bideak, A.; Blaut, A.; Hoppe, J.M.; Muller, M.B.; Federico, G.; Eltrich, N.; Grone, H.J.; Locati, M.; Vielhauer, V. The atypical chemokine receptor 2 limits renal inflammation and fibrosis in murine progressive immune complex glomerulonephritis. Kidney Int. 2018, 93, 826–841.
53. Sjoberg, E.; Meyrath, M.; Milde, L.; Herrera, M.; Lovrot, J.; Hagerstrand, D.; Frings, O.; Bartish, M.; Rolny, C.; Sonnhammer, E.; et al. A Novel ACKR2-Dependent Role of Fibroblast-Derived CXCL14 in Epithelial-to-Mesenchymal Transition and Metastasis of Breast Cancer. Clin. Cancer Res. 2019, 25, 3702–3717.
54. Chevigne, A.; Fievez, V.; Szpakowska, M.; Fischer, A.; Counson, M.; Plesseria, J.M.; Schmit, J.C.; Deroo, S. Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1 infection. Biochim. Biophys. Acta 2014, 1843, 1031–1041.
55. Ahuja, S.K.; Murphy, P.M. The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophilactivating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J. Biol. Chem. 1996, 271, 20545–20550.
56. Wolf, M.; Delgado, M.B.; Jones, S.A.; Dewald, B.; Clark-Lewis, I.; Baggiolini, M. Granulocyte chemotactic protein 2 acts via both IL-8 receptors, CXCR1 and CXCR2. Eur. J. Immunol. 1998, 28, 164–170.
57. Scholten, D.J.; Canals, M.;Wijtmans, M.; de Munnik, S.; Nguyen, P.; Verzijl, D.; de Esch, I.J.; Vischer, H.F.; Smit, M.J.; Leurs, R. Pharmacological characterization of a small-molecule agonist for the chemokine receptor CXCR3. Br. J. Pharm. 2012, 166, 898–911.
58. Berchiche, Y.A.; Sakmar, T.P. CXC Chemokine Receptor 3 Alternative Splice Variants Selectively Activate Different Signaling Pathways. Mol. Pharm. 2016, 90, 483–495.
59. Bonecchi, R.; Borroni, E.M.; Anselmo, A.; Doni, A.; Savino, B.; Mirolo, M.; Fabbri, M.; Jala, V.R.; Haribabu, B.; Mantovani, A.; et al. Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation. Blood 2008, 112, 493–503.
60. Savino, B.; Borroni, E.M.; Torres, N.M.; Proost, P.; Struyf, S.; Mortier, A.; Mantovani, A.; Locati, M.; Bonecchi, R. Recognition versus adaptive up-

regulation and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence. J. Biol. Chem. 2009, 284, 26207–26215.
61. Mortier, A.; Gouwy, M.; Van Damme, J.; Proost, P.; Struyf, S. CD26/dipeptidylpeptidase IV-chemokine interactions: Double-edged regulation of inflammation and tumor biology. J. Leukoc. Biol. 2016, 99, 955–969.
62. 62. Proost, P.; Schutyser, E.; Menten, P.; Struyf, S.; Wuyts, A.; Opdenakker, G.; Detheux, M.; Parmentier, M.; Durinx, C.; Lambeir, A.M.; et al. Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties. Blood 2001, 98, 3554–3561.
63. Bonecchi, R.; Locati, M.; Galliera, E.; Vulcano, M.; Sironi, M.; Fra, A.M.; Gobbi, M.; Vecchi, A.; Sozzani, S.; Haribabu, B.; et al. Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. J. Immunol. 2004, 172, 4972–4976.
64. Kleist, A.B.; Getschman, A.E.; Ziarek, J.J.; Nevins, A.M.; Gauthier, P.A.; Chevigne, A.; Szpakowska, M.; Volkman, B.F. New paradigms in chemokine receptor signal transduction: Moving beyond the two-site model. Biochem. Pharm. 2016, 114, 53–68.
65. Reynders, N.; Abboud, D.; Baragli, A.; Noman, M.Z.; Rogister, B.; Niclou, S.P.; Heveker, N.; Janji, B.; Hanson, J.; Szpakowska, M.; et al. The Distinct Roles of CXCR3 Variants and Their Ligands in the Tumor Microenvironment. Cells 2019, 8, 613.
66. Karin, N.; Razon, H. Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. Cytokine 2018, 109, 24–28.
67. Soejima, K.; Rollins, B.J. A functional IFN-gamma-inducible protein-10/CXCL10-specific receptor expressed by epithelial and endothelial cells that is neither CXCR3 nor glycosaminoglycan. J. Immunol. 2001, 167, 6576–6582.
68. Neote, K.; Darbonne,W.; Ogez, J.; Horuk, R.; Schall, T.J. Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. J. Biol. Chem. 1993, 268, 122247–122249.
69. Szpakowska, M.; Dupuis, N.; Baragli, A.; Counson, M.; Hanson, J.; Piette, J.; Chevigne, A. Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7. Biochem. Pharm. 2016, 114, 14–21.
70. Gosling, J.; Dairaghi, D.J.; Wang, Y.; Hanley, M.; Talbot, D.; Miao, Z.; Schall, T.J. Cutting edge: Identification of a novel chemokine receptor that binds dendritic and T cell-active chemokines including ELC, SLC, and TECK. J. Immunol. 2000, 164, 2851–2856.

Discussion and outlook

The number of publications reporting the involvement of chemokines and their receptors in diseases has been increasing over the years since their discovery. Chemokine receptors and their endogenous ligands exert important functions in many vital physiological cellular processes, such as cell migration, proliferation, and differentiation. However, alternations in their expression, such as an enhanced or a reduced expression could derail the chemokine receptor network possibly leading to abnormal cell mobilization or proliferation resulting in some cases in the development of particular diseases, including autoimmune and vascular pathologies, inflammatory diseases and cancer. Although being important therapeutic targets for these diseases, not many therapeutic molecules targeting these receptors are present on the market to date.

One chemokine can recognize multiple chemokine receptors and vice versa. However, each chemokine-receptor interaction induces specific cellular functions depending on their spatial and temporal expression that results in appropriate physiological responses. The latter indicates that the chemokine receptor network is non-redundant. Moreover, the involvement of a particular chemokine-receptor axis in homeostasis or disease state is often overestimated. Multiple chemokine receptors can be expressed on similar cells at the same time, however, dependent on the cellular context a chemokine receptor will trigger a cellular function that drives the overall response compared to the other chemokine receptors axis present in the same cellular context. For example, CCR1 and CCR2 promote the migration of monocytes, macrophages and T lymphocytes to the synovial fluids of patients with rheumatoid arthritis. As both chemokine receptors are expressed on these cell types, it was first believed that both contributed equally to the disease development. However, additional studies discovered that the attraction of these immune cells to the inflamed joints is primarily mediated by CCR1, and not by CCR2, suggesting that blocking CCR1 would be able to reduce the inflammation but not CCR2. Additionally, one should also compare the contribution of a particular chemokine-receptor axis in each animal model when translating the drug candidate from one model to another. For example, CXCR1 is the main driver of neutrophil recruitment in human, but mice lack the gene encoding for this chemokine receptor. Instead, mice use CCR1 to migrate neutrophils to substitute for the absence of CXCR1. Therefore, administering an anti-CCR1

drug for the attenuation of neutrophil migration in mice will not have the predicted and desired outcome in neutrophil-driven human diseases [1]. Moreover, the broad, open, and similar ligand binding pockets of chemokine receptors prove to be demanding targets to develop receptor-specific and effective small molecule drugs [2]. This non-redundant crosstalk between chemokine receptors and their ligands, the impact of each chemokine receptor axis in a particular cellular context and model, and the open binding pocket contribute to what makes them so challenging to modulate therapeutic targets.

The CXCR3 chemokine receptor binds three chemokines: CXCL11, CXCL10, and CXCL9. Together, they mediate the trafficking of immune cells, particularly T lymphocytes, for immunosurveillance. In inflammatory conditions, the three CXCR3 ligands are upregulated by the pro-inflammatory cytokines INF-γ and TNF-□ and attract activated T cells to the damaged tissue to regulate the inflammation. However, a higher expression of CXCR3 and their ligands also contribute to disease development, including cancer proliferation and metastasis [3]. Interestingly, this chemokine receptor can be transcribed to three variants: CXCR3-A, CXCR3-B, and CXCR3-Alt. Each CXCR3 chemokine receptor variant differs in their amino sequences and displays different characteristics. CXCR3-A is referred to as the reference receptor variant, CXCR3-B shows a 51 amino acid extension at its N terminus [4], and CXCR3-Alt lacks two transmembrane helices and has an alternated C terminal region [5]. Although these variants have been discovered for almost two decades, much research focused on elucidating the role of CXCR3-A. Consequently, most of CXCR3-A's biology, from its cellular effect and downstream signaling pathways to in vivo implication, has been described. The binding of all three ligands to CXCR3-A triggers the activation of $G_{\alpha i/o}$ proteins, which leads to the migration of immune cells. The study of the other two variants has been mostly neglected as a result of which, today, their underlying mechanisms for their molecular and functional properties have not been fully mapped out. Stimulation of CXCR3-B induces opposite cellular responses compared to CXCR3-A, such as inhibition of cell proliferation and migration, however, data on its signaling properties remains unclear [4]. Even fewer reports are available on the CXCR3-Alt receptor variant. CXCR3-Alt is known to bind all CXCR3 ligands and activation of this receptor variant leads to its internalization; however, its signaling properties are still undiscovered. Moreover, recently, this receptor, together with CXCL11, has been shown to be important players in the attenuation of bladder cancer upon chemotherapy [6, 7].

At the start of the project, CXCR3-B's signaling and functional characteristics were poorly understood as conflicting results were reported. Additionally, the exact contributions of both CXCR3 variants and their ligands in the tumor microenvironment that leads to their observed cellular effect could not be easily found as the available reviews and publications often referred to the term CXCR3 and did not distinguish between CXCR3-A and CXCR3-B. Therefore, this PhD project started with two objectives: the first one was to investigate and elucidate the molecular and functional differences between the CXCR3 variants, CXCR3-A

and CXCR3-B, and elucidating the roles of each player in the CXCR3 axis in the tumor microenvironment to validate them as possible valuable drug targets. Besides the fundamental research, the second objective of this project was more applied and translational. It consisted in the development of probes allowing to discriminate the expression of CXCR3-A from CXCR3-B at the protein level in vitro and in vivo, and possibly use these probes as possible therapeutic molecules to counter CXCR3-driven pathologies, such as cancer development and metastasis, rheumatoid arthritis and transplant rejection.

Reviews on the contribution of CXCR3-A, CXCR3-B or their ligands in the tumor bed were difficult to find at the beginning of the project. Moreover, the available reports did not make the distinction between both CXCR3 variants but rather referred to CXCR3 overall. Therefore, an extensive literature review with focus on CXCR3-A and CXCR3-B, as two distinct chemokine receptors, and their shared ligands in the tumor microenvironment was performed at the beginning of my PhD (**chapter 1**). During this study, we found cancer cells increase CXCR3-A's expression at the cell surface which contributes to cancer development following receptor activation. Additionally, cancer cells hijacked this chemokine receptor' ability to induce cell migration to spread to distant tissues in collaboration with the upregulated CXCR3 ligands. On the other hand, the expression of CXCR3-B on these cells attenuated tumor development by inhibiting cancer cell proliferation and angiogenesis.

These interesting opposing cellular effects of CXCR3-A and CXCR3-B, summarized in our review, led us to investigate the functions and signaling properties of these two CXCR3 variants in vitro. To compare the two CXCR3 variants at the molecular, cellular and functional levels, new assays and tools were implemented or developed, including cell lines expressing stably CXCR3-A or -B, G protein recruitment and dissociation assays, internalization as well as chemokine uptake assays as well as other techniques to measure different secondary messengers and the related downstream signaling pathways. Overall, the molecular and functional comparison of CXCR3-A and CXCR3-B revealed that CXCR3-B displays many attributes of an atypical chemokine receptor (**chapter 2**). CXCR3-B showed a drastic reduction of its ability to induce G protein-mediated signaling following activation yet maintained β-arrestin recruitment and demonstrated a distinct intracellular localization in absence of chemokine. Moreover, it had contrasting receptor trafficking in contrast to CXCR3-A. Furthermore, we were able to show that the N terminal extension of CXCR3-B lies at the basis of its atypical properties as truncations of its N terminus allowed recoupling of G proteins to this CXCR3 variant.

The recent publication of the interplay between two cxc3 receptors, cxcr3.2 and cxcr3.3, in zebra fish described cxcr3.3 to have properties of ACKRs and regulate cxcr3.2-mediated cellular effect may corroborate our results and could highlight the importance of having these two receptor variants with opposing signaling outcomes in humans [8].

ACKR3 is known to bind CXCL11 [21] and our screening of all the presently known CXC chemokines on ACKR2 led to the identification of ACKR2 as a scavenger for CXCL10. Taking these new observations into account, one might question the interest of CXCR3-B being another ACKRs bind and internalize CXCR3 ligands. The importance of CXCR3-B could be revealed in inflammatory conditions. ACKR3 is continuously present on the cell surface of endothelial cells (EC) in homeostatic conditions to regulate the extracellular CXCL12 and is upregulated in inflammatory conditions [22]. CXCR3 endogenous ligands, CXCL11, CXCL10, and CXCL9, are induced and expressed during inflammation [23] and CXCR3-B is omnipresent on EC in normal and inflammatory conditions [11]. One might postulate that ACKR2, ACKR3 and CXCR3-B are needed to ensure proper regulation of the different chemokine levels, including CXCL12, CXCL11, CXCL10 and CXCL9, present in the extracellular space in inflammatory conditions. However, bearing in mind that the chemokine receptor network is non-redundant, functional selective, and that the expression of each chemokine and receptor is temporal and spatially induced, studying the interactions between ACKRs and their shared ligands in more complex in vitro experiments, and in vivo, would provide a better understanding of how the chemokines are scavenged by which ACKR and would shed light on their possible function and inter-complementarities.

Investigation of the CXCR3-A and CXCR3-B in vitro and in vivo proved to be a difficult task. One of the major hurdles of studying CXCR3 and its variants is the lack of tools to investigate them. To date, no antibodies are available that can distinguish all CXCR3 variants as the primary antibodies used today recognize CXCR3-A's N-terminus sequence, a motif present in all variants. Although a few CXCR3-B antibodies have been generated, using CXCR3-B's 51 AA extension, they are not wildly used as its applications are limited to Western Blot or ELISA. Therefore, the generation of variants specific tools would enable us to visualize and investigate the expression of the CXCR3 variants at the cell surface in vivo more in-depth. Mice are excellent models to investigate the impact of chemokines and their receptors by knocking out the genes of interest. While CXCR3-A mice knockouts are available, CXCR3-B does not exist in mice as the proposed alternative splicing of CXCR3-B, using the published mouse cxcr3 genomic sequence, generates an in-frame stop codon resulting in the termination of CXCR3-B's translation [9]. The latter makes it even more difficult to prove its existence as well as its cellular effect in vivo. The fact that CXCR3 variants recognize the same endogenous chemokines makes it also difficult to generate molecules that specifically modulate one variant. Conformational antibodies could help in that regard as the different cellular effects of CXCR3-A and -B probably lies in different conformational states. During this PhD, I invested a lot of my time in the generation of tools, including cell lines, production of CXCR3 chemokines with different tags, and assays, to generate and isolate antibody fragments that were able to distinguish and modulate both CXCR3 variants. This project was in collaboration with a private company, but due to many difficulties related to CXCR3 listed above and the COVID-19 pandemic, this part of my PhD project had to be put on hold and I was not able to valorize this work with the isolation of

CXCR3 specific antibody fragments. Furthermore, due to the confidentiality of this part of the project, the results are not presented and discussed in this thesis manuscript.

The expression of CXCR3-A on immune cells and its function are widely acknowledged. However, the existence of CXCR3-B in vivo in humans is still a matter of debate. Presently, data is available on the expression of CXCR3-B, which described this receptor to be present on EC and other barrier cells type, using ex vivo tissue samples or variant specific primers [4, 10, 11]. Yet, in vitro CXCR3-B is absent on the surface of these cells in culture. The latter suggests a loss of expression possible due to the in vitro non-physiological conditions in which the cells are cultured or epigenetic control [9]. The emergence of new sequencing techniques like RNA- and q-PCR-seq based techniques will allow to generate more robust and conclusive data on its expression. It might also be of interest to be able to induce this CXCR3-B variant on different cell types using pro-inflammatory cytokines. Finding the right media conditions to express CXCR3-B on endothelial cells in culture would be of great help to elucidate its molecular signatures and function at a physiological expression level.

To train me in the molecular and cellular and assays available in the group at the start of this project, I first contributed to the comparative study of the roles of the two cysteine residues present in the N terminal extracellular domain of ACKR3 and CXCR3 on ligand binding and activation. The two cysteines motif of ACKR3, separated by four residues, were shown to form an atypical arch which is not observed in all other known chemokine receptors. CXCR3, however, contains two adjacent cysteines in its N-terminus that possibly form a disulphide bridge and add some rigidity to its N terminus. To assess the impact of these cysteines in CXCR3 ligand binding and activation, a CXCR3-A mutant was generated, in which the N-terminal cysteines were substituted for serines, resulting in the disruption of the possible cysteine bridge (**chapter 3**) [13]. Although the substitution of the cysteine motif did not change CXCR3-A's functionality towards its endogenous ligands, it increased its expression at the cell surface. This first research work allowed me to get first-hand experience on various cellular assays, especially the β-arrestin recruitment assay.

This β-arrestin recruitment assay, based on the complementation of a split Nanoluciferase protein, was used throughout this thesis and all related publications. This assay was instrumental to identify CXCL10, previously reported to bind to CXCR3 only, as a new chemokine ligand for ACKR2 and shed light on the implication of this atypical chemokine receptor in the regulation of CXCR3 related ligand (**chapter 5**) [14]. This assay was also key for the pairings of CCL20 and CCL22 as two new ligands for ACKR4 that we identify during a systematic reassessment of chemokine-receptor pairings towards the different ACKRs (**chapter 4**) [15]. Additionally, the screening of all known chemokines on CXCR3-A and CXCR3-B disproved CXCL4 and revealed vCCL2 as a ligand of CXCR3-A and CXCR3-B (**chapter 2**). This screening effort attests that systematic reassessment of

chemokine-receptor pairing is critically needed as important interactions may remain unexplored. Even though this β-arrestin recruitment assay would technically allow us to screen all known chemokines on all chemokine receptors, it might anyway overlook G protein-biased or constitutively arrestin-recruiting receptors. The latter has been recently reported for the newly described ACKR GPR182. This receptor does not recruit β-arrestin recruitment upon ligand stimulation but downregulation of arrestin production results in decreased basal arrestin recruitment levels suggesting that this receptor is a high constitutively active receptor in absence of its kindred ligands [16]. Furthermore, chemokine receptor's function might be β-arrestin independent, as described for ACKR1 [17], and ACKR3 [18, 19] and ACKR4 [20] suggesting that this highly sensitive β-arrestin based assay, although being a very powerful tool, has some limitations and other complementary assays are needed to identify new or confirm ligands that do not induce β-arrestins recruitment. Additionally, molecules that weakly trigger the recruitment of β-arrestins, such as CXCL9 and CXCL10 for CXCR3-B, also attest to the limitations of this assay.

In conclusion, in this thesis, new molecular, signaling and functional properties for CXCR3-B have been described. Our results show that the N-terminal extension of CXCR3-B drastically affects receptor features, modifying the receptor cellular localization and preventing G protein coupling, while preserving β-arrestin recruitment and chemokine uptake capacities. Moreover, we also demonstrate that gradual truncation of the N terminus leads to progressive recovery of surface expression and recoupling to G protein. Our study clarifies the molecular basis underlying the divergent cellular effects of CXCR3 variants that now may be regarded as opposing effectors in analogy to classical and atypical chemokine receptors. We propose CXCR3-B to be an intrinsically biased beta-arrestin chemokine receptor and therefore an atypical chemokine receptor. This new concept, supported by our dataset, changes our perception of the role of CXCR3-B in homeostatic and diseases state and possibly explains the contrasting functional biological effects described in the literature. Lowering extracellular CXCR3 chemokine levels by CXCR3-B could directly regulate CXCR3-A-mediated cellular effects, which could present themselves as cell anti-proliferative or migration inhibitory physiological effects as observed in the tumor microenvironment. A similar mode of action of ACKRs modulating classical chemokine receptors-driven cellular effect has been described for ACKR2 and ACKR4: these ACKRs regulate the bioavailability of extracellular chemokines for their related chemokine receptors resulting in the inhibition of tumor growth and metastasis [24]. Moreover, with CXCR3-B being present on endothelial cells (EC) in homeostatic conditions, like ACKR3 and ACKR4, we can postulate that CXCR3-B mediates the migration of CXCR3-A+ immune cells by regulating extracellular chemokine levels. The proposal of CXCR3-B as atypical chemokine receptors can be further strengthened by the presence of two homologous CXCR3 chemokine receptor variants, cxcr3.2 and cxcr3.3, in zebra fish, which was recently described.

Finally, while most of my work focused on CXCR3, as a group effort, we also discovered several chemokine ligands for the atypical chemokine receptors ACKR2 and ACKR4. These findings contribute to the expansion of the chemokine receptor network (see figure 2) that might lead to novel insights into their implication in several diseases and the generation of new modulators. Below, I summarized the highlight of each publication and my perspective on the following research questions that flow from this PhD results and that need to be answered to validate our findings or to further expand our understanding of the chemokines receptors network.

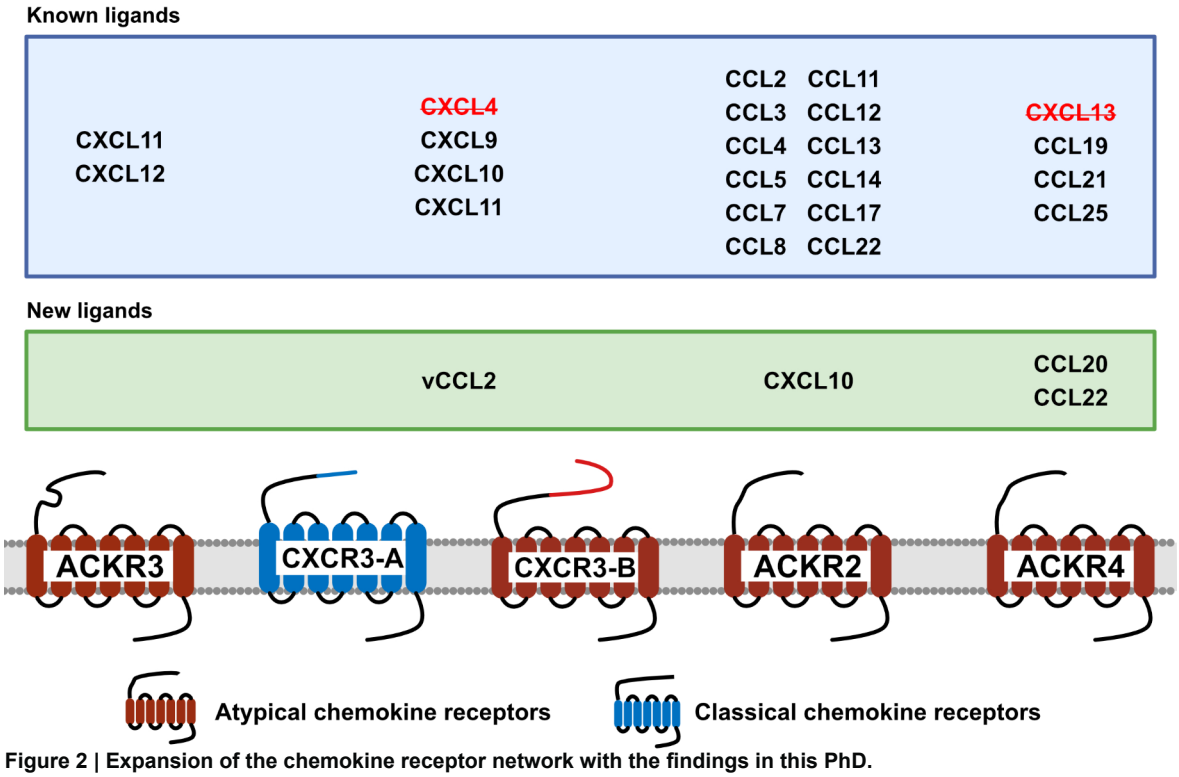

Figure 2 | Expansion of the chemokine receptor network with the findings in this PhD.

Highlights of PhD thesis and future research questions

Key findings per chapter

Chapter 1:

- CXCR3-A and -B display opposing cellular effects within the tumor microenvironment.
- CXCL11, CXCL10 and CXCL9 are upregulated under inflammatory conditions. They attract leukocytes to the tumor bed to regulate the tumor's progression, but can also contribute to cancer development and metastasis.

Chapter 2:

- CXCR3-B is a β-arrestin biased receptor, which is localized intracellularly and takes up extracellular CXCR3 ligands.
- CXCR3-B's atypical features arise from its N-terminal extension, which limit its ability to interact efficiently with G proteins.

Chapter 3:

- Removal of ACKR3's extracellular arch, by disrupting disulphide bridges, shows alternations in ligand binding capabilities, but not in the receptor's functionality.
- CXCR3-A's N-terminal cysteines limit its expression at the cell surface but are not essential for the activation of the receptor.

Chapter 4:

- CCL20 and CCL22, but not CXCL13, trigger β-arrestin recruitment towards ACKR4.

Chapter 5:

- ACKR2 recognizes and scavenges extracellular CXCL10, but with a different binding and activation mode compared to its cognate CC chemokines.

Unanswered questions and outlook

- **What are the structural differences between CXCR3-A and CXCR3-B in absence and presence of its endogenous ligands?** Answering this research question would enable us to determine the underlying structural differences between CXCR3-A and CXCR3-B and would complement the pharmacological data acquired during this PhD.
- **Are ACKR2, ACKR3 and CXCR3-B expressed simultaneously in inflammatory conditions?** Acquiring more data on ACKRs' expression *in vivo* together with studying the interactions between these ACKRs and their shared ligands in more complex *in vitro* experiments would provide a better understanding of how the chemokines are scavenged by which ACKR and would shed light on their possible function *in vivo*.
- **Developing probes that distinguish and modulate CXCR3-A and CXCR3-B specifically would allow us to investigate these receptors *in vivo* and validate them as possible therapeutic targets.**

References

1. Schall, T.J. and A.E. Proudfoot, Overcoming hurdles in developing successful drugs targeting chemokine receptors. Nat Rev Immunol, 2011. 11(5): p. 355-63.
2. Zhao, S., B. Wu, and R.C. Stevens, Advancing Chemokine GPCR Structure Based Drug Discovery. Structure, 2019. 27(3): p. 405-408.
3. Reynders, N., et al., The Distinct Roles of CXCR3 Variants and Their Ligands in the Tumor Microenvironment. Cells, 2019. 8(6).
4. Lasagni, L., et al., An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. J Exp Med, 2003. 197(11): p. 1537-49.
5. Ehlert, J.E., et al., Identification and partial characterization of a variant of human CXCR3 generated by posttranscriptional exon skipping. J Immunol, 2004. 173(10): p. 6234-40.
6. Berchiche, Y.A. and T.P. Sakmar, CXC Chemokine Receptor 3 Alternative Splice Variants Selectively Activate Different Signaling Pathways. Mol Pharmacol, 2016. 90(4): p. 483-95.
7. Vollmer, T., et al., The intratumoral CXCR3 chemokine system is predictive of chemotherapy response in human bladder cancer. Sci Transl Med, 2021. 13(576).
8. Sommer, F., et al., Frontline Science: Antagonism between regular and atypical Cxcr3 receptors regulates macrophage migration during infection and injury in zebrafish. J Leukoc Biol, 2020. 107(2): p. 185-203.
9. Campanella, G.S., R.A. Colvin, and A.D. Luster, CXCL10 can inhibit endothelial cell proliferation independently of CXCR3. PLoS One, 2010. 5(9): p. e12700.
10. Feil, C. and H.G. Augustin, Endothelial cells differentially express functional CXC-chemokine receptor-4 (CXCR-4/fusin) under the control of autocrine activity and exogenous cytokines. Biochem Biophys Res Commun, 1998. 247(1): p. 38-45.
11. Garcia-Lopez, M.A., et al., CXCR3 chemokine receptor distribution in normal and inflamed tissues: expression on activated lymphocytes, endothelial cells, and dendritic cells. Lab Invest, 2001. 81(3): p. 409-18.
12. Veazey, R.S., et al., Topically applied recombinant chemokine analogues fully protect macaques from vaginal simian-human immunodeficiency virus challenge. J Infect Dis, 2009. 199(10): p. 1525-7.
13. Szpakowska, M., et al., Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists. Biochem Pharmacol, 2018. 153: p. 299-309.
14. Chevigne, A., et al., CXCL10 Is an Agonist of the CC Family Chemokine Scavenger Receptor ACKR2/D6. Cancers (Basel), 2021. 13(5).
15. Meyrath, M., et al., Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22. J Leukoc Biol, 2021. 109(2): p. 373-376.
16. Le Mercier, A., et al., GPR182 is an endothelium-specific atypical chemokine receptor that maintains hematopoietic stem cell homeostasis. Proc Natl Acad Sci U S A, 2021. 118(17).
17. Chakera, A., et al., The duffy antigen/receptor for chemokines exists in an oligomeric form in living cells and functionally antagonizes CCR5 signaling through hetero-oligomerization. Mol Pharmacol, 2008. 73(5): p. 1362-70.
18. Montpas, N., et al., Ligand-specific conformational transitions and intracellular transport are required for atypical chemokine receptor 3-mediated chemokine scavenging. J Biol Chem, 2018. 293(3): p. 893-905.
19. Saaber, F., et al., ACKR3 Regulation of Neuronal Migration Requires ACKR3 Phosphorylation, but Not beta-Arrestin. Cell Rep, 2019. 26(6): p. 1473-1488 e9.
20. Comerford, I., et al., The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. Eur J Immunol, 2006. 36(7): p. 1904-16.
21. Naumann, U., et al., CXCR7 functions as a scavenger for CXCL12 and CXCL11. PLoS One, 2010. 5(2): p. e9175.
22. Santagata, S., et al., CXCR4 and CXCR7 Signaling Pathways: A Focus on the Cross-Talk Between Cancer Cells and Tumor Microenvironment. Front Oncol, 2021. 11: p. 591386.
23. Metzemaekers, M., et al., Overview of the Mechanisms that May Contribute to the Non-Redundant Activities of Interferon-Inducible CXC Chemokine Receptor 3 Ligands. Front Immunol, 2017. 8: p. 1970.
24. Lokeshwar, B.L., G. Kallifatidis, and J.J. Hoy, Atypical chemokine receptors in tumor cell growth and metastasis. Adv Cancer Res, 2020. 145: p. 1-27.