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INVESTIGATING THE CHEMOKINE RECEPTOR NETWORK: FROM MOLECULAR ASPECTS OF CXCR3 TO A PATIENT-BASED STUDY IN GLIOMA

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Affidavit

I hereby confirm that the PhD thesis entitled "**Investigating the chemokine receptor network: from molecular aspects of CXCR3 to a patient-based study in glioma**." has been written independently and without any other sources than cited.

Esch-sur-Alzette, 18/03/2024,

Giulia D'Uonnolo

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Table of content

List of illustrations

Illustrations 2, 3, 4, 5, 7, 8, 10, 11 and 13 were created with BioRender.com.

Abbreviations

AC-like	astrocyte-like
ACKR	atypical chemokine receptor
ADM	adrenomedullin
ALA	aminolevulenic acid
ATRX	α-thalassemia mental retardation X-linked
BBB	blood-brain barrier
BRAF	v-raf murine sarcoma viral oncogene homolog B
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CCL	CC motif chemokine ligand
CCR	CC motif chemokine receptor
CD26	cluster differentiation 26
CDK4	cyclin-dependent kinase 4
CDKN2A/B	cyclin-dependent kinase inhibitors 2A and 2B
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
CNS	central nervous system
CRS	chemokine recognition site
CT	cellular tumour
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CXCL	CXC motif chemokine ligand
CXCR	CXC motif chemokine receptor
CX3CL	CX3C motif chemokine ligand
CX3CR	CX3C motif chemokine receptor
DARC	Duffy antigen/receptor for chemokines
DBP	Duffy binding proteins
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
ECL	extracellular loops
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FDA	US Food and Drug Administration
FFA	free fatty acid (receptor)
GAG	glycosaminoglycan
GBM	glioblastoma
GDP	guanidine diphosphate
GPCRs	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
GSC	glioma stem cell
GTP	guanidine triphosphate
GTEx	Genotype-Tissue Expression

HCMV	human cytomegalovirus
HEK	human embryonic kidney
HUMEC	human microvascular endothelial cells
ICL	intracellular loops
IL	interleukin
IDH	isocitrate dehydrogenase
IFN	interferon
IP3	inositol trisphosphate
IT	infiltrating tumour
LE	leading edge
LGG	low grade glioma
LPS	lipopolysaccharide
MGMT	O6-methylguanine DNA methyltransferase
MET	met tyrosine-protein kinase
MES-like	mesenchymal-like
MVP	microvascular proliferation
NA	not available
NF1	neurofibromatosis type 1
NK	natural killer
Nluc	nanoluciferase
NPC-like	neural-progenitor-like
mAb	monoclonal antibody
MDSC	myeloid-derived suppressor cells
MFI	mean fluorescence intensity
MOR	mu opioid receptor
OPC-like	oligodendrocyte-progenitor-like
PAMP	proadrenomedullin N-terminal 20 peptide
PAN	pseudo palisading around necrosis
PBMC	peripheral blood mononuclear cells
PD-1	programmed cell death protein 1
PD-L1	programmed cell death protein ligand 1
PDGFRA	platelet-derived growth factor receptor-α
PEI	polyethylenimine
PI3K	phosphoinositide 3- kinase
PLC	phospholipase C
PPI	protein-protein interaction
PTM	post-translational modification
rGBM	relapsed GBM
RNA	ribonucleic acid
RSEM	RNA-Seq by Expectation Maximization
scRNAseq	single-cell RNA sequencing
SD	standard deviation
SEM	standard error of the mean
TAM	tumor-associated macrophage

TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor-β
TIL	tumour-infiltrating lymphocytes
TM	transmembrane domain
TME	tumour microenvironment
TMZ	temozolomide
TNF	tumour necrosis factor
TP53	tumour protein p53
TPM	transcripts per million
T reg	regulatory T cells
XCL	XC Motif chemokine ligand
XCR	XC Motif chemokine receptor
WHO	World Health Organization
WT	wildtype
1p/19q codel	co-deletion of chromosome 1p and 19q whole arms

List of publications and manuscripts

The present thesis is based on the following publications:

- Book chapter publication (shared first-author)

Luís R, **D'Uonnolo G**, Palmer CB, Meyrath M, Uchański T, Wantz M, Rogister B, Janji B, Chevigné A, Szpakowska M. *Nanoluciferase-based methods to monitor activation, modulation and trafficking of atypical chemokine receptors.* Methods Cell Biol. 2022; 169:279-294. https://doi.org/10.1016/bs.mcb.2022.03.002

- Book chapter publication (shared first-author)

Palmer CB, **D'Uonnolo G**, Luís R, Meyrath M, Uchański T, Chevigné A, Szpakowska M. *Nanoluciferase-based complementation assay for systematic profiling of GPCR–GRK interactions.* Cell Biol. 2022; 169:309-321.https://doi.org/10.1016/bs.mcb.2022.04.001

- Research publication (shared first-author)

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- Research publication (co-author)

Chevigné A, Janji B, Meyrath M, Reynders N, **D'Uonnolo G**, Uchański T, Xiao M, Berchem G, Ollert M, Kwon YJ, Noman MZ, Szpakowska M. *CXCL10 Is an Agonist of the CC Family Chemokine Scavenger Receptor ACKR2/D6.* Cancers (Basel). 2021 Mar 2;13(5):1054. https://doi.org/10.3390/cancers13051054

- Review publication (co-author)

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- Review publication (shared first-author)

Isci D, **D'Uonnolo G**, Wantz M, Rogister B, Lombard A, Chevigné A, Szpakowska M, Neirinckx V. *Patient-Oriented Perspective on Chemokine Receptor Expression and Function in Glioma.* Cancers (Basel). 2021 Dec 28;14(1):130. https://doi.org/10.3390/cancers14010130

- Research manuscript (shared first-author)

D'Uonnolo G, Isci D, Nosirov B, Kuppens A, Wantz M, Nazarov PV, Golebiewska A, Rogister B, Chevigné A, Neirinck V, Szpakowska M. *Patient-based multilevel transcriptomic analysis highlights relevant chemokines and chemokine receptor axes in glioblastoma.* Under review in Cell Communication and Signaling.

Additional manuscripts that constitute important outcomes of my PhD studies, which are currently under preparation or out of the scope of this thesis:

- Research manuscript (first-author)

D'Uonnolo G, Youssef N, Luís R, Alonso Bartolomé A, Hoffman C, Szpakowska M, Chevigné A. *Different requirements and interaction modes of β-arrestin isoforms for basal and ligand-induced interactions with CXCR3.* Manuscript under preparation.

- Research manuscript (co-author)

Luís R, Albano F, **D'Uonnolo G,** Bonecchi R, Szpakowska M, Chevigné A. *Development and validation of LIH222 as a selective high-affinity chimeric chemokine for the modulation and detection of the scavenging receptor ACKR2/D.* Manuscript under finalization.

- Research manuscript (co-author)

Isci D, Kuppens A, Scalisi J, Cokaiko J, **D'Uonnolo G,** Wantz M, Szpakowska M, Chevigné A, Rogister B, Neirinckx V. *Heterogeneous expression of the atypical chemokine receptor ACKR3 in glioblastoma patient-derived tissue samples and cell cultures.* Under review in Cancer Cell International.

Abstract

Chemokines and their cognate receptors are responsible for the migration and positioning of target cells in specific sites, in physiological and pathological conditions. The chemokine interaction network is extremely complex, with the presence of homeostatic and pro-inflammatory chemokines that are able to bind and activate both signalling receptors (CKRs) and non-signalling receptors (ACKRs). Chemokine receptors are a large subgroup of G protein-coupled receptors (GPCRs). Despite the fact that approximately one-third of all FDA-approved drugs target GPCRs, very few of them are directed against chemokine receptors, which is likely, at least in part, due to the complexity of their interaction network.

The focus of this thesis was therefore to contribute to a better understanding of the intricate interplay between chemokines and their receptors by investigating their regulation at different levels and biological contexts.

The chemokine receptor CXCR3, which is highly important for effective immune responses, was analysed in depth at molecular level. Among others, we undertook a thorough comparison of its two major isoforms, CXCR3-A and CXCR3-B, and highlighted the atypical features of the latter. CXCR3 was also used as model GPCR to investigate the molecular determinants driving the interactions with the two β-arrestins, under basal conditions and following ligand stimulation.

Moreover, several players of the chemokine system are implicated in tumour development and dissemination. With the aim of gaining a better understanding on their differential involvement in cancer, we investigated their expression in adult-type diffuse gliomas, with a particular interest in glioblastoma, the most severe brain malignancy. From a translational perspective, using publicly available datasets from patient-derived material, we assessed expression levels of the chemokines and their receptors and revealed the most abundant ligand–receptor crosstalks.

Aims

Chemokine receptors together with their ligands play a crucial role in leukocyte migration and positioning. They represent potential valuable therapeutic targets for many diseases in which immune cells are implicated, including cancer, autoimmune and inflammatory diseases. However, many aspects governing the chemokine system remain unclear.

The aim of this PhD thesis, was to better understand the interaction networks between the ligands and receptors, both classical and atypical, forming the complex chemokine system.

To allow these investigations, efforts needed to be invested first into the design and development of versatile tools to study various aspects of GPCR activation (detailed in *Chapter I*). Comprehensive guidelines to methodologies to systematically investigate the atypical chemokine receptors, for which the traditional assays monitoring G protein-dependent events are not suited, are provided in the first section *(Chapter I.i)*. The second section *(Chapter I.ii)* describes methods to evaluate the interactions between receptors and G protein-coupled receptor kinases.

We then undertook an in-depth characterisation of the chemokine receptor CXCR3, with a specific goal to better understand one of its enigmatic isoforms, CXCR3-B, showing ambiguous signalling and functions. C*hapter II*, presents a comparative analysis of CXCR3-B with the main receptor isoform, CXCR3-A. We provide insights into the molecular features responsible for the β-arrestin bias of the isoform CXCR3-B and its multiple features characteristic of atypical chemokine receptors in contrast to the classical isoform CXCR3-A.

As the atypical chemokine receptors have the fundamental role of regulating the availability of the ligands for other signalling receptors, we wished to examine new ligand–receptor pairings within this receptor family. *Chapters III and IV*, focus on how the ACKR family is expanding, highlighting a potential novel member of the family with clinical relevance.

To better understand the clinical significance of the chemokine system we also undertook an analysis evaluating the most abundant components of the network in glioma and glioblastoma patient-derived samples, both from the point of view of the receptors in C*hapter V.i* and the chemokines in C*hapter V.ii.*

SYNOPSIS

1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) constitute the largest membrane protein family, with over 800 members. They localize on cellular membranes, in the majority of cases on the plasma membrane. Upon activation by the respective ligands, they are able to transduce extracellular signals inside the cells. Endogenous GPCR ligands encompass a wide variety of molecules, including lipids, nucleotides, hormones, peptides, and larger proteins. GPCRs are categorized into classes based on their sequence and evolutionary similarities[1], specifically: class A (rhodopsin), class B (secretin and adhesion), class C (glutamate), and class F (Frizzled)[2,3]. This classification groups together GPCRs with common physiological ligands, as the specific region where receptors differ the most, is the one responsible for ligand selectivity[4]. GPCRs are involved in several pathophysiological processes, for example, chemokine receptors are crucial in immune responses, free fatty acid (FFA) receptors are critical for gut metabolism, and opioid receptors are important players in pain and emotional behaviours. Because of GPCRs' participation in the regulation of diverse pathophysiological processes and their accessibility on the cell surface, which facilitates their pharmacological targeting, GPCRs are largely considered promising drug targets. The latest comprehensive reports identified 475 approved drugs targeting 165 GPCRs, accounting for around one-third of the total US Food and Drug Administration (FDA)-approved drugs[2,3] (Illustration 1).

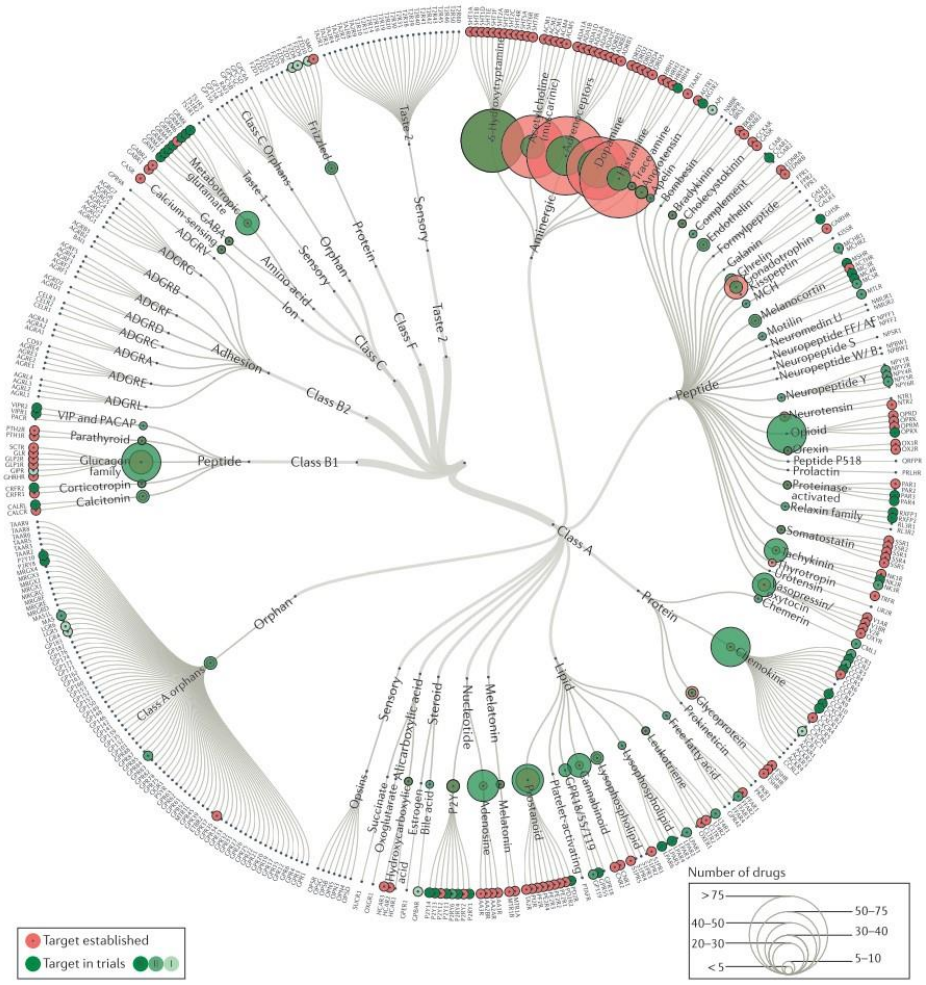

Illustration 1 - GPCR drug targets

GPCRs are clustered in classes highlighting ligand types and receptor families. GPCR targets of FDA-approved molecules for individual receptors are shown in red, and of molecules in clinical trials in green (outer ring). For receptor families (inner ring), two concentric circles are presented: the red circle indicates the number of approved drugs (with an established target in that family), whereas the green circle indicates the number of agents under clinical trials for GPCR targets from that family. From Hauser et al[2].

1.1 GPCRs structure

Despite having variable amino acid sequences, GPCRs show a conserved three-dimensional structure. They share three specific regions: the extracellular region, the transmembrane region, and the intracellular region (Illustration 2). The extracellular one is composed of the N terminus and three extracellular loops (ECLs). Typically, this portion is involved in the recognition of ligands and therefore considered as the ligand-binding module of the GPCRs. It shows large diversity across GPCR families and is considered to undergo smaller conformational changes. The intracellular region comprises three intracellular loops (ICLs) and the C terminus, which bear multiple interaction determinants and sites subject to post-translational modifications fundamental for downstream signalling. Therefore, this region may be considered as the receptor signalling module. It is fairly similar among different

GPCR families but it is where receptors show larger conformational changes. As for the transmembrane region (TM), it is composed of seven α-helices, which cross the cell membrane and connect the extracellular loops to the intracellular loops[4,5]. GPCRs are thus also often referred to as seven-transmembrane (7TM) receptors. This portion is responsible for transducing information about ligand binding to the signalling module via conformational changes. It includes conserved motifs and comprises a network of inter-transmembrane contacts among amino acids forming the structural scaffold of the receptor[5,6].

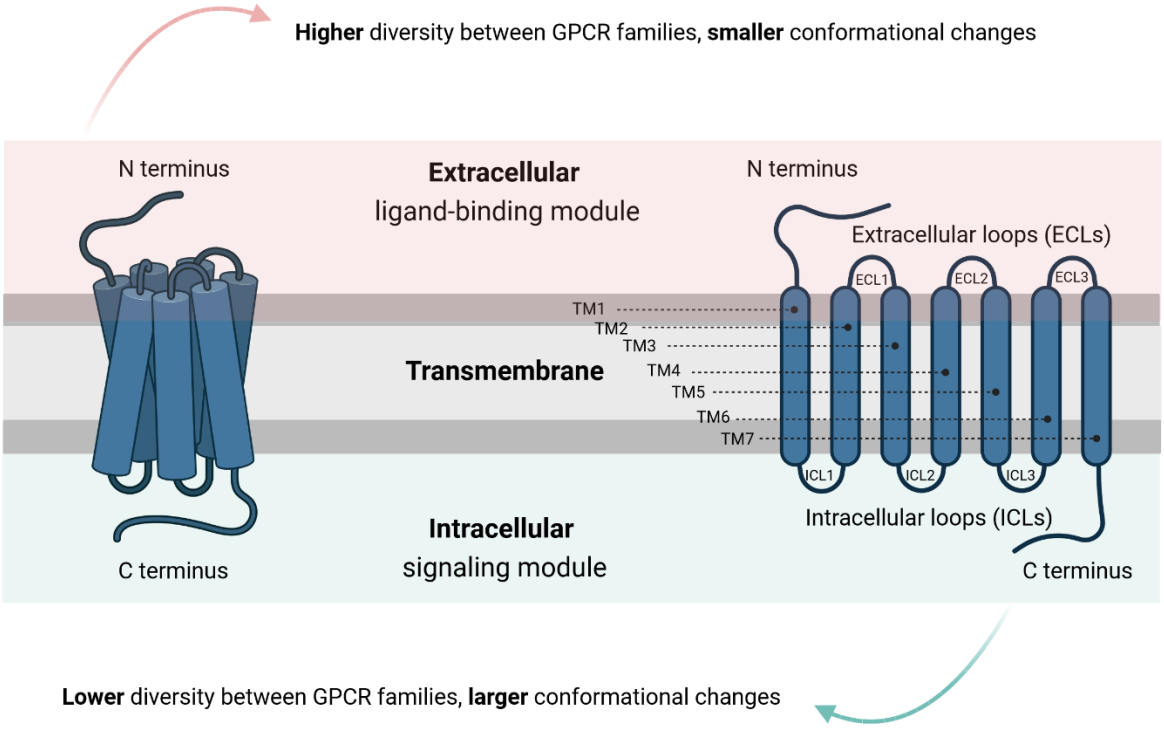

Illustration 2 – Overview of GPCR structure
General GPCR structure and related modules. Illustration based on Katritch et al[4].

1.2 GPCRs activation

GPCRs are highly dynamic and can undergo important conformational changes. In basal conditions, they transition between different states of activation. However, ligand binding is generally responsible for a shift into an active state by promoting or stabilising structural rearrangements[5,6]. Those conformational changes in GPCRs modify the binding interfaces of the transmembrane domains, resulting in a narrower binding pocket and wide changes at the intracellular side[5,6]. Such molecular events determine the recruitment and activation of heterotrimeric G protein complexes; for instance GPCRs show outward rotation of TM helix H6, upon ligand stimulation, resulting in the exposure of the G protein-binding site[7]. When the G protein complex interacts with the receptor, it undergoes conformational changes and

becomes activated, GTP binds to the Gα-subunit and the Gβγ dimer dissociates from the trimer. Both GTP-bound Gα and free Gβγ can mediate intracellular signalling. The human genome encodes for 16 Gα, 5 Gβ and 12 Gγ subunits, theoretically creating hundreds of distinct combinations[8]. Heterotrimeric G proteins are normally divided into four subfamilies according to the Gα subunits ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$) triggering different downstream signalling outcomes (e.g. cAMP modulation, IP3 accumulation, increase of intracellular Ca^{2+} and Rho activation)[9] (Illustration 3). Little is known about the relevance of Gβγ diversity, however a recent study suggested it to be linked to unique translocation to various cellular organelles[8]. GPCRs can couple to more than one specific G protein complex, leading to several distinctive cellular events[9].

GPCRs are regulated by the arrestin family, with the majority of receptors interacting with β-arrestins (arrestin-2, also known as β-arrestin-1, and arrestin-3, also known as β-arrestin-2), which serve as scaffold proteins regulating receptor signaling in time and space[10,11]. Visual arrestins (arrestin-1 and arrestin-4) are also part of the arrestin family but they are generally involved in GPCR regulation in the visual system.

β-arrestins were initially described for their ability to induce the termination of G protein signaling and were also shown to facilitate receptor internalization by recruiting other proteins (Illustration 3). Most GPCRs interact with β-arrestins upon phosphorylation at multiple sites (“phosphosites”), mainly serine and threonine residues (Ser/Thr) present in the intracellular region of the receptor, including the C terminus and intracellular loops. The G protein-coupled receptor kinases (or GRKs) are extremely important in driving the phosphorylation-dependent events[9,12]. The GRK family is composed of seven members. Four of them are ubiquitously expressed (GRK2, 3, 5 and 6), whereas others have a tissue-specific distribution with GRK1 and GRK7 being part of the visual system and GRK4 being mostly expressed in the testis[13-16]. Remarkably, combinations of six ubiquitously expressed regulatory proteins (the two β-arrestin isoforms and four GRKs, GRK2, 3, 5 and 6) orchestrate the regulation of all non-visual GPCRs[10]. The action of β-arrestin proteins allow receptor desensitization and endocytosis through the action of accessory proteins like clathrin, adaptor protein 2 (AP2) and phosphoinositides. β-arrestin interaction with downstream transducers are facilitated by conformational changes in β-arrestin that take place upon binding to the phosphorylated activated GPCRs[11,17,18]. Of note, it has been proposed that for some receptors, β-arrestins are dispensable for their internalization.

Moreover, it was shown that signaling may still occur from the endosomal compartment following receptor internalization[19,20].

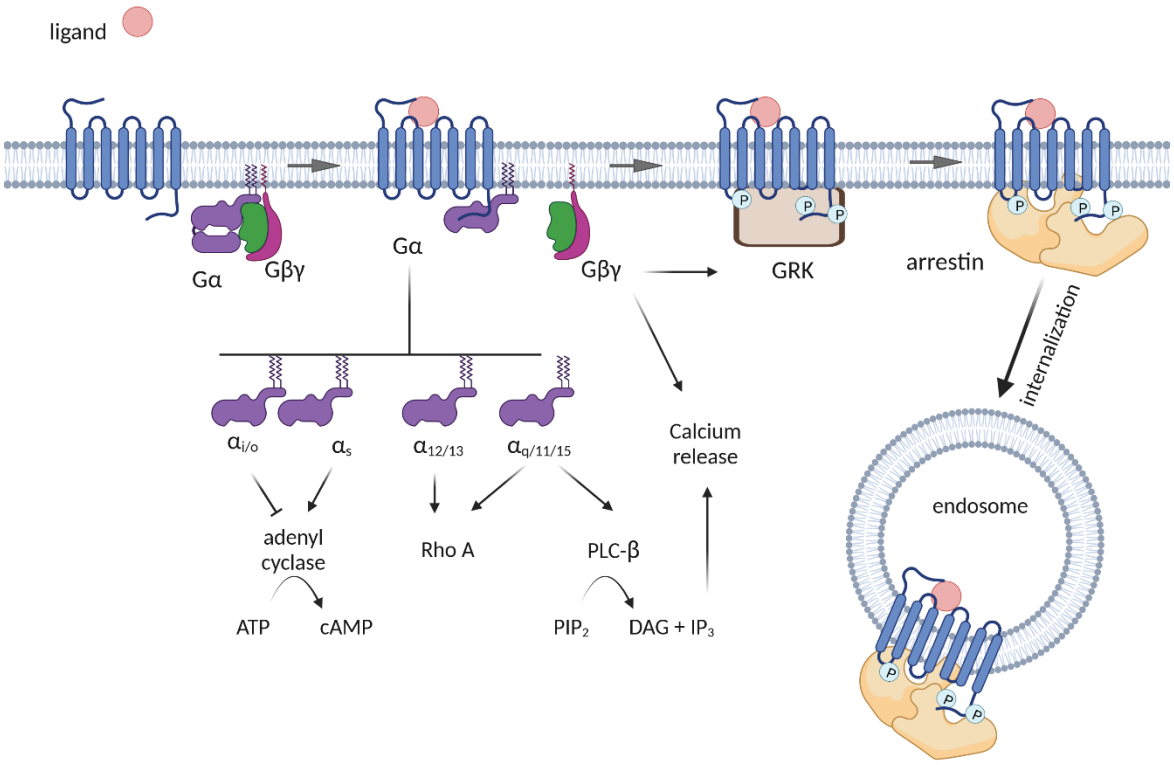

Illustration 3 – Overview of GPCR activation cycle
Schematic representation of the activation cycle of GPCRs. Upon ligand binding, the receptor undergoes conformational changes, which stimulates G protein activation and subsequent dissociation of the Gα subunit from the Gβγ dimer. Engagement of specifics Gα subunits will determine activation of different effector proteins resulting in different cellular events. The free Gβγ dimer can bind to GRKs and promote their recruitment to the GPCR. GRKs phosphorylate the intracellular phosphosites of the receptors. Subsequently, β-arrestins are recruited to the GPCRs and act as scaffold platforms for the recruitment of proteins with several functions, including some supporting GPCR internalization into endosomal structures.

1.3 Biased signalling of GPCRs

A single GPCR can be activated by multiple ligands, which often have different affinities for the receptors and can promote different conformational changes. Based on the effect they elicit on the receptor, the endogenous ligands can be typically divided into full agonists, characterized by the highest detected efficacy, and partial agonists, which show a reduced maximal response compared to the full agonist. Different ligands can also preferentially activate one intracellular signalling pathway over another, this ability being defined as biased signalling. One example of biased signalling is the ability of different molecules, be it endogenous ligands or synthetic molecules, to stimulate distinct G protein subfamilies[21]. The idea of selectively triggering specific signalling events with the activation of a given GPCR is rather appealing. In particular, biased signalling generates the possibility to aim to develop

pharmacological modulators able to dissociate the favourable therapeutic effects from the unfavourable side effects, by selective activation of defined signalling pathways. The distinct cellular responses observed have been proposed as consequences of biased ligands stabilizing different receptor conformations, which retain distinct affinities for transducer proteins[22-25]. The dynamics of the relationship between GPCR conformation and the precise signalling deriving from it are not fully delineated. To assess biased signalling it is important to monitor transducers directly interacting with the receptors like G proteins, GRKs and β-arrestins, rather than focusing on the outcomes of the signalling cascade, like changes in cAMP levels or ERK phosphorylation. This is because the activity of proteins downstream of the signalling pathway can be influenced by systemic responses or tissue-specific events[25].

Ligand bias is a widely studied form of biased signalling. However, the receptor itself may also be at the origin of biased signalling and preferentially activate a pathway over another in response to a "balanced" ligand. Additionally, tissue bias may be observed with different effects for the same ligand–receptor pair depending on where they are expressed[26].

1.4 GPCR isoforms

Different GPCR-mediated signalling effects can be due to variations in receptor sequence or expression, accounting for an additional source of bias. Single receptors can be expressed as diverse functional isoforms characterized by distinct tissue distribution, particular signalling abilities or modified ligand selectivity[27,28]. Alternative splicing as well as alternative transcription start and termination sites are the sources of GPCR isoforms, which could also result in different receptor structures and functionalities. Splicing machineries and transcription regulatory elements contribute to the definition of distinct cell subsets and tissue-specific expression of the receptor isoforms. The exact impact of GPCR isoforms is still largely unknown. However, a recent analysis, with the use of data integration, explored the structural diversity and functional significance of GPCR isoforms[27] and identified isoforms for several receptors, 363 different GPCRs and 625 distinct isoforms, spanning different GPCR classes. Additionally, the majority of the non-reference isoforms have altered N terminus, which was linked to modified ligand-binding abilities. Alteration of the intracellular region is also common in non-reference isoforms, which was suggested to be associated with altered receptor signalling (transducer coupling, GPCR internalization and membrane trafficking)[27]. As concerns the tissue distribution of receptor isoforms, it is rather heterogeneous. Of note, different tissues have variable presence of multiple GPCR isoforms and the reference isoform is not necessarily the one most abundantly expressed[27]. Isoform

diversity could have important implications and such phenomenon should not be underestimated in the perspective of pharmacological GPCR targeting.

2. The chemokine receptor family

The chemokine receptor family is a subgroup of class A GPCRs. Chemokine receptors are largely expressed on immune cells and allow their migration down the concentration gradient of the related ligands, chemokines. Chemokines and their receptors have therefore an important role in orchestrating the precise positioning of immune cells[29-31].

2.1 Chemokine receptors and their ligands

Chemokines are small soluble proteins (8–14 kDa), also defined as chemotactic cytokines, which regardless their sequences share the same three-dimensional structure. Chemokine structure comprises a flexible N terminus, the characteristic cysteine-containing motif, the "N-loop", one antiparallel β-sheet with three strands, interconnected by the "30s loop" and "40s loop", and a C-terminal α-helix[32]. The human genome encodes for around 50 different chemokines, which are categorized into four main subfamilies based on the type of the cysteine motif (CC, CXC, CX3C and XC, illustration 4). Those cysteine residues form disulphide bridges with cysteine residues further downstream, being crucial for chemokine tertiary structure and thus receptor interaction. Chemokines can also be classified depending on their biological role and expression profile. Most of the chemokines are heavily produced in inflammatory conditions and therefore defined as pro-inflammatory. A minority of chemokines show constitutive expression, suggesting a role in the maintenance of normal immune functions and are therefore defined as homeostatic. Interestingly, some chemokines are reported to be present in homeostatic conditions as well as being highly expressed upon inflammation. Overall, their primary function is to promote and regulate directional cell migration, a process largely known as chemotaxis[31,33,34].

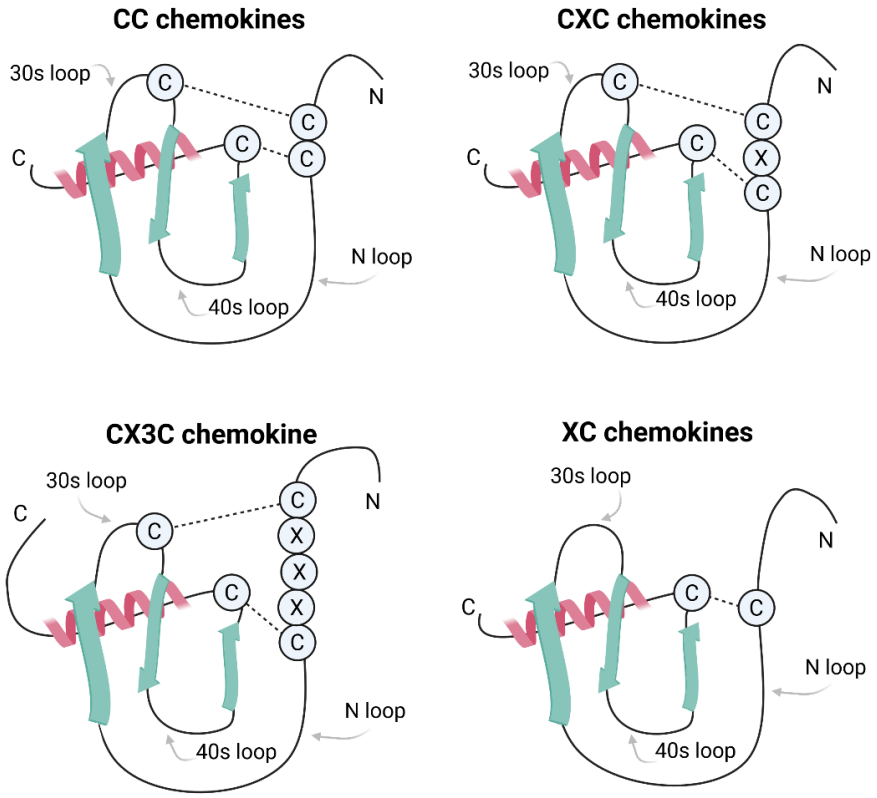

Illustration 4 – Schematic representation of the chemokine subfamilies
Graphical representation of the chemokine classification based on the type of N-terminal cysteine motif (CC, CXC, CX3C and XC). Disulphide bridges are represented as dashed lines, the three anti-parallel β-sheets are shown in green and the C-terminal α-helix in red. Grey arrows indicate the N loop, the 30s and 40s loops. Illustration adapted from Vinader et al[35].

Chemokines interact and activate a subfamily of GPCRs, the chemokine receptors. These receptors can be divided into two classes based on their different properties and functionality. The *classical chemokine receptors*, are mainly expressed by leukocytes and characterized by conventional GPCR signalling (described in section 2.2). They are subdivided into four subfamilies, CCR, CXCR, XCR and CX3CR, according to the class of chemokines they interact with. In humans, 18 classical chemokine receptors have been described (CCR1–10, CXCR1–6, XCR1 and CX3CR1)[25,32,35,36]. The *atypical chemokine receptors* (ACKRs, described in further detail in section 2.4), are mainly present on endothelial and barrier cells and have currently four representatives (ACKR1–4).

The chemokine interaction network is highly intricate: chemokines can bind to one or more classical chemokine receptors and vice versa. An additional layer of complexity is given by the atypical chemokine receptors, which are typically quite promiscuous[37] (illustration 5).

Some chemokine receptors are preferentially expressed by certain cell subsets, like CXCR2 and CXCR6, being mainly found on neutrophils and activated T-cells, respectively, and

others, like CXCR4, show a more ubiquitous expression (hematopoietic stem cells and several subtypes of myeloid and lymphoid cells)[29,30]. In other cases, the receptors are not specifically restricted to cell subsets but the ligand–receptor axis is mainly involved in defined areas, describing a determined function. For example, the homeostatic axes CXCL13─CXCR5 and CCL19─CCR7 are particularly abundant in secondary lymphoid organs, with the former being associated with the organization of B-cell areas for interaction and maturation of subsets of B- and T-cells. The second axis is crucial for the homing of naïve T cells and dendritic cells in the lymph nodes for the process of antigen presentation, a crucial event for the initiation of the immune responses[30,38].

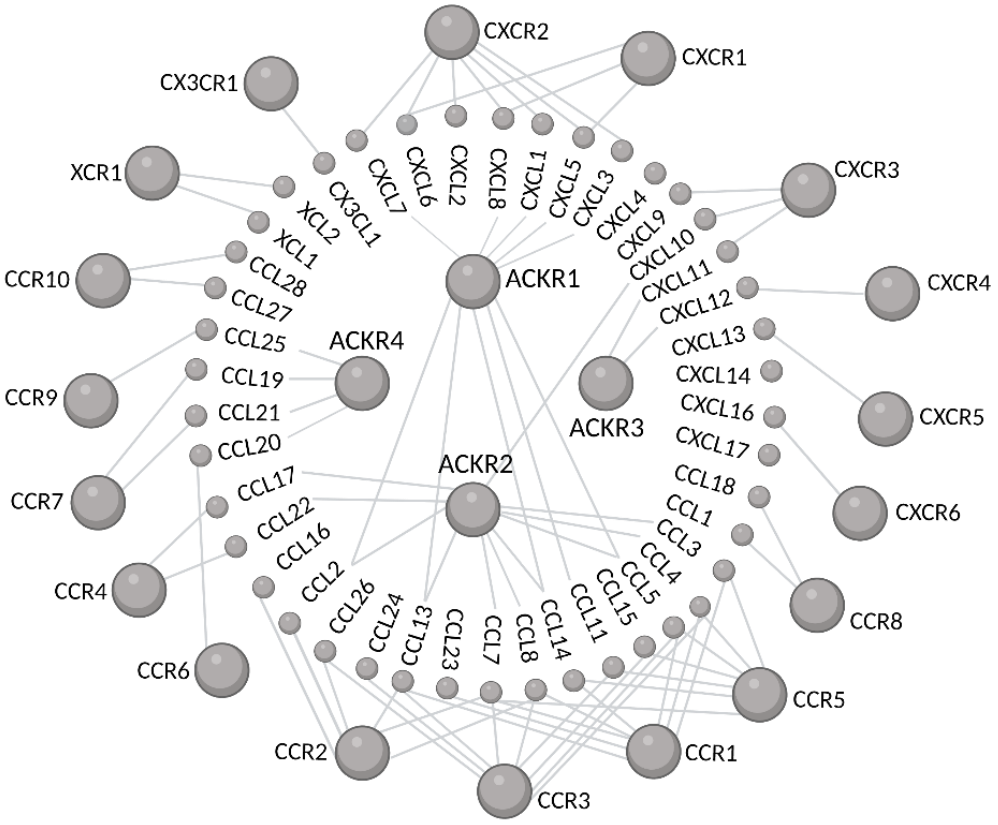

Illustration 5 – Schematic representation of the chemokine–receptor interaction network
Chemokines (intermediate ring) can interact with classical (external ring) and atypical chemokine receptors (centre). Known ligand–receptor interactions are indicated with connecting lines. Illustration based on Sommer et al[37].

2.2 Chemokine–chemokine receptor interactions and related signalling

Biochemical, molecular and structural studies revealed critical to shed light on the dynamics and modalities of chemokine–chemokine receptor interactions[39-43]. The initially described "two-site model" illustrated the importance of the binding of the receptor N terminus (also defined as chemokine recognition site – CRS1) to a groove between the chemokine N-loop and the β3-strand[44]. This first step of interaction was suggested to dictate part of ligand–

receptor specificity. The second step, crucial for receptor activation and subsequent induction of conformational changes, occurs between the chemokine N terminus and the receptor transmembrane domains exposed to the extracellular environment (CRS2)[39,40]. Thanks to advancements in structural studies, it is now possible to appreciate that the chemokine–receptor interactions are more complex than described in this model, with contiguous binding interfaces rather than spatially separated regions and an important role detained by the receptor ECLs[45,46].

Upon activation, chemokine receptors mainly signal via the $G\alpha_{i/o}$ subfamily, which leads to the inhibition of adenylyl cyclase resulting in reduced levels of cAMP within the cells. After chemokine-induced G protein activation, increased Ca^{2+} ions are detected, normally linked to the activity of Gβγ dimer, which triggers phospholipase C-β activity (PLC-β), that catalyses the conversion of phosphatidylinositol-4,5–bisphosphate (PIP_2) to diacylglycerol (DAG) and inositoltriphosphate (IP_3)[47 32,42] Free IP_3 is the direct mediator of calcium ion release from the endoplasmic reticulum. Some chemokine receptors were shown to be able to activate $G\alpha_q$ signalling as an alternative pathway, which acts via the modulation of intracellular calcium levels[48-50]. The downstream cascade encompasses activation of ERK and several other kinases, altogether causing cytoskeletal re-arrangement and cell migration.

Similar to other GPCRs, chemokine receptor activation is regulated by GRK-mediated phosphorylation at the intracellular sites. Importantly, serine and threonine residues from the receptor C terminus and intracellular loops constitute GRK-targeted receptor phosphosites. This event is followed by β-arrestins recruitment to the receptors, receptor internalization and delivery to the endosomal compartment[12,32,34,42].

2.3 Chemokine regulation

The activity of the chemokines is highly regulated at different levels. In addition to gene expression and transcription regulation, there are post-translation modifications (PTM) that can potentiate or hinder their activity. Enzymatic cleavage by protease is an important PTM that affects different chemokines[51-53]. Different proteins are responsible for this, matrix-metalloproteases and dipeptidyl peptidases are among the most studied. Dipeptidyl peptidase IV or CD26 is a membrane glycoprotein that cleaves chemokines at the N-terminus upon recognition of specific N-terminal amino acids (X1-P2↓-X3). The physiological outcome of CD26-shortened ligands is variable and largely depends on the chemokines and the receptor considered: some are variable like CCL5, which was found unchanged, inactivated, or with modified receptor specificity. Other chemokines lose their activity, like CXCR3

ligands or CXCL12 with respect to CXCR4[53-58]. Chemokines are subjected also to other PTMs like C-terminal truncation, glycosylation, nitration or citrullination, resulting in a wide range of effects[51,59-62].

Of note, chemokines are reported to interact also with glycosaminoglycans (GAGs) leading to the formation of a chemokine gradient on endothelial layers and extracellular matrix, further contributing to precise leukocyte migration and positioning[53,63]. GAGs-chemokines interaction can also be protective towards PTMs, which affect the binding of the chemokines to both chemokine receptors and GAGs[55,61,63]. Chemokines were also recently described to bind membrane phospholipids, such as phosphatidylserine, where they retain receptor-activating abilities[64].

An additional mechanism of chemokine regulation is embodied by a small subfamily of chemokine receptors, the atypical chemokine receptors (ACKRs).

2.4 Atypical chemokine receptors (ACKRs)

ACKRs constitute a distinct group of chemokine receptors, currently composed of four members: ACKR1, previously known as DARC; ACKR2, or D6; ACKR3, formerly CXCR7, and ACKR4, alternatively called CCRL1 or CCX-CKR. Over the years additional receptors have been proposed as members of this family (CCRL2, PITPNM3, and more recently GPR182), however, their validation is still pending[65-71]. ACKRs play an important role in chemokine regulation. Indeed, they were initially described as decoy or scavenger receptors regulating the availability of their ligands in the extracellular environment and therefore controlling the activation of classical chemokine receptors[33,34,36,72].

ACKRs are expressed on different cell subsets than their classical counterparts. They are mostly found in non-hematopoietic cells and only rarely in leukocytes. They are largely present at barrier sites with enrichment in lymphatic and blood endothelial cells, stromal cells and, to some extent, epithelial cells. These atypical receptors are grouped together based on their inability to induce the classical G protein-related signalling upon binding to their ligands, leading to their consideration as non-signalling receptors. Most of them (ACKR2, ACKR3, ACKR4) are able to interact with the regulatory proteins, β-arrestins, upon stimulation with related ligands, and, ACKR2 in particular, constitutively[73-78] (Illustration 6). Some reported the possibility of G protein-independent signalling, however, this topic is still largely debated in the field[26,79-81].

Atypical chemokine receptors are responsible for chemokine internalization, also defined as uptake, which can then be transported to other sides of the cells (like ACKR1) or targeted to lysosomal degradation (ACKR2, ACKR3 and ACKR4)[33,34,65,66,72,82,83]. Due to this ability to internalize their targets, they are crucial for the control of extracellular concentrations of the chemokines, therefore acting as important players in the regulation of classical chemokine receptors by maintaining and shaping the chemokine gradients. Those atypical receptors can regulate both homeostatic and inflammatory chemokines. In animal models, their absence has been associated with alterations of physiological processes. For example, genetic elimination of ACKR3 results in perinatal death due to developmental defects, whereas, in absence of ACKR2, animals show deregulated immune functions that are particularly evident in the case of uncontrolled inflammatory processes.

ACKRs can bind a large spectrum of chemokines, with the exception of ACKR3 that only binds two endogenous CXC chemokines. Some atypical receptors can bind chemokines from different families, although to date, XC and CX3C chemokines are not reported to interact with ACKRs[33,66]. Over the recent years, novel chemokine ligands have been identified for different atypical receptors[75,76,84]. Interestingly, non-chemokine ligands were discovered to bind to ACKR3[77,78].

Atypical chemokine receptors also retain a particular cellular distribution. In contrast to the conventional receptors, typically present on the plasma membrane, they are predominantly found on intracellular membranes. Some members, like ACKR2 and ACKR3, are also described by the capacity of continuous cycling between the cell surface and the cytoplasm, defined as constitutive trafficking (Illustration 6).

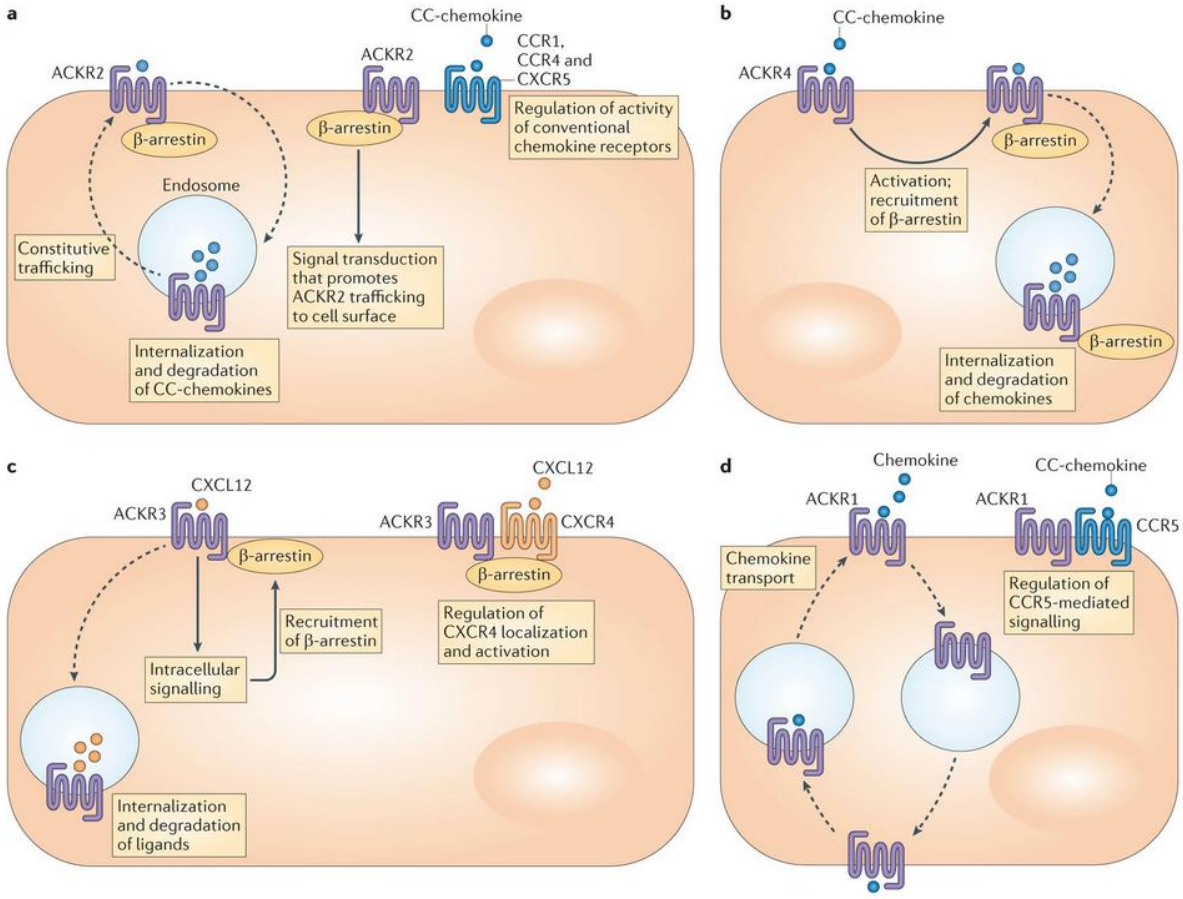

Illustration 6 – Schematic representation of ACKR functionalities
Description of ACKR2 (a), ACKR4 (b), ACKR3 (c) and ACKR1 (d) activities upon interaction with their cognate chemokine ligands. Illustration from Nibbs et al[83].

3. CXCR3

The chemokine receptor CXCR3 is a classical chemokine receptor activated by three inflammatory chemokines CXCL11, CXCL10, and CXCL9, formerly known as I-TAC (IFN-inducible T cell α chemoattractant), IP-10 (IFN-γ-inducible protein of 10 kDa), and Mig (monokine induced by IFN-γ), respectively[85-87]. CXCR3 is abundantly expressed on activated immune effector cells like T-lymphocytes, particularly present on $CD8^+$ T cells and Th1 $CD4^+$ T cells but also on other T helper subsets and T regulatory cells. In addition, it is also found on NK cells, NKT cells, and specific types of dendritic cells and B cells. There is also evidence of CXCR3 presence on non-leukocyte cells like fibroblasts, endothelial, and epithelial cells[86-91]. This receptor is considered a crucial player in adaptive immune responses by coordinating the positioning of the abovementioned leukocytes in the inflamed sites. Its relevance in inflammation is confirmed by animal models, where genetic ablation of CXCR3 leads to a reduced recruitment of T cells and alters T cell subsets composition during infections, chronic inflammations, or autoimmune diseases[87,92-94].

3.1 CXCR3 and related ligands

CXCR3 ligands CXCL9, CXCL10 and CXCL11 belong to the family of IFN-inducible chemokines. IFN-γ induces the production of all three ligands, but other pro-inflammatory molecules are also able to do so, including IFN-β for CXCL9 or IFN-α and IFN-β for CXCL10. TNF can also trigger the production of all three chemokines but to a lower extent[87,95] (Illustration 7). *In vivo*, the effects induced by CXCR3 ligands are non-redundant suggesting different biological contexts of receptor activation[95]. This could be associated with the different promoter elements of CXCL11, CXCL10, and CXCL9, therefore being upregulated in response to different inflammatory stimuli or depending on the cell types (tissue bias)[87,88,95]. These IFN-inducible chemokines are secreted by a large variety of cells, including human microvascular endothelial cells (HUMEC), keratinocytes, fibroblasts, peripheral blood mononuclear cells (PBMC), monocytes, macrophages, and T cells[96-99] (Illustration 7).

As concerns the mechanisms of ligand–receptor interaction, the three ligands seem to engage different CXCR3 regions[100,101]. The structure of CXCR3 in an active state with CXCL11 was resolved only very recently[102]. The importance of the N terminus of CXCR3 in the initial binding of CXCL11 was shown, specifically through a conserved Pro42-Cys43 (PC) motif, also termed chemokine recognition site 1.5 (CRS1.5) found to stretch into the groove formed by β2, β3 and N-loop of CXCL11. Furthermore, the N terminus (residues 1–8) of CXCL11, was reported to insert deeply into the central binding pocket (chemokine recognition site 2, CRS2) of CXCR3, rather in agreement with "two-site model" (detailed in section 2.2)[102]. In line with the current notion of more complex chemokine–receptor interactions, characterized by contiguous binding interfaces with the involvement of receptor ECLs, the authors observed important interactions between the 30s loop of CXCL11 and ECL2 of CXCR3 in receptor activation[102]. The ligand–receptor binding are now clarified for CXCL11, but they are yet to be established for CXCL10 and CXCL9.

These three chemokines bind to CXCR3 with different affinities, with CXCL11 being the strongest and CXCL9 the weakest ligand, respectively[87,95,103,104]. Their abilities to stimulate calcium mobilization and β-arrestin recruitment reflect the respective affinities[87,95,105]. In some studies, the CXCR3 ligands are considered to be responsible for biased signalling, inducing diverse downstream signalling events or showing different efficacy and potency in β-arrestin recruitment and receptor internalization [26,106-108].

CXCR3 ligands largely interact with glycosaminoglycans (GAG), which are involved in chemokine accumulation in specific sites and support directional leukocyte migration. Binding of these chemokines to GAGs was reported to be crucial *in vivo* and may also protect them from inactivating PTMs, like the one induced by CD26[55,109-111].

In addition to CXCL9, CXCL10 and CXCL11, CXCR3 was suggested to bind the platelet-derived CXCL4 and CXCL4L1, triggering angiostatic functions[112-115]. Those chemokines are considered to mediate leukocyte recruitment to inflamed endothelium. Despite their ability to define immune cell positioning being largely established, the specific receptor(s) through which they act remains unclear. In addition to CXCR3, CXCL4 and CXCL4L1 were proposed to bind different chemokine receptors over the years, like CCR1[112-114,116]. Additionally, they were described to indirectly interact with different chemokine receptors in form of heterodimers with chemokines like CCL5 and CXCL8[117-119]. Noteworthy, CXCL4 and CXCL4L1 are characterized by the presence of several positively charged amino acids that explain their high affinity for GAGs. A recent study proposes CXCL4 as an unconventional chemokine that can induce immune cell recruitment without activation of chemokine receptors, but rather by interaction with GAGs. Such a mode of action could explain the variety of leukocyte subsets that have been shown to transmigrate when exposed to these ligands[120].

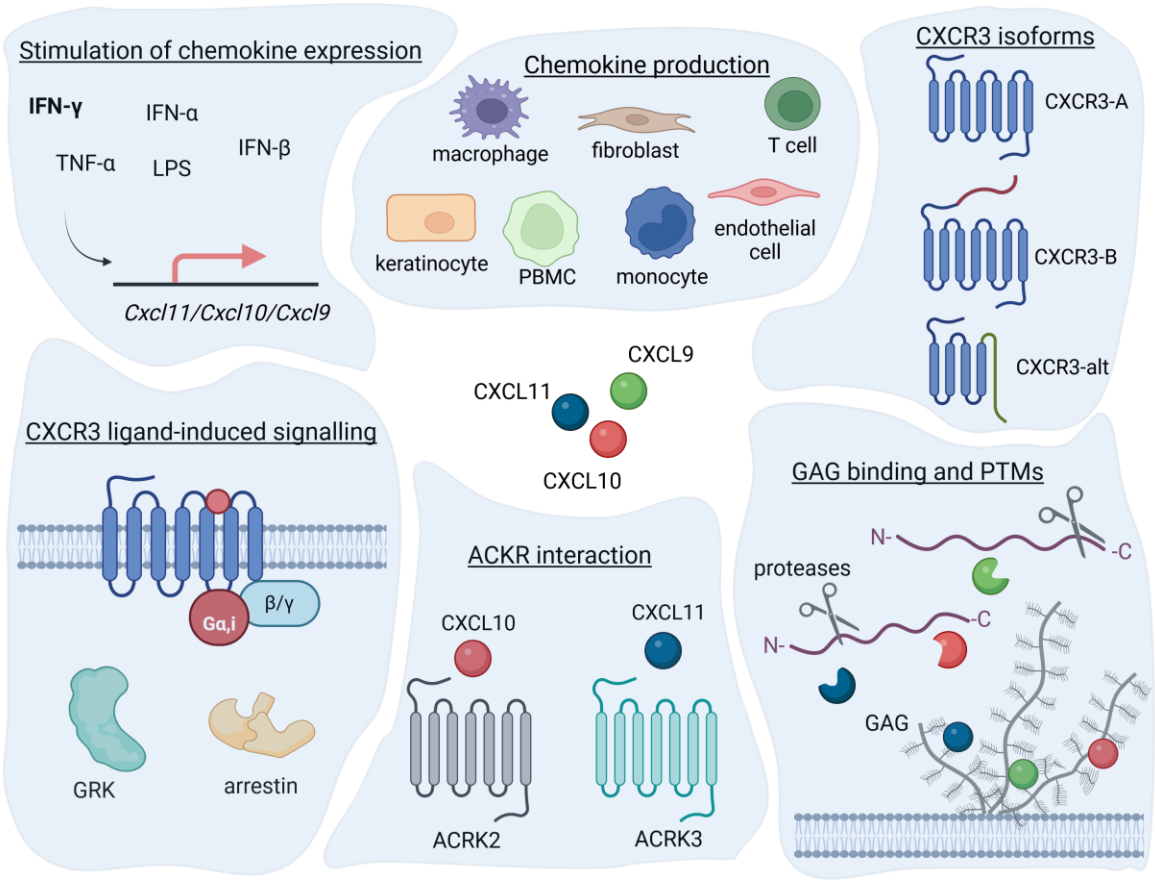

Illustration 7 – CXCR3 and its ligands
CXCL11, CXCL10, and CXCL9 are CXCR3 ligands produced upon specific inflammatory stimuli from a large variety of cells. They are also regulated at the level of GAG binding and PTMs. Their availability is regulated by atypical chemokine receptors ACKR2 and ACKR3. When interacting with CXCR3, those chemokines trigger the classical chemokine receptor signalling characterized by Gαi protein activation, subsequent phosphorylation by GRKs and interaction with β-arrestins. An additional layer of complexity is given by the presence of CXCR3 splicing isoforms: CXCR-3-A, CXCR3-B and CXCR3-alt, which possess differential features. Illustration based on Metzemaekers et al[87].

3.2 Molecular aspects of CXCR3

There has been a growing interest over the years in therapeutic targeting of CXCR3 or its ligands in inflammation. To overcome the scarce success of such efforts, the idea of biased agonists able to selectively stimulate the desired effects was considered as an appealing strategy[24,106,121-123]. To better understand the outcomes of CXCR3 activation by its three endogenous ligands (CXCL11, CXCL10, CXCL9), different *in vitro* and *in vivo* studies have investigated their molecular and functional properties[69,124-127].

The binding of any of these three ligands to CXCR3 triggers PTX-sensitive Gαi activation to initiate the signalling cascade leading to cell migration[128,129]. It was recently shown that activated CXCR3 could also trigger G protein signalling from the endosomal compartment[124]. Concerning receptor internalization, CXCL10- and CXCL9-induced

internalization were suggested to mainly rely on receptor C terminus whereas CXCL11 shows a partial dependency on the intracellular loops[130]. The relevance of β-arrestins for receptor internalization is debated; some reports suggest it to be β-arrestin independent[130,131], while another study supports their dependencies[124]. The precise contribution of GRK-mediated phosphorylation in β-arrestin recruitment to CXCR3 is still largely unexplored. In 2023, an analysis, assessing the impact of CXCR3 C terminus on biased signalling, revealed agonist-induced GRK2 and GRK3, but not GRK5 or GRK6 recruitment to the chemokine receptor[69].

3.3 CXCR3 isoforms

CXCR3 encodes for a protein of 368 amino acids and it is the only variant present in mice. In 2003 and 2004, two additional variants were described in humans[112,132]. Upon identification of the novel isoforms, the well-characterized CXCR3 was proposed to be renamed CXCR3-A, and the newly discovered versions CXCR3-B and CXCR3-alt[112,132]. In comparison to the reference isoform, CXCR3-B (415 amino acids) has an extended N terminus in which as result of alternative splicing, the first four residues found in CXCR3-A are "replaced" by different 51 amino acids[112]. CXCR3-alt is characterized by strong structural alterations: because of exon skipping leading to a 337-bp deletion, the receptor has a predicted structure retaining only four or five transmembrane domains (267 amino acids)[132] (Illustrations 7 and 8).

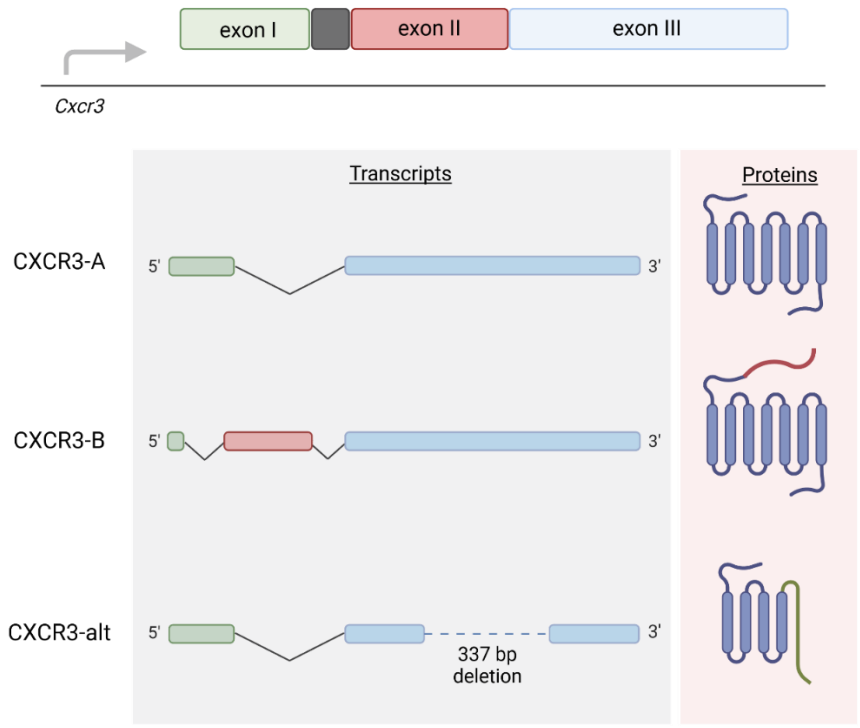

Illustration 8– CXCR3 isoforms
Overview of CXCR3 isoforms at transcriptional level and protein level. Following alternative splicing, three different transcripts can be generated that will result in three structurally and functionally different proteins, CXCR3-A, CXCR3-B and CXCR3-alt. Illustration based on Metzemaekers et al[87].

Tissue distribution of the receptor isoforms was mainly assessed at transcriptomic level[27,112,133,134]. CXCR3-A is mainly found on immune cells, CXCR3-B was suggested to be co-expressed but at a lower level[135]. Moreover, CXCR3-B is described as selective isoform in endothelial cells[112]. However, a detailed definition of isoform-specific expression at the protein level remains complex due to the scarcity of reliable antibodies selective for the different receptor isoforms.

Regarding the ligand selectivity of the alternative isoforms, CXCR3-B is suggested to share the ligands CXCL11, CXCL10, and CXCL9 with the canonical isoform[33], whereas CXCR3-alt was reported to only bind CXCL11[132]. CXCL4 was originally shown as an exclusive ligand for CXCR3-B isoform[112], subsequently described to similarly interact with both CXCR3-A and CXCR3-B[113,136] and ultimately proposed to mediate leukocyte migration via GAG binding instead of chemokine receptors[120].

Considering the signalling properties of CXCR3 isoforms, it appears that CXCR3-B is able to recruit β-arrestin-1 and β-arrestin-2 upon stimulation with the related ligands, albeit with reduced potency and efficacy. Additionally, it seems to have differential G protein signalling

compared to CXCR3-A[127,137]. One of the few molecular analyses of CXCR3-alt revealed conflicting signalling abilities for this isoform. No cAMP modulation or β-arrestin recruitment could be detected after ligand stimulation, however a faint induction of ERK phosphorylation was observed[137].

CXCR3 and related ligands are important in different diseases[138-142], such as cancer, where their role is rather complex and debated[90,143-147]. Overall, neither the receptor nor the chemokines can be considered as universal favourable or unfavourable markers and depending on the tumour type they can have different prognostic implications. CXCL10 was shown to reduce cancer cell growth and invasiveness in melanoma[148]. In breast cancer, CXCL9 is considered as a good prognosis marker whereas CXCL10 is indicative of a negative prognosis[147,149]. In a mouse model of the same cancer type, the presence of CXCR3 was reported as a biomarker for the efficacy of PD-1-based immunotherapy[150]. In the same year, another study supported the role of CXCR3-B in promoting cancer stem cells in breast tumours[151]. Additional studies across different cancer types, including colon cancer, melanoma, breast cancer, renal cell carcinoma and many types of leukaemia highlighted conflicting links with CXCR3, varying from favourable outcome to association with highly aggressive malignancy[90,145,152]. It was proposed that in some cases CXCR3 could mediate the recruitment of tumour-suppressive immune cells, and be responsible for an anti-tumour effect, whereas in other instances CXCR3 is tumour-supportive and promotes cancer cell growth and metastasis formation[145]. The distinct receptor isoforms were also considered as bearing opposing roles in the tumour context with CXCR3-A being the pro-tumoural isoform and CXCR3-B having a tumour-controlling role[134,153-161]. Additional studies are needed to better elucidate to what extent the opposing roles of CXCR3 in cancer can be attributed to the presence of the two isoforms.

4. Glioma

Diffuse gliomas constitute the most common type of primary brain tumour in adults (~80%). They originate from glial cells and are aggressive and heterogeneous neoplasms characterized by high mortality rate[162-165]. Appropriate classification is essential to discriminate different glioma types in groups sharing similar features. Because of morphological aspects, histology-based, tumour sections presenting nuclear atypia and increased mitotic activity are considered low-grade gliomas (LGG), whereas the presence of microvascular proliferation and necrotic regions is used to discriminate the high-grade gliomas[166,167]. Glioma patients have a broad age range, however, an important distinction

has to be made between adult-type and paediatric diffuse gliomas. They have been shown to be largely different malignancies, with distinct developmental origins, and variable biological and molecular features[168-170]. The grouping of gliomas based on specific phenotypic and genetic alterations is also crucial for therapy selection, trying to target the effects of the mutations identified[170]. In 2016, the fourth edition of the WHO classification of Tumours from the Central Nervous System (WHO CNS4) introduced the use of molecular biomarkers, in addition to the long-established histological profiling, for the specific clinical diagnosis. The importance of the molecular features has been reinforced and expanded in 2021 with the updated WHO CNS5 classification[170,171]. Genetic alteration of isocitrate dehydrogenase (IDH) and co-deletion of chromosome 1p and 19q whole arms (1p/19q codel) are now used to classify adult-type diffuse gliomas in three entities: oligodendroglioma (IDH mut, 1p/19q codel, grade 2,3), astrocytoma (IDH mut, grade 2,3,4) and glioblastoma (IDH wt, grade 4)[171]. Additional genes are often found mutated or silenced in CNS neoplasms and used for accurate diagnosis (α-thalassemia mental retardation X-linked, *ATRX*; tumour protein p53, *TP53*; cyclin-dependent kinase inhibitors 2A and 2B, *CDKN2A/B;* promoter of telomerase reverse transcriptase, *TERT promoter;* copy number alteration of chromosomes 7 or 10; epidermal growth factor receptor, *EGFR*; platelet-derived growth factor receptor-α, *PDGFRA*, Met tyrosine-protein kinase, *MET*; V-raf murine sarcoma viral oncogene homolog B, *BRAF;* phosphoinositide 3-kinase, *PI3K;* neurofibromatosis type 1, *NF1*; cyclin-dependent kinase 4, *CDK4*)[172].

4.1 Glioblastoma

Glioblastoma (GBM) is the most common and aggressive primary brain malignancy representing around 48% of all primary cancers of the central nervous system, with a median overall survival of about 15 months[166,173]. Despite remarkable advances in the development of effective therapies against many cancer types, the standard of care for GBM has not changed over the last two decades and consists of maximal tumour resection, followed by localized radiotherapy in combination with the chemotherapeutic agent temozolomide (TMZ)[165,173]. GBM shows a particularly strong tumour cell infiltration, which renders complete resection tremendously difficult to achieve[174]. Additionally, this tumour is characterized by the presence of hypoxic areas, which can support niches of glioma stem cells, largely considered as responsible for malignancy relapse[165-167,174,175] (Illustration 9).

Glioblastoma is still incurable and, in all cases, the disease will progress or relapse (rGBM). There are several options for the treatment of recurrent GBM, the two most common being

the use of a different chemotherapy drug, lomustine, for patients with MGMT methylation, or EGFR inhibitors, like bevacizumab[163,176-178]. A potential option could be immunotherapy aiming to transform a "cold tumour" into a "hot tumour"[174,179] i.e. switch the tumour microenvironment from scarcely to highly infiltrated with activated immune cells. Despite the use of immune checkpoint inhibitors proved to be an efficacious treatment in patients with melanoma brain metastasis[180,181], this therapy does not increase GBM patient's survival[182].

Glioblastoma is characterized by an important heterogeneity, both intertumoural and intratumoural[173,174,183]. Integration of bulk and single-cell RNA sequencing data allowed the identification of four cellular states for tumour cells in GBM: neural-progenitor-like (NPC-like), oligodendrocyte-progenitor-like (OPC-like), astrocyte-like (AC-like), and mesenchymal-like (MES-like) states[184]. Differently from how previously considered, these cellular states are found within the same tumour[185,186]. However, the distribution of cells among the four distinct states varies between different GBM samples and each state is characterized by genetic alterations in CDK4 (NPC-like), PDGFRA (OPC-like), EGFR (AC-like) and NF1 (MES-like)[184].

Other important factors such as the tumour site and the strong immunosuppressive tumour microenvironment have a negative impact in terms of therapeutic efficacy. The brain has been considered an immune-privileged site for long. Recent evidence suggests the presence of an active mechanism responsible for maintaining immune regulation[173,174]. Accumulation of adaptive immune cells, and in particular effector T cells, remains a big challenge. The blood-brain barrier (BBB) is a protective element of the CNS and it may interfere with the delivery of drugs like chemotherapeutic agents and limit the recruitment and infiltration of the immune cells. In healthy brains the large majority of molecules are filtered by the BBB, however, in GBM those capillaries are greatly altered (Illustration 9). The tumour-associated capillary bed is characterized by increased permeability, upregulation of transporter proteins, and formation of new vessels feeding the cancer cells[174,187].

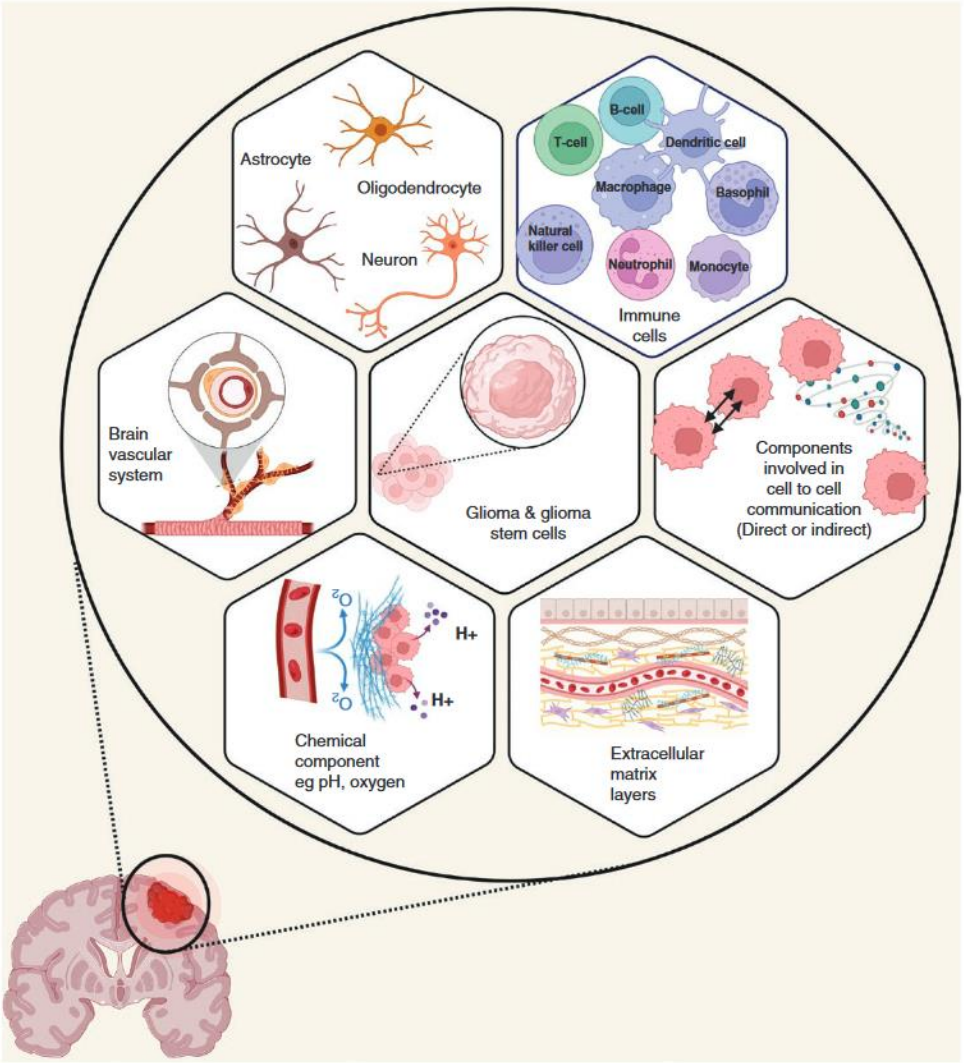

Illustration 9 – Glioma tumour microenvironment (TME)
Schematic representing the components of glioma TME, characterized by the presence of brain cells, immune cell infiltration, hypoxia, remodelling of the extracellular matrix, tumour cells subverting the surrounding cells, vascular alteration and glioma stem cells. Illustration from Sharma et al[188].

GBM tumour microenvironment is strongly immunosuppressive, with an extremely scarce presence of neoantigens able to boost anti-tumour immune reaction[173]. The presence of infiltrating T cells is very limited and, when present, it was shown to positively correlate with favourable clinical outcome[189]. The great majority of immune cells present in GBM belong to innate immunity and are tumour-supportive[188]. Microglia, macrophages and monocytes can constitute even half of the whole GBM mass and secrete several molecules favouring tissue remodelling and modifications of the extracellular matrix under the influence of the cancer cells[173,188]. In addition to immune cells, GBM can interact and subvert cellular types of the brain like neurons, astrocytes and, oligodendrocytes (Illustration 9).

4.2 The chemokine system in gliomas

The chemokine network is responsible for the correct positioning of leukocytes. They also orchestrate immune cell recruitment in the TME. In the context of cancer, they play a role in intercellular communications with the involvement of immune cells but also cancer cells and stromal cells[190-194]. The chemokine system can contribute to anti-tumour response with recruitment of activated effector cells and their presence would favour immune-mediated tumour eradication. In some cases, chemokine–receptor interactions mediate tumour-supportive functions, by enhancing an immunosuppressive phenotype of the leukocytes and tissue remodelling[190-194]. Several descriptions highlighted the presence and relevance of chemokines and their receptors in gliomas, GBM included[195-198]. The importance of the CXCR4–CXCL12 axis in this disease is well established and its activity contributes to the carcinogenic process and disease relapse[199-201]. There is growing interest in the possibility of therapeutic targeting of chemokine receptors as an alternative approach to overcome the inevitable recurrence of these deadly malignancies[195,197,201,202].

Research findings

Chemokines binding to cognate receptors are responsible for the induction of migration and the positioning of target cells in specific sites, in physiological and pathological conditions. The chemokine interaction network is extremely complex. Chemokines are able to interact with both signalling receptors (CKRs) and non-signalling receptors (ACKRs). Chemokine receptors are a subgroup of G protein-coupled receptors. While approximately one-third of FDA-approved drugs are directed against GPCRs, very few chemokine receptors are currently targeted in clinic. The complexity of the chemokine network is in part accountable for this discrepancy. To better understand the entanglement of this system, in this thesis, different aspects and settings of the chemokine system have been investigated.

Methodologies were developed and implemented to monitor the activation and trafficking of chemokine receptors, with a particular focus on ACKRs (Chapters I.i and Chapter I.ii). Thanks to technological advances, new ligand–receptor axes have been identified amongst the atypical chemokine receptors[75-78,203,204]. We systematically reviewed the novel pairings proposed, highlighting the importance of methodical reassessment of chemokine–chemokine receptor interactions (Chapter IV).

The chemokine receptor CXCR3, which is crucial for effective immune responses, was analysed in depth at the molecular level (Chapter II and in the annexes). We undertook a thorough molecular and functional comparison of the two major isoforms CXCR3-A and CXCR3-B, and highlighted the atypical features of the latter (Chapter II).

Several players of the chemokine system are largely present in tumours. To obtain a more precise overview of their involvement in cancer, we focused on chemokines and chemokine receptors evaluation in gliomas, with a particular interest in glioblastoma, the most severe brain malignancy. With a translational perspective, using information from patient-derived material, we assessed gene expression levels of the chemokines (Chapter VI.ii) and their receptors (Chapter VI.i) from publicly available datasets, focusing on GBM tissue-derived transcriptomic data.

Chapter I.i - NanoLuc-based methods to study ACKRs

In the last two decades, a subfamily of atypical chemokine receptors (ACKRs) gained attention. The different members identified so far, are grouped because of their shared inability to activate G protein signalling upon interaction with their ligands. The vast majority of assays to study chemokine receptors, or GPCRs in general, relies on the assessment of these pathways, therefore the lack of G protein activation hinders ACKR characterization with the use of canonical techniques. The main function of ACKRs is to remove the chemokines from the extracellular environment by ligand sequestration/internalization[33]. Molecular approaches are now available to investigate protein–protein interactions (PPI) and can constitute alternative strategies to monitor ACKR activation. They allow the detection of receptor activation by assessing their interaction with transducers like β-arrestins and GRKs or their trafficking by monitoring various events such as the disappearance from the cell surface and the subsequent delivery to the endosomal compartment (Illustration 10). The PPI-based assays developed and described in this thesis rely on the bioluminescent protein Nanoluciferase (NLuc) that is able to emit light in the presence of its substrate furimazine[205]. NLuc can be split into two subunits, a small one (SmBiT) and a larger one (LgBiT), which have a very low relative affinity towards each other (K_D= 190 µM). By fusing them to the proteins of interest, in presence of the substrate, it is possible to detect NLuc complementation and reconstitution. When the targeted proteins come in very close proximity, functional NLuc can be reconstituted and generates bioluminescence. NLuc can also be used as donor molecule for BRET (Bioluminescence Resonance Energy Transfer)-based assays, where, in the presence of the substrate, the light emitted will excite a fluorescent acceptor protein resulting in fluorescence emission of a specific wavelength. The bioluminescent donor and fluorescent acceptor are fused to the proteins of interest and energy transfer will take place if those proteins are in close proximity (<10 nm).

In this thesis, different NLuc complementation or NanoBRET-based analyses are described as models to assess the activation and trafficking of two atypical chemokine receptors, ACKR2 and ACKR3[206]. These NLuc-based methodologies revealed to be reliable tools to characterize activity, ligand selectivity and intracellular trafficking of seven-transmembrane receptors uncoupled from G proteins.

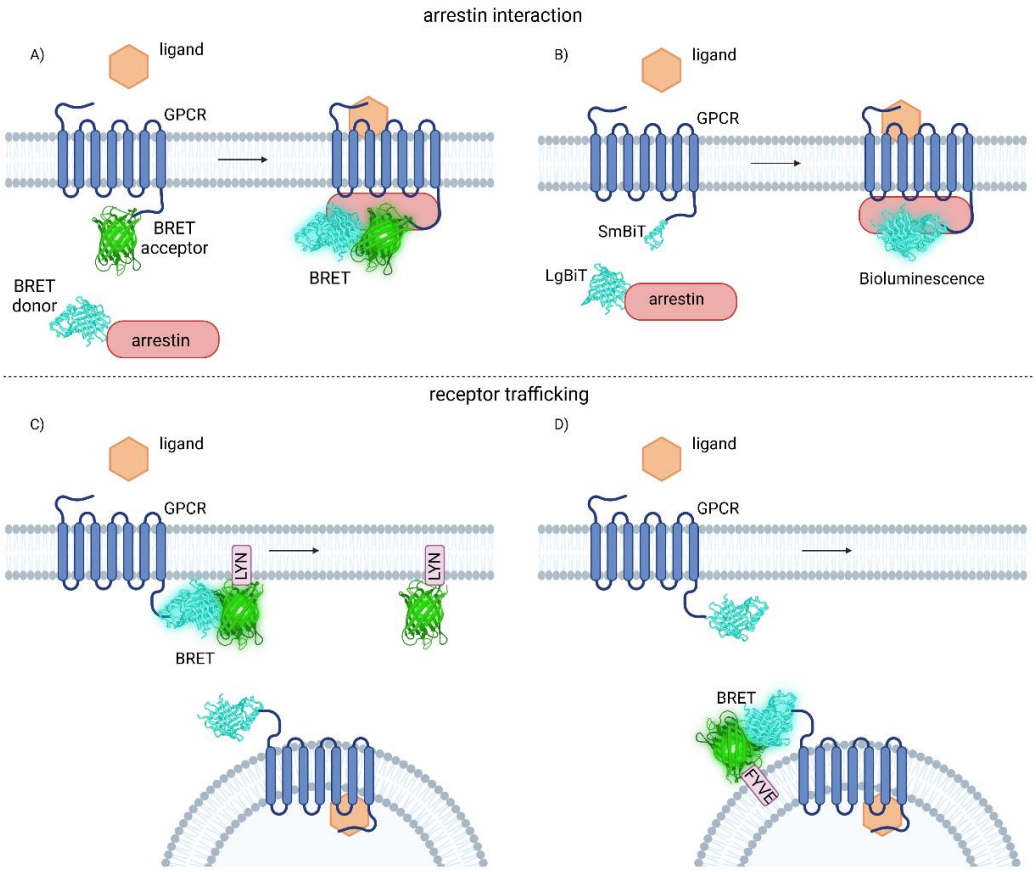

Illustration 10 – Schematic representation of NLuc-based assays to investigate receptor activation
Graphics describing the principles of β-arrestin recruitment analysis by BRET technology (A) and NLuc complementation (B). Receptor trafficking can be monitored by BRET-based plasma membrane dissociation assay with the use of the plasma membrane marker LYN-mNeongreen (C) or with endosomal delivery assay with the early endosome marker FYVE-mNeongreen (D).

Chapter I.ii - NanoLuc-based method to study GPCR-GRK interaction

GPCR stimulation is characterized by the activation of specific G protein subsets and related signalling. Some receptors can be engaged without inducing those signalling events. A common feature among signalling and non-signalling GPCRs is their phosphorylation by GPCR kinases (GRKs) and subsequent β-arrestin recruitment. Recent years have witnessed an increasing interest in the "barcode" hypothesis regulating GPCR–β-arrestin interactions, where β-arrestins can recognize different receptor phosphorylation states to induce determined function. The GRK family includes seven protein members and it is the major group of kinases responsible for receptor phosphorylation at the intracellular sites. GRKs may show specific tissue distribution, like GRK1 and GRK7, which are confined to the visual system, and are mostly expressed in the retina or GRK4, largely found in the testis. Others like GRK2-3-5-6 are ubiquitously expressed and considered to regulate most of the non-visual GPCRs[207]. Phylogenetically, these latter four isoforms can be grouped into the GRK2-3 subfamily and the GRK5-6 subfamily. GRK2-3 show cytoplasmic distribution

while GRK5-6 are membrane-anchored because of the presence of a membrane-binding domain[208,209]. Thanks to the establishment of cellular models genetically ablated for one or more GRK variants[210,211], it is currently possible to investigate their functional impact. So far, they have been largely used to assess specific GRK relevance for receptor internalization or GPCR–arrestin interaction[207,210-212]. However, with those approaches, the precise GPCR–GRK dynamics remain unexplored, especially in terms of kinetics and cellular processes other than β-arrestin interaction and receptor internalization. In our analysis, we methodologically describe the versatility of NLuc complementation assay to monitor GPCR–GRK interaction for several GPCRs[213]. We included distinct types of receptors, including signalling and non-signalling, binding different types of ligands. Our systematic profiling approach revealed the main interactions with the four ubiquitous GRKs (GRK2-3-5-6), with primary recruitment of GRK2 and GRK3 observed shortly following receptor stimulation. The membrane-anchored GRK5 and GRK6 retain variable profiles for different receptors. Some GPCRs show a positive recruitment for GRK5 and GRK6, but in the majority of cases, a progressive reduction of interaction is detected.

Chapter II - CXCR3-B and its atypical features

The chemokine receptor CXCR3 is largely present on different immune cells, mostly activated T-cells but also on NK cells. It is responsible for the migration and positioning of these cells in inflammatory sites[86,95]. Due to alternative splicing, CXCR3 can be expressed as different receptor isoforms: CXCR3-A, CXCR3-B and CXCR3-alt. CXCR3-A is the largely characterized canonical isoform. It is strongly conserved across different species and it can induce cell migration upon receptor activation[87,90,95]. CXCR3-B presents an extended N terminus in comparison to CXCR3-A, that accounts for a difference of 51 amino acids and it was found on human endothelial and barrier cells, in opposition to CXCR3-A which is mostly expressed by leukocytes[90,112]. CXCR3-alt presents important structural variations: it lacks at least two transmembrane domains, which results in important alteration of the receptor structure[132]. This isoform is the least characterized and its relevance is highly debated. From a functional point of view, CXCR3-A and CXCR3-B have been proposed to have opposing functions, with CXCR3-A isoform able to stimulate cell migration and proliferation and CXCR3-B inhibiting such processes[90,154,214]. To better understand the enigmatic CXCR3-B isoform we undertook a detailed molecular analysis in comparison to CXCR3-A[105] (Illustration 12). Activation properties were assessed by evaluating the abilities of the two isoforms to promote intracellular signalling events. Specifically, we monitored G

protein recruitment, activation, and dissociation as well as related downstream signalling events and reported important alterations for CXCR3-B. Despite detrimental impairment in G protein coupling, CXCR3-B was still able to recruit β-arrestin. We also demonstrated that the two isoforms have the same ligand selectivity. In light of these findings, CXCR3-B was hypothesized as having features in analogy with ACKRs, which are characterized by their lack of G protein signalling. To further delineate the unique traits of CXCR3-B, the subcellular localization and ligand internalization abilities were investigated and revealed that CXCR3-B is largely distributed intracellularly and is able to mediate chemokine uptake, therefore adding additional features in common with ACKRs (Illustration 11). Moreover, we found that the N-terminal extension is the determinant for those differential characteristics and therefore contributes to the atypical features of the receptor. Of note, several CXCR3 isoforms were also reported in zebrafish. In this model, they are suggested to have opposite functionalities in the induction of cell migration and proposed as classical and atypical chemokine receptors[215].

Overall, this study delineates CXCR3-B as β-arrestin-biased receptor and highlights the several characteristics that CXCR3-B has in common with atypical chemokine receptors. Given the strong sequence similarity of the two isoforms, the assessment of their endogenous expression remains technically challenging and for this reason, the related distribution is still debated. The possibility of co-expression by the same cells is a tempting speculation, with CXCR3-A being the signalling receptor showing elevated chemokine affinity and the CXCR3-B a possible scavenger, that, due to its reduced affinity, is intervening in case of excessive presence of the related shared ligands.

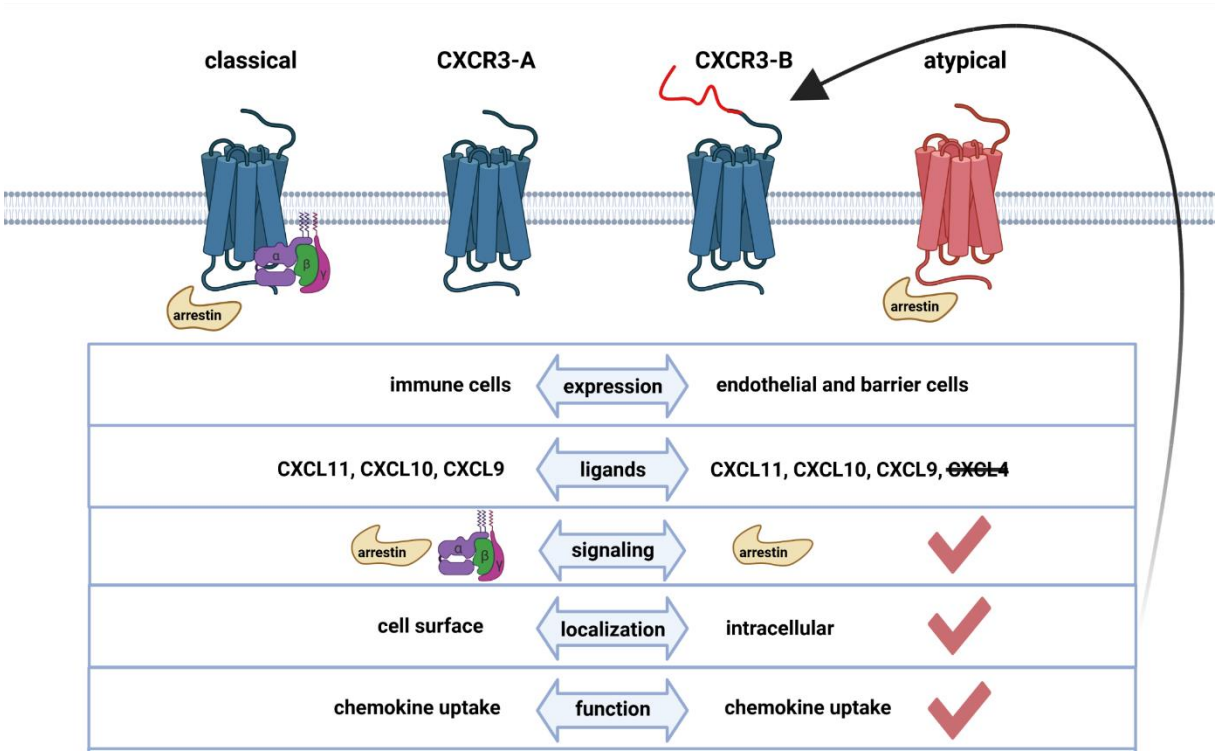

Illustration 11 – Molecular comparison of CXCR3-A and CXCR3-B
Schematic summarizing the differences and similarities of the main two isoforms of CXCR3, that emerged in our detailed profiling[105] at the level of ligand selectivity, signalling pathways, receptor distribution and functionality.

Chapter III – ACKR2 is a scavenger for CXCL10

The atypical chemokine receptor ACKR2 was first discovered in 1997 and suggested as a selective scavenger for pro-inflammatory CC chemokines only (CCL2–8, CCL11–13, CCL17 and CCL22)[71]. ACKR2 is abundantly expressed by endothelial cells and, to a lower extent, by some leukocyte subsets[71,216,217]. Its genetic ablation in mice promotes deregulated inflammatory reaction culminating in exacerbated inflammation[71,216]. ACKR2 is therefore an important regulator of the immune response. In this analysis[75], we identified the IFN-induced CXCL10 as an additional ligand for ACKR2. To date, CXCL10 was only described to activate CXCR3, which promotes recruitment of activated immune cells to inflammatory sites[87]. With the use of NLuc-based PPI assays (described in Chapter I.i), CXCL10–ACKR2 interaction was fully characterized. This study revealed that CXCL10 induces β-arrestin recruitment to ACKR2 with higher potency than to CXCR3. Additionally, ACKR2 was shown to specifically internalize CXCL10 and remove it from the extracellular environment, thereby regulating the activity of the chemokine receptor CXCR3. These findings demonstrate that ACKR2 acts as a decoy for both inflammatory CC and CXC chemokines, adding a layer of complexity to the already elaborated chemokine system. This discovery strengthens the potential of ACKR2 modulation to finely tune inflammatory reactions.

Chapter IV - New pairings among ACKRs

Atypical chemokine receptors constitute a subfamily of chemokine receptors that was initially described as decoy or scavenger receptors. Their distinctive feature is the inability to trigger cell migration or/and G protein signalling[218]. In 2013, ACKR1 (DARC), ACKR2 (D6), ACKR3 (CXCR7) and ACKR4 (CCRL1) have been officially approved as a distinct class of chemokine receptors[33,65]. Since then, several new ligand–receptor pairings have been proposed for these receptors including some non-chemokine ligands. With our short review, we systematically assessed and commented on the novel interactions identified in this area (Illustration 12[82]).

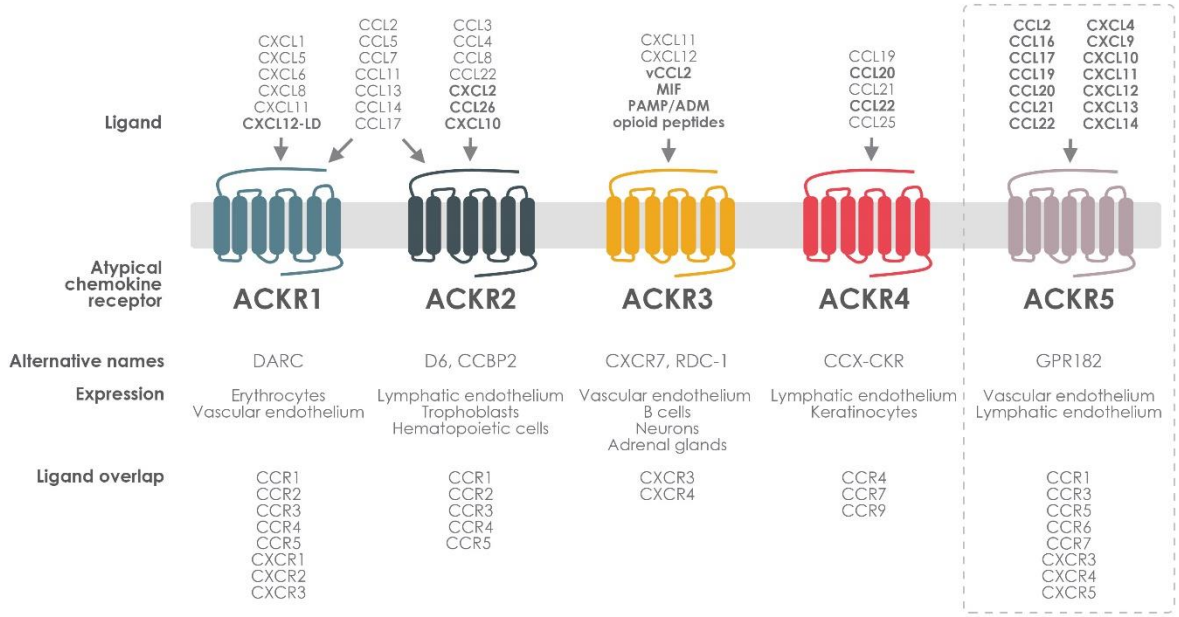

Illustration 12 - ACKRs

Schematic representation of ACKRs showing their expression profile, ligand selectivity and indirect regulation of classical chemokine receptors[82]. The recently de-orphanized GPR182/ACKR5 has been proposed as a new member of the atypical chemokine receptor family and its validation is still waiting for official approval. Illustration from Szpakowska et al[82].

ACKR1 is reported as the most promiscuous chemokine receptor, able to bind chemokines from CC and CXC families. It is unique also among ACKRs in that it does not target its ligands to degradation but regulates their availability for the classical chemokine receptors by ligand sequestration or transcytosis. ACKR1 was recently shown to interact with the dimeric form of CXCL12[204]. ACKR2 is described to scavenge several pro-inflammatory chemokines and it is therefore particularly important in containing and resolving inflammatory reactions[218,219]. The first CXC ligand for ACKR2 was identified in our laboratory in 2021[75] (Chapter V). ACKR3 originally discovered as decoy receptor for the chemokines CXCL11 and CXCL12[33,65], is now considered as a broad-spectrum atypical

receptor able to bind different types of ligands[77,78,203,220], including opioid peptides and a viral CC chemokine vCCL2/vMIP-II. ACKR4 was suggested to bind CCL19, CCL21, CCL25 and CXCL13[33,65]. Novel studies confirmed the specificity of the CC ligands, identifying in addition CCL20 as an ACKR4 chemokine[76,82]. CXCL13 was however not confirmed[76,82]. GPR182 was until recently classified as orphan receptor showing phylogenetic similarity to ACKR3[221]. Three independent studies propose it as a promiscuous atypical chemokine receptor[67,68,70] although the precise set of chemokines able to activate it remains unclear. This analysis highlights the importance of the reassessment of ligand–receptor interactions to gain a more precise understanding of this highly intricate interaction network.

Chapter V – Brain malignancies – chemokine receptors and their ligands in glioma

Chemokines and chemokine receptors are crucial for leukocytes positioning in inflammatory sites, for maintenance and development of lymphoid organs and they are involved in different phases of immune responses. Their deregulation is observed in several pathologies like auto-immune diseases and numerous cancers[190,192,222,223]. In neoplasms, the chemokine system is responsible for the crosstalk between tumour cells and the tumour microenvironment but it also directly influences cancer cell proliferation, migration and invasion[197,198]. Gliomas are severe malignancies of the central nervous system and among those, glioblastoma is one of the deadliest tumour types, characterized by an extremely poor overall survival (less than 15 months)[170,224]. Such diseases are defined by constant recurrence, which has been associated with important tumour heterogeneity, a strong connection between malignant cells and the surrounding brain tissue, and a scarcely infiltrating tumour microenvironment, which remains largely immunesuppressive[175,183,184,225]. To improve our understanding of the chemokine system in GBM, especially taking into account their potential as targets for combined therapies[183,226], we evaluated the most abundantly expressed chemokines (Chapter V.ii) and chemokine receptors (Chapter V.i) in gliomas, with a particular interest in GBM. Considering the recent progress in the meticulous collection and analysis of tumour samples together with the related clinical data from patients affected by different cancer types, we exploited publicly available datasets including brain tumour material[184,227-230]. These studies aim to generate a comprehensive patient-based handbook expanding our understanding of chemokine receptors and cognate ligands. To do this, the expression of chemokines and their receptors

was investigated at transcriptomic level across different glioma types, in separate GBM tissue areas, and distinct cell types from the GBM tumour microenvironment.

To study the different glioma types, tumour entities were classified by molecular features reflecting a progressive increase in disease severity, giving the opportunity to assess if and which players of the chemokine system are particularly enriched in GBM. With this approach, we could delineate the most relevant chemokine–receptor axis in these malignancies (Illustration 13).

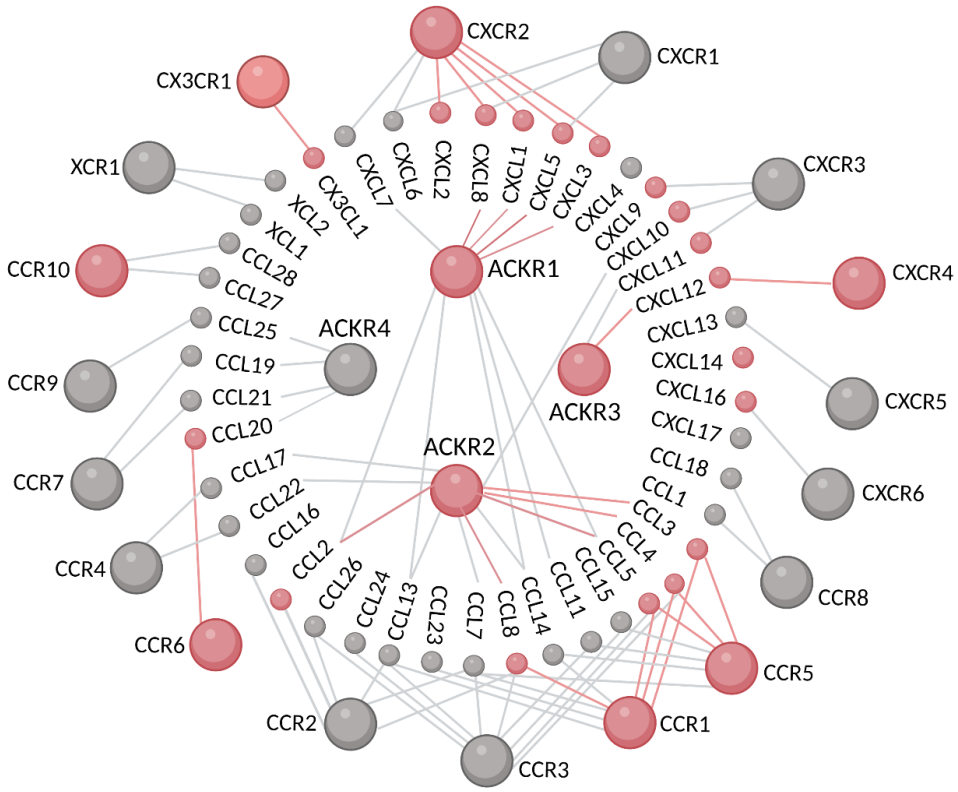

Illustration 13 – Chemokine–receptor interaction network in glioma
Representative scheme highlighting the chemokines and chemokine receptors found abundantly expressed in gliomas in our data analysis (Chapter VI.i and Chapter VI.ii).

Gene expression was also analysed in different sub-regions of the GBM mass. This profiling revealed heterogeneous distribution for some ligands and a couple of receptors. Selective expression in restricted areas was detected for a few others, as indication of determined functions, like supportive of angiogenesis or hypoxic environment. With the use of a publicly available resource[230], harmonizing single-cell RNA sequencing data from GBM patients encompassing 16 different studies, the expression of chemokines and their receptors was inspected according to different cell subsets. The same collection allowed the building of a probability interaction map to infer tumour intercellular crosstalk centred on the chemokine system. This study represents the first patient-based guide detailing expression profiles and

signalling axis of the chemokine network in severe brain malignancies. Our findings confirm the relevance of specific elements of this system that are currently evaluated in clinical trials as potential targets of combined therapy. Of note, other chemokine–receptor axes, still poorly characterized in GBM, emerged as important, paving the way to further studies aiming to better characterize them, confirm their importance and ultimately assess their targeting for therapeutic opportunities.

MATERIAL AND METHODS

Chemokines and antibodies

- Chapters I.i and I.ii for native chemokines;
- Chapter II and III, for native chemokines, fluorescently labelled chemokines, custom chemokines, CD26-processed chemokines and antibodies;

Cell culture

- Chapter I.i, I.ii, II, III, commercially available HEK293T cell line;
- Chapter II and III, HEK293T-derived stable cell lines established in the lab and commercially available U-87 MG cell line;
- Chapter III, commercially available B16F10 melanoma cell line;

Nanoluciferase complementation assay

- Chapter I.i, for β-arrestin or GRK3 interaction with ACKRs;
- Chapter I.ii, for GPCR─GRK interaction;
- Chapter II, to monitor interactions between engineered Gα proteins or β-arrestin with the chemokine receptors and to evaluate intracellular calcium mobilization;
- Chapter III and IV, for GPCR─β-arrestin interaction;

BRET (Biolumiescence Resonance Energy Transfer) assay

- Chapter I.i and III, to study β-arrestin interaction with ACKRs and receptor trafficking, namely plasma membrane mobilization and endosomal delivery;
- Chapter II, to monitor heterotrimeric G-protein dissociation and GPCR─β-arrestin interaction;

Additional Nanoluciferase-based techniques

Chapter II: cAMP measurements, transcriptional Nanoluciferase reporter assays, surface Nanoluciferase complementation (HiBiT);

Flow cytometry

Chapter II and III to validate stable cell lines, study receptor expression and assess chemokine binding or uptake;

Confocal microscopy

Chapter II and III to verify receptor expression and evaluate chemokine uptake;

ELISA

Chapter III to monitor chemokine scavenging;

Gene expression profiling from publicly available datasets

Chapter V, evaluation of chemokines (V.ii) and chemokine receptors (V.i) expression at transcriptomic level from publicly available patient-derived data.

1. The Cancer Genome Atlas (TCGA – *Ceccarelli et al.*);
2. TCGA-GTEx comparison;
3. Ivy Glioblastoma Atlas Project (*Puchalski et al.*);
4. GBMseq (*Darmanis et al.*) and Single Cell Portal (*Neftel et al.*) in Chapter V.i and GBmap (*Ruiz-Moreno et al.*) in Chapter V.ii;

RESULTS

Chapter I – Methodologies

i) Nanoluc-based methods to study ACKRs

My contribution to this chapter:

This book chapter represents an important outcome of the thesis and describes the use of cellular assays allowing to investigate various aspect of receptor biology exemplified by the characterization of the atypical chemokine receptor family. Applying such assays was critical to delineate the atypical features of CXCR3-B isoform (Chapter II) and better understand the interactions and function a new ligand for ACKR2 (Chapter III). I was involved in the experimental design, data generation and analysis of NanoBiT and NanoBRET ACKR3 experiments monitoring β-arrestin and GRK recruitment (Figure 1, panels C, E, H, K). I also contributed to the generation of the related schematics (Figure1, panels A, F, I). I performed confocal microscopy experiments and analyzed associated images that are included for representative purposes (Figure 2, panels A, D). Moreover, I actively contributed to the writing of the the book chapter.

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Highlights & aims

Atypical chemokine receptors influence the activity of classical receptors by tightly regulating the extracellular levels of their ligands. In opposition to their canonical counterparts, ACKRs are unable to trigger G protein signaling, which precludes the use of standard assays to assess their activation. There is a need for alternative approaches to monitor ACKR activation, modulation and trafficking. In this book chapter, we describe Nanoluciferase-based methodologies in a detailed manner to characterize these non-canonical receptors. We illustrate how Nanoluciferase Binary Technology (NanoBiT) and Nanoluciferase Bioluminescence Resonance Energy Transfer (NanoBRET) are reliable methods to monitor ACKR2 and ACKR3 activation. In particular, ACKR activation is measured by assessing β-arrestin and GRK recruitment, and/or receptor trafficking by monitoring their disappearance from the plasma membrane and subsequent delivery to early endosomes.

Nanoluciferase-based methods to monitor activation, modulation and trafficking of Atypical Chemokine Receptors.

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Abstract

Chemokines regulate directed cell migration, proliferation and survival and are key components in various physiological and pathological processes. They exert their functions by interacting with seven-transmembrane domain receptors that signal through G proteins (GPCRs). Atypical chemokine receptors (ACKRs) play important roles in the chemokine–receptor network by regulating chemokine bioavailability for the classical receptors through chemokine sequestration, scavenging or transport. Presently, this subfamily of receptors comprises four members: ACKR1, ACKR2, ACKR3 and ACKR4. They differ notably from the classical chemokine receptors by their inability to elicit G protein-mediated signaling, which precludes the use of classical assays relying on the activation of G proteins and related downstream secondary messengers to investigate ACKRs. There is therefore a need for alternative approaches to monitor ACKR activation, modulation and trafficking. This chapter details sensitive and versatile methods based on Nanoluciferase Binary Technology (NanoBiT) and Nanoluciferase Bioluminescence Resonance Energy Transfer (NanoBRET) to monitor ACKR2 and ACKR3 activity through the measurement of β-arrestin and GRK recruitment, and receptor trafficking, including internalization and delivery to early endosomes.

1. Introduction

Chemokine receptors belong to the class A of the G protein-coupled receptor (GPCR) family, presenting a typical seven-transmembrane structure. Over 20 different receptors have been identified in humans and are classified as CCR, CXCR, CX3CR and XCR, according to the chemokine family they bind[1]. The N terminus and the extracellular loops of these receptors interact with chemokine ligands and determine their specificity, while the C terminus and the intracellular loops are involved in the recruitment of signaling and regulatory proteins[2,3]. Classical chemokine receptors bind heterotrimeric G proteins consisting of $G_{\alpha i/o}$, G_{β} and G_{γ} subunits. Upon activation, these subunits dissociate into $G_{\alpha i/o}$ and $G_{\beta\gamma}$, which triggers downstream cellular responses that range from chemotaxis to proliferation, adhesion and gene expression[4]. Subsequently, G protein-coupled receptor kinases (GRKs) are recruited to the receptor and phosphorylate multiple sites of the C terminus, triggering β-arrestin recruitment[5]. The receptor is then delivered to early endosomes with two possible outcomes: receptor dissociation from the ligand and recycling to the surface or targeting for lysosomal degradation[6].

In recent years, a new subfamily of chemokine receptors with important regulatory functions has been described and coined atypical chemokine receptors (ACKRs) due to their inability to signal through G proteins[7]. This biased signaling of ACKRs renders more difficult the unravelling of their function and pathways, since many of the techniques currently available for the assessment of receptor–ligand interactions and downstream signaling rely directly or indirectly on their signaling through G proteins[8]. Current knowledge underlines that the important physiological and pathological regulatory roles of ACKRs rely on their effective ability to internalize chemokines and to target them for lysosomal degradation or to transport them to opposite sides of the cell[9]. Among ACKRs, ACKR2 (formerly D6) is able to scavenge multiple inflammatory chemokines, including CCL2, CCL5 and CXCL10, and plays an important role in the resolution phase of inflammation by preventing exacerbated immune responses as well as in the tumor bed regulating chemokine-driven infiltration of immune cells[10-16]. Another example is ACKR3 (formerly CXCR7), which acts as a scavenger for CXCL11 and CXCL12[17-19], and which has recently been shown to also bind a broad-spectrum of endogenous opioid peptides, among which BAM22[20-22]. Given the important functions of ACKRs in pathological conditions, it is crucial to have robust, reliable and reproducible assays to monitor the activation, modulation and intracellular trafficking

of ACKRs in order to further investigate their biological function or validate novel activity modulators[9].

Over the last five years, NanoLuc Binary Technology (NanoBiT) and NanoBRET (Bioluminescence Resonance Energy Transfer) have emerged as highly sensitive, versatile and complementary approaches to study complex transient and stable protein–protein interactions [23,24]. Both methods rely on the Nanoluciferase (NLuc), a small 19-kDa enzyme identified in the deep-sea shrimp *Oplophorus gracilirostris*, which emits bioluminescence in the presence of its physiological substrate, furimazine[25]. NanoBiT is based on the complementation of two subunits of NLuc: the Large BiT (LgBiT) with a size of 18 kDa and the small BiT (SmBiT) with a size of 1.3 kDa (11 amino acids). The putative interactive partners are tagged with one of the subunits and upon interaction, the complemented enzyme is able to produce a bioluminescent signal in the presence of the substrate. Since the subunits present a very low affinity (K_D = 190 µM) towards each other, luminescence is only emitted if the affinity of the proteins drives the complementation of the NLuc[26]. The simplicity, sensitivity and specificity of the assay constitute its major strengths, making it an excellent option for high-throughput screenings. NanoBRET is another method to study protein–protein interactions: NLuc is fused to one protein of interest and the other is labeled with a fluorescent partner. Upon close interaction (<10 nm) and in the presence of the NLuc substrate, energy emitted by NLuc is transferred to the fluorescent partner, which in turn emits a fluorescent signal. Importantly, this technique relies exclusively on proximity of the proteins and thus does not require a physical interaction between the subunits[23]. Moreover, with NanoBRET, it is possible to easily determine the relative expression of each pair through the measurement of the bioluminescence of the donor and the pre-excited fluorophore alone.

In this chapter, we describe different methodologies based on NanoBiT and NanoBRET to monitor the activation, modulation, bias and trafficking of two model atypical chemokine receptors, namely ACKR2 and ACKR3, by monitoring the recruitment of β-arrestins or GRKs, the disappearance from the plasma membrane (internalization) and delivery to the early endosomes[27,28]. These non-exhaustive descriptions exemplify the potential and versatility of Nanoluciferase-based approaches to monitor the different steps of ACKRs cycle and give better insights into their functions and to develop modulators.

2. Materials

2.1. Cell culture and transfection

1. Human embryonic kidney 293T (HEK293T) cells (CRL-3216, ATCC, USA)
2. Cell culture flasks (Greiner Bio-One, Germany)
3. Dulbecco's modified Eagle medium (DMEM, GIBCO, USA) supplemented with 0.04 mM phenol red, 1 mM sodium pyruvate, 4 mM L-glutamine, 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS, Merck, Germany) and 50 units/mL of penicillin/streptomycin (GIBCO, USA)
4. 10-cm cell culture dishes (Corning, USA)
5. Opti-MEM, Reduced Serum Medium (GIBCO, USA)
6. Polyethylenimine (PEI, 1 mg/ml, Merck, Germany)
7. GPCR-C-SmBiT and LgBiT-N-β arrestin1/2 expression constructs – for β-arrestin recruitment in NanoBiT.
8. GPCR-C-mNeonGreen and NLuc-N-β-arrestin1/2 expression constructs – for β-arrestin recruitment in NanoBRET
9. GPCR-C-SmBiT and LgBiT-N-GRK expression constructs – for GRK recruitment
10. GPCR-C-NLuc and Lyn-C-mNeonGreen or CAAX-N-mNeonGreen expression constructs – for internalization
11. GPCR-C-NLuc or GPCR + NLuc-N-arrestin and mNeonGreen-N-FYVE expression constructs – for endosomal delivery

(see section 2.3 for amino acid sequences of constructs listed in 7-11)

2.2. Sample preparation and reading

2.2.1. NanoBiT-based assays

1. Phosphate-buffered saline (PBS, GIBCO, USA)
2. Versene (GIBCO, USA)
3. 15-mL conical tubes (Greiner Bio-One, Germany)
4. White flat-bottom LUMITRAC 96-well plates, medium binding (Greiner Bio-One, Germany)
5. Transparent U-bottom 96-well plates (Greiner Bio-One, Germany)
6. 12-channel pipets: 2–20 µL range and 20–200 µL range (Mettler Toledo, Germany)
7. CASY Automated cell counter (OMNI Life Science, Germany)
8. NanoLuc substrate: Furimazine (Nano-Glo® Luciferase Assay Substrate, Promega, USA) or coelenterazine H (Regis Technologies, USA)
9. Luminescence plate reader: Mithras LB940 (Berthold Technologies, Germany)

2.2.2. NanoBRET-based assays

1. Phosphate-buffered saline (PBS, GIBCO, USA)
2. Versene (GIBCO, USA)
3. 15-mL conical tubes (Greiner Bio-One, Germany)
4. Black flat-bottom FLUOTRAC 96-well plates, medium binding (Greiner Bio-One, Germany)
5. 12-channel pipets: 2–20 µL range and 20–200 µL range (Mettler Toledo, Germany)
6. CASY Automated cell counter (OMNI Life Science, Germany)
7. NanoLuc substrate: Furimazine (Nano-Glo® Luciferase Assay Substrate, Promega, USA) or, alternatively, coelenterazine H (Regis Technologies, USA)

8. Plate reader equipped with 450/10 filter for donor luminescence emission and 530 LP filter for acceptor fluorescence emission: GloMax Explorer plate reader (Promega, USA)

2.3. Sequences of tagged protein partners (see note 1)

2.3.1. GPCR-C-SmBiT

SmBiT (VTGYRLFEEIL) inserted with a flexible linker (GSSGGGGSGGGGSSG) at the C terminus of the receptor.

2.3.2. LgBiT-N-effector

LgBiT

(MVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSVTPIQRIVRSGEN
ALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGTLVIDGVTPN
MLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTIN)
inserted with a flexible linker (GSSGGGGSGGGGSSG) at the N terminus of the human β-arrestin 1, β-arrestin 2 or GRK3.

2.3.3. GPCR-C-mNeonGreesn

mNeonGreen

(MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQGTGNPNDGYEELNLKST
KGDLQFSPWILVPHIGYGFHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFED
GASLTVNYRYTYEGSHIKGEAQVKGTGFPADGPVMTNSLTAADWCRSKKTYP
NDKTIISTFKWSYTTGNGKRYRSTARTTYTFAKPMAANYLKNQPMYVFRKTE
LKHSKTELNFKEWQKAFTDVMGMDELYK) inserted with a flexible linker (GSSGGGGSGG-GGSSG) at the C terminus of the receptor.

2.3.4. NLuc-N-effector

NLuc
(VFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENG
LKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNM
IDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGW
RLCERILA) inserted with a flexible linker (GSS) at the N terminus of the human β-arrestin 1 or β-arrestin 2.

2.3.5. Lyn-C-mNeonGreen

mNeongreen
(MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQGTGNPNDGYEELNLKST
KGDLQFSPWILVPHIGYGFHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFED
GASLTVNYRYTYEGSHIKGEAQVKGTGFPADGPVMTNSLTAADWCRSKKTYP
NDKTIISTFKWSYTTGNGKRYRSTARTTYTFAKPMAANYLKNQPMYVFRKTE
LKHSKTELNFKEWQKAFTDVMGMDELYK) inserted with a flexible linker (GSSGGGGSGG GGSSG) at the C terminus of the first 11 residues (MGCIKSKGKDS) of the human Lyn-kinase sequence.

2.3.6. mNeonGreen-N-CAAX

mNeonGreen (MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQGTGNPNDGYEELNLKST KGDLQFSPWILVPHIGYGFHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFED GASLTVNYRYTYEGSHIKGEAQVKGTGFPADGPVMTNSLTAADWCRSKKTYP NDKTIISTFKWSYTTGNGKRYRSTARTTYTFAKPMAANYLKNQPMYVFRKTE LKHSKTELNFKEWQKAFTDVMGMDELYK) inserted with a flexible linker (GSSGGGGSGG GGSSG) at the N terminus of the polybasic sequence and prenylation CAAX box (GKKKKKKSKTKCVIM) of the human KRas sequence.

2.3.7. mNeonGreen-N-FYVE

mNeonGreen (MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQGTGNPNDGYEELNLKST KGDLQFSPWILVPHIGYGFHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFED GASLTVNYRYTYEGSHIKGEAQVKGTGFPADGPVMTNSLTAADWCRSKKTYP NDKTIISTFKWSYTTGNGKRYRSTARTTYTFAKPMAANYLKNQPMYVFRKTE LKHSKTELNFKEWQKAFTDVMGMDELYK) inserted with a flexible linker (GSSGGGGSGG GGSSG) at the N terminus of the FYVE domain of the human endofin (residues from Q739 to K806, MQKQPTWVPDSEAPNCMNCQVKFTFTKRRHHCRACGKVFCGVCCNRK CKLQYLEKEARVCVVCYETISK).

2.3.8. GPCR-C-NLuc

NLuc (VFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENG LKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNM IDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGW RLCERILA) inserted with a flexible linker (GSSG) at the C terminus of the receptor sequences.

3. Methods

3.1. Cell preparation and transfection

All the protocols are executed in sterile conditions under a laminar flow hood.

3.1.1. Receptor activation – β-arrestin and GRK recruitment

1. Maintain HEK293T cells in culture medium at 37°C with 5% CO_2.
2. Seed cells 100 000 cells/cm^2 in a 10-cm cell culture dish with 10 mL of culture medium (*see note 2*).
3. Incubate the cells at 37°C with 5% CO_2 for 24 h.
4. Dilute 12 µL of PEI in 400 µL of Opti-MEM. In a separate tube, add 400 ng of GPCR-encoding vectors, 40 ng of effector-encoding vectors and 3.6 µg of irrelevant DNA (e.g. empty pcDNA 3.1) to 400 µL of Opti-MEM (*see note 2, 3 and 4*) and mix thoroughly. Use plasmids coding for GPCR-C-SmBiT and LgBiT-N-effector for NanoBiT or GPCR-C-mNeonGreen and NLuc-N-effector for NanoBRET assays.
5. Add the PEI-Opti-MEM mix to the DNA and pipette up and down.

6. Incubate the mix for 20 min at room temperature.
7. During this period, remove the medium from the dishes and add 10 mL of fresh pre-warmed medium.
8. Add the mix to the cells in a dropwise manner and ensure even distribution over the surface.
9. Incubate the cells at 37°C with 5% CO_2 for 24 h.

3.1.2. Receptor trafficking – internalization and endosomal delivery

1. Maintain HEK293T cells in culture medium at 37°C with 5% CO_2.
2. Seed cells at 100 000 cells/cm^2 in a 10-cm cell culture dish with 10 mL of culture medium (*see note 2*).
3. Incubate the cells at 37°C with 5% CO_2 for 24 h.
4. If the interest lies in internalization, dilute 12 µL of PEI in 400 µL of Opti-MEM. In a separate tube, add 400 ng of receptor-encoding DNA and 3.6 µg of Lyn construct to 400 µL of Opti-MEM (*see notes 2 and 4*). Use plasmids coding for GPCR-C-NLuc and Lyn-C-mNeonGreen.
5. If the interest lies in endosomal delivery, dilute 12 µL of PEI in 400 µL of Opti-MEM. In a separate tube, add 40 ng of receptor-encoding DNA and 4 µg of FYVE construct to 400 µL of Opti-MEM (*see notes 2 and 4*). Use plasmids coding for GPCR-C-NLuc and mNeonGreen-N-FYVE.
6. Mix thoroughly and add the PEI-Opti-MEM mix to the DNA by pipetting up and down.
7. Incubate the mix for 20 min at room temperature.
8. During this period, remove the old medium from the dishes and add 10 mL of fresh pre-warmed medium.
9. Add the mix to the cells in a dropwise manner and ensure even distribution over the surface.
10. Incubate the cells at 37°C with 5% CO_2 for 24 h.

3.2. Sample preparation and reading

3.2.1. Receptor activation – β-arrestin and GRK recruitment in NanoBiT

1. Aspirate the culture medium and wash the cells with 5 mL of PBS.
2. Add 1 mL of Versene to the dish and incubate at 37°C for 5 min.
3. Collect the cells in 9 mL of Opti-MEM by pipetting up and down and transfer the cells to a 15-mL conical tube.
4. Centrifuge the cells for 5 min at 300 x *g* at room temperature.
5. Discard the supernatant and resuspend the cells in Opti-MEM at a concentration of 1.1x10^6 cells/mL.
6. Add Nanoluciferase substrate: furimazine, prepared according to the manufacturer's instructions (final dilution of 1:100). Alternatively, coelenterazine H at 10 nM can be used (*see note 6*).
7. Incubate the cells at 37°C for 20 min.
8. In a transparent U-bottom 96-well plate, prepare the ligands and dilutions to be tested at a concentration of 10X in Opti-MEM.
9. Distribute 90 µL of the cell suspension per well (=100.000 cells/well, *see note 5*) in a white flat-bottom 96-well plate.
10. Add 10 µl of the 10X concentrated ligands with a multichannel pipet and measure directly the luminescence with a plate reader for 25 cycles, over 25 min.

11. Analyze the results considering the different possibilities presented in note 7.

3.2.2. Receptor activation – β-arrestin recruitment in NanoBRET

1. Aspirate the culture medium and wash the cells with 5 mL of PBS.
2. Add 1 mL of Versene to the dish and incubate at 37°C for 5 min.
3. Collect the cells in 9 mL of Opti-MEM by pipetting up and down and transfer the cells to a 15-mL conical tube.
4. Centrifuge the cells for 5 min at 300 × *g* at room temperature.
5. Discard the supernatant and resuspend the cells in Opti-MEM. Adjust the volume to a concentration of 1.25×10^6 cells/mL.
6. Distribute 80 µL of the cell suspension per well (=100.000 cells/well, *see note 5*) in a black flat-bottom 96-well plate and incubate at 37°C for 10 min.
7. Meanwhile, in a transparent U-bottom 96-well plate, prepare the ligands and dilutions to be tested at a concentration of 10X in Opti-MEM.
8. In a transparent U-bottom 96-well plate, prepare a row with the Nanoluciferase substrate (100 µL/well): furimazine, prepared according to the manufacturer's instructions or a coelenterazine H at 100 nM (*see note 6*).
9. Add 10 µL of the 10X concentrated ligands with a multichannel pipet.
10. Add 10 µL of the diluted furimazine or coelenterazine H with a multichannel pipet.
11. Read NanoBRET (i.e. 450/10 filter for donor luminescence and 530 LP filter for fluorescent acceptor signal) directly with a BRET-compatible plate reader for 7 cycles, over 30 min.
12. Analyze the results considering the different possibilities presented in note 7.

3.2.3. Receptor trafficking – internalization and endosomal delivery

1. Aspirate culture medium and wash the cells with 5 mL of PBS.
2. Add 1 mL of Versene to the dish and incubate at 37°C for 5 min.
3. Collect the cells in 9 mL of Opti-MEM by pipetting up and down and transfer the cells to a 15-mL conical tube.
4. Centrifuge the cells for 5 min at 300 × *g* at room temperature.
5. Discard the supernatant and resuspend the cells in Opti-MEM. Adjust the volume to have a cell concentration of 1.25×10^6 cells/mL (*see note 5*).
6. Plate 80 µL of the cell suspension per well (=100.000 cells/well) in a black flat-bottom 96-well plate and incubate at 37°C while preparing the ligands.
7. On a transparent U-bottom 96-well plate, prepare the ligands and dilutions to be tested at a concentration of 10X in Opti-MEM.
8. On a transparent U-bottom 96-well plate, prepare a row with the Nanoluciferase substrate: furimazine, prepared according to the manufacturer's instructions or coelenterazine H at 100 nM (*see note 6*).

<u>For internalization or mobilization to plasma membrane (Lyn/CAAX)</u>

9a. Add 10 µL of the 10X concentrated ligands with a multichannel pipet.
10a. Add 10 µL of the diluted furimazine or coelenterazine H with a multichannel pipet.
11a. Read NanoBRET (i.e. 450/10 filter for donor luminescence and 530 LP filter for fluorescent acceptor signal) directly with a BRET-compatible plate reader for 7 cycles, over 30 min.
12a. Analyze the results considering the different possibilities presented in note 7.

For endosomal delivery (FYVE) (*see note 8*)

9b. Add 10 µL of the 10X concentrated ligands with a multichannel pipet.
10b. Incubate at 37°C for 2 h.
11b. Add 10 µL of the diluted furimazine or coelenterazine H with a multichannel pipet.
12b. Read NanoBRET (i.e. 450/10 filter for donor luminescence and 530 LP filter for fluorescent acceptor signal) directly with a BRET-compatible plate reader for 7 cycles, over 30 min.
13b. Analyze the results considering the different possibilities presented in note 7.

4. Controls and additional considerations

For NanoBRET several different fluorescent partners may be used. In this chapter, we used mNeonGreen as the acceptor fluorescent protein, however HaloTag accommodating different fluorescent ligands (e.g ligand 618) and Venus are equivalent partners commonly used along NLuc [29]. Nevertheless, choosing a fluorescent acceptor with a reduced overlap of the NLuc emission spectra and a high quantum yield is pivotal.

Another critical aspect to account for in all the tools presented is the orientation of the NLuc/mNeonGreen or SmBiT/LgBiT relative to the GPCR and sensor. An example of this can be observed in figure 2G, where keeping NLuc at the C terminus of ACKR3 but changing mNeonGreen from the N to the C terminus of FYVE invalidates the results obtained.

Finally, an important but often overlooked aspect is ligand preparation. If several plates are to be read, ligands should be freshly prepared, since degradation may occur over time.

5. Concluding remarks

In our particular examples, we sought to characterize two ACKRs, which have different receptor selectivity and show slightly different trafficking properties. As shown in Figure 1, the ligands of ACKR2 and ACKR3 induce the recruitment of β-arrestins (Figure 1, panels B-E and G-H) and GRK3 (panels J-K) specifically. Furthermore, Figure 2 demonstrates the ability of the receptors to be mobilized to the plasma membrane, internalized or addressed to the early endosomes upon ligand stimulation. Overall, the examples shown in this methodological description reveal the complementarity of both NLuc-based methods to study the GPCR interactome. Furthermore, we observed a higher specificity of NanoBiT compared to NanoBRET in the described setup, particularly when assessing β-arrestin recruitment. Nevertheless, the NanoBRET assay strength is revealed when conducting receptor trafficking assays or saturation curves.

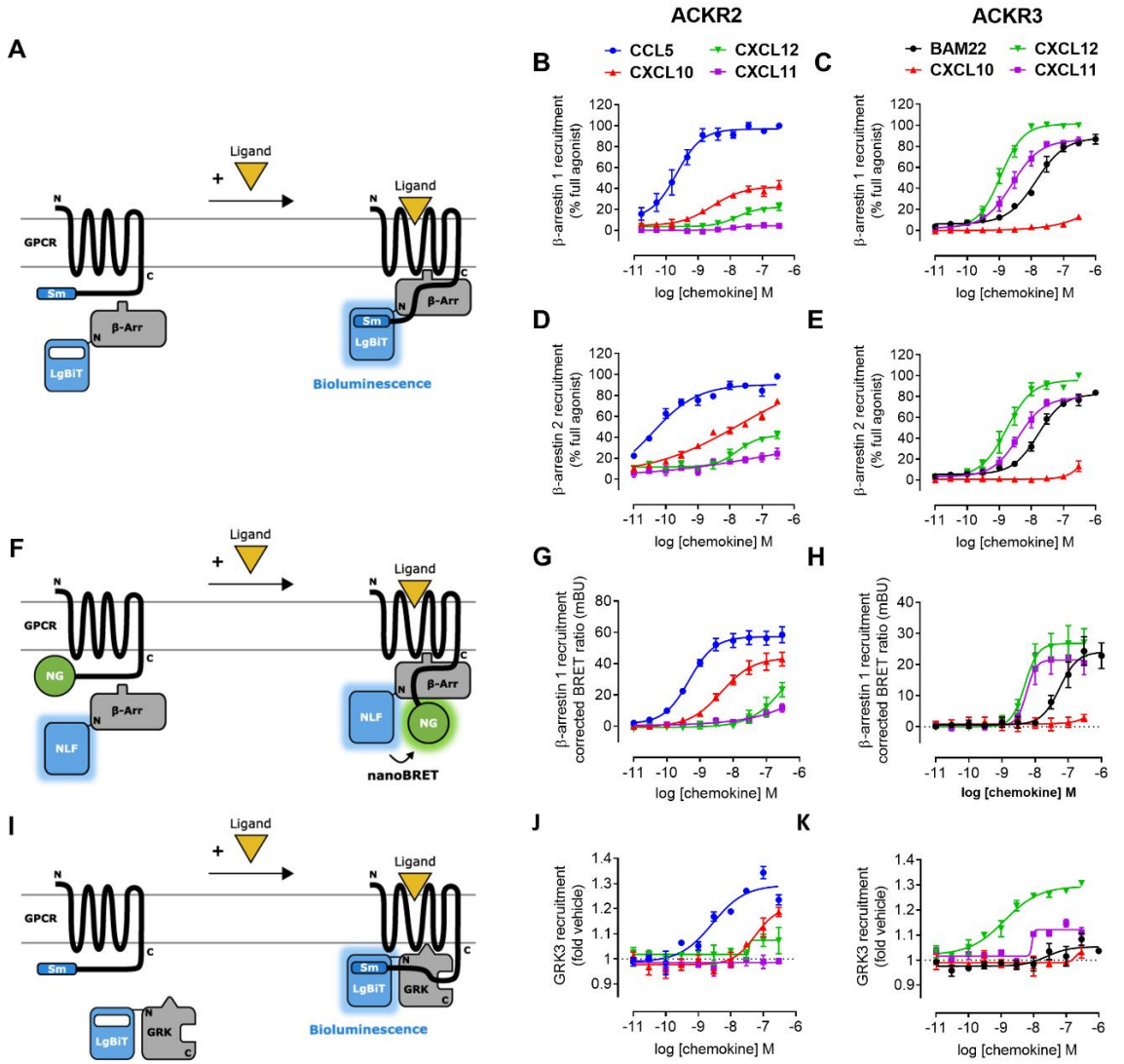

Figure 1. ACKR2 vs ACKR3 β-arrestins and GRK recruitment using NLuc-based methodologies.

(A) Schematic representation of Nanoluciferase complementation assay with tagged GPCRs and β-arrestins**.** (**B, D**) Recruitment of β-arrestin 1 **(B)** and β-arrestin 2 **(D)** to ACKR2 induced by CCL5, CXCL10 and CXCL12 monitored by NanoBiT, showing the concentration–response relationship. CXCL11 was used as negative control. (**C, E**) Recruitment of β-arrestin 1 **(C)** and β-arrestin 2 **(E)** to ACKR3 induced by BAM22, CXCL11 and CXCL12 monitored by NanoBiT, showing the concentration–response relationship. CXCL10 was used as negative control. **(F)** Schematic representation of Nanoluciferase Bioluminescence Resonance Energy Transfer with tagged GPCR and β-arrestin. (**G**, **H**) β-arrestin 1 recruitment to ACKR2 (**G**) and ACKR3 (**H**) monitored by NanoBRET. All NanoBiT and NanoBRET assays were conducted in HEK293T cells. Data points represent mean ± SEM of three independent experiments. (**I**) Schematic representation of Nanoluciferase complementation assay (NanoBiT) with tagged GPCR and GRK. (**J**) GRK3 recruitment to ACKR2 induced by CCL5, CXCL10 and CXCL12, showing the concentration–response relationship. CXCL11 was used as negative control. (**K**) GRK3 recruitment to ACKR3 by BAM22, CXCL11 and CXCL12, showing the concentration–response relationship. CXCL10 was used as negative control. All assays were conducted in HEK293T cells. Data points represent mean ± SEM of three independent experiments. NLF: Nanoluciferase; NG: mNeonGreen.

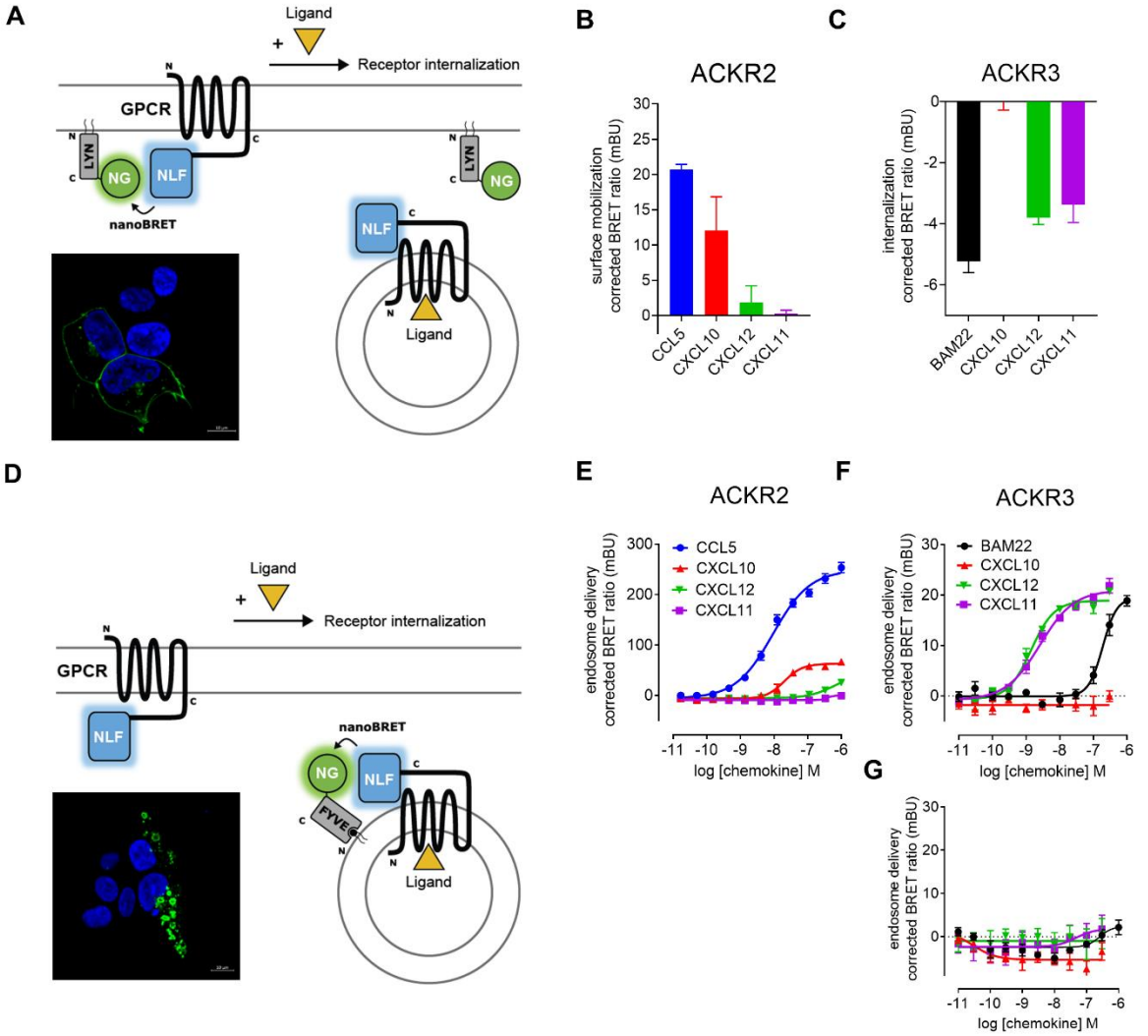

Figure 2 ACKR2 vs ACKR3 trafficking monitored by NanoBRET-based assays.

(A, D) Schematic representation and confocal pictures of methods to assess receptor trafficking. (A) Surface GPCR-NLuc can be monitored using Lyn-mNeonGreen or CAAX-mNeonGreen **(D)** GPCR delivery to the endosomes can be monitored using FYVE-mNeonGreen and NLuc tagged directly to the receptor. Alternatively, NLuc-tagged β-arrestin can be used to monitor receptor–β-arrestin complex trafficking. **(B)** ACKR2 mobilization to the plasma membrane or **(C)** ACKR3 internalization induced by chemokine ligands (100 nM). **(E)** ACKR2/β-arrestin 1 complex delivery to the early endosomes induced by CCL5, CXCL10 and CXCL12, showing the concentration–response relationship. CXCL11 was used as negative control. **(F, G)** ACKR3 delivery to the early endosomes induced by BAM22, CXCL10 and CXCL12, showing the concentration–response relationship. CXCL11 was used as negative control. FYVE was tagged with mNeonGreen at the C terminus **(F)** or the N terminus **(G)**. NLF: Nanoluciferase; NG: mNeonGreen.

6. Notes

Note 1: All vectors described in this chapter are available upon request (andy.chevigne@lih.lu). The 7 GRKs and β-arrestin 1/constructs tagged with LgBiT in both N and C orientations are also available.

Note 2: Cell density was optimized for HEK293T but other cell lines as well as primary cells can be used. Other cell culture vessels may be used with adapted medium volumes, transfection mixes and the DNA:PEI ratio of 3:1 (w/w) maintained. We have found that 1.5 µg of DNA and 4.5 µg of PEI in 300 µl Opti-MEM are optimal for transfection of cells in 6 well-plates.

Note 3: The transfection ratios for GRK recruitment were not optimized for the purpose of this book chapter. We used a ratio of 1:10 GPCR-C-SmBiT and 1:100 LgBiT-N-GRK for the transfection of HEK293T cells (400 ng GPCR and 40 ng GRK) topped up to 4 µg with irrelevant DNA. These ratios should be considered as starting points and may be optimized for different receptors, plasmid promoters and cellular backgrounds.

Note 4: In this chapter, we use plasmids under a CMV promoter. Considering the strength of both the promoter and the NLuc signal, diluting the DNA leads to more physiological receptor and effector expression. CRISPR/Cas9 technology can also be used to assess interactions between endogenously expressed partners, without the possible artifacts derived from overexpression[30,31]. NanoBiT and NanoBRET plasmids encoding NLuc and fragments with the respective linkers and multiple cloning sites are commercially available from Promega. NanoBiT plasmids are under a HSV-TK promoter, while NanoBRET plasmids are under a CMV promoter. Promega plasmids require the use of HaloTag as a fluorescent acceptor which requires coupling with different ligands.

Note 5: We found that 100 000 HEK293T cells/well provides consistent results. This number can be modified, but consistency of the results should be confirmed by comparison. Furthermore, if using different cell lines, the number of cells per well should also be adapted.

Note 6: Other substrates can be used to replace furimazine (e.g. coelenterazine H). However, it should be considered that coelenterazine H is prone to chemical instability primarily through auto-oxidation, which may generate a higher background luminescence and decrease the sensitivity of the assays.

Note 7: For analysis, controls including unstimulated/vehicle treated cells should be included to determine the average baseline signal. The results shown in this chapter were analyzed to reflect the different analysis possibilities. The common step to all methods shown was to systematically average the peak signals for each condition. For normalization to the percentage of maximum response of the full agonist, the baseline signal is subtracted from each condition. Then, the highest concentration of the agonist is considered as 100%, and all other conditions are normalized accordingly (Figure 1, panels B, C, D and E). To analyze the results in terms of fold to background/vehicle, the value of each condition is divided by the baseline (Figure 1, panels J and K). For corrected BRET normalization, the ratio of the baseline control is subtracted from the ratio of each stimulated condition (Figure 1, panel G and H, and Figure 2).

Note 8: For certain receptors, direct tagging with NLuc did not result in sufficient BRET signal. In these cases, receptor–β-arrestin-NLuc complex was used as an indirect way to assess receptor endosomal delivery. Other subcellular markers such as Rab proteins can be used as alternatives to assess receptor trafficking with the methodologies described[28,29].

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Chapter I – Methodologies

ii) NanoBiT-based method to study GPCR-GRK interaction

My contribution to this Chapter:

This work represents an important part of my PhD thesis. I took part in this study by contributing to the generation of molecular tools necessary for cellular assays (see Figure 1, panel C) and optimizing the protocol detailed in the Methods section. I contributed to the profiling of the eight receptors by taking part in the generation, analysis and interpretation of data described in Figure 2. I was also involved in the writing of each section of the manuscript.

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Highlights & aims

G protein-coupled receptor kinases (GRKs) are important regulators of receptor activity by catalyzing phosphorylation of its C terminus. Upon ligand stimulation, GRK-mediated receptor phosphorylation is essential for arrestin binding, initiation of GPCR desensitization and ultimately internalization. This book chapter describes a standardized method, that relies on the complementation of the split Nanoluciferase (NanoBiT), for systematic assessment of agonist-induced interactions between GPCRs and the seven GRK isoforms.

With this approach, we characterize six canonical receptors representing different receptor classes: the opioid receptor MOR, the serotonin receptor 5-HT1A, the vasopressin receptor, AVPR2, the aminergic receptor B2AR, the calcitonin receptor CGRPR and the classical chemokine receptors CXCR3, as well as two β-arrestin-biased receptors ACKR2 and ACKR3. The set of receptors analyzed show different GRK recruitment profiles.

Nanoluciferase-based complementation assay for systematic profiling of GPCR–GRK interactions

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Keywords: B2AR, GRK2, GRK3, GRK5, GRK6, ACKR3, kinase, NanoBiT, arrestin, MOR, opioids

Abstract

G protein-coupled receptor kinases (GRKs) are a family of seven soluble receptor-modifying enzymes which are essential regulators of GPCR activity. Following agonist-induced receptor activation and G protein dissociation, GRKs prime the receptor for desensitization through phosphorylation of its C terminus, which subsequently allows arrestins to bind and initiate the receptor internalization process. While GRKs constitute key GPCR-interacting proteins, to date, no method has been put forward to readily and systematically determine the preference of a specific GPCR towards the seven different GRKs (GRK1-7). This chapter describes a simple and standardized approach for systematic profiling of GRK1-7–GPCR interaction relying on the complementation of the split Nanoluciferase (NanoBiT). When applied to a set of eight GPCRs (MOR, 5-HT_{1A}, AVPR2, B2AR, CGRPR, CXCR3), including 2 intrinsically β-arrestin-biased receptors (ACKR2 and ACKR3), this methodology yields highly reproducible results highlighting different GRK recruitment profiles. Using this assay, further characterization of MOR, a crucial target in the development of analgesics, reveals not only its GRK fingerprint but also related kinetics and activity of various ligands for a single GRK.

1. Introduction

The G protein-coupled receptors (GPCRs) represent the largest family of signaling receptors in humans and are involved in a plethora of physiological and pathological processes. As such, they constitute essential signaling pivots and are the target of over a third of FDA-approved compounds[1]. These receptors activate distinct heterotrimeric G proteins leading to the modulation of downstream cellular responses such as an increase or decrease in cyclic AMP (cAMP) concentration, intracellular Ca^{2+} influx, or phosphorylation of the mitogen-activated protein kinase (MAPK) family[2]. G protein-coupled receptor kinases (GRKs) act as important relay between GPCR signaling, desensitization and trafficking. They have proven essential to manage the fine-tuned choreography of GPCR phosphorylation and subsequent arrestin recruitment, which are then responsible for the "arrest" of G protein-dependent signaling[3,4], receptor internalization and its later fate (**Fig. 1A**). While the GPCR family is made up of about 800 members in humans alone, they are regulated by a surprisingly restricted number of effector proteins, including seven GRKs (GRK1-7) and four arrestins. This numerical discrepancy between receptors and effectors has gave rise to the barcode theory, whereby the stabilized conformation of a ligand-bound receptor will result in a

specific phosphorylation pattern of its C-terminal tail by GRKs, leading to different arrestin binding and conformations, ultimately dictating different receptor fates[5].

A distinction is often drawn within the GPCR field between the visual and non-visual receptors, as the former are typically regulated by the so-called "visual" arrestins 1 and 4, and GRK1 (previously coined rhodopsin kinase) and GRK7[6]. Indeed, these show a specific tissue distribution restricted to the retina, while GRK4 is mostly expressed in the testis[7-10]. Most non-visual GPCRs are phosphorylated by the ubiquitously expressed GRK2, GRK3, GRK5 and GRK6 with different preferences, which then recruit arrestins 2 and 3, commonly referred to as β-arrestin-1 and β-arrestin-2[3].

GRKs can be separated in three subfamilies based on sequence homology: GRK1 (1 and 7), GRK2 (2 and 3) and GRK4 (4, 5 and 6). Four of them are membrane-anchored, namely GRK1 and GRK7, which are prenylated[11,12] and GRK4 and GRK6, which are palmitoylated[13,14]. GRK2 and GRK3 are recruited through their pleckstrin homology (PH) domain, which binds the $G_{\beta\gamma}$ dimer, made accessible following G protein activation and dissociation[15-17]. GRK5 interacts with membrane lipids directly through its polybasic/hydrophobic domain[18].

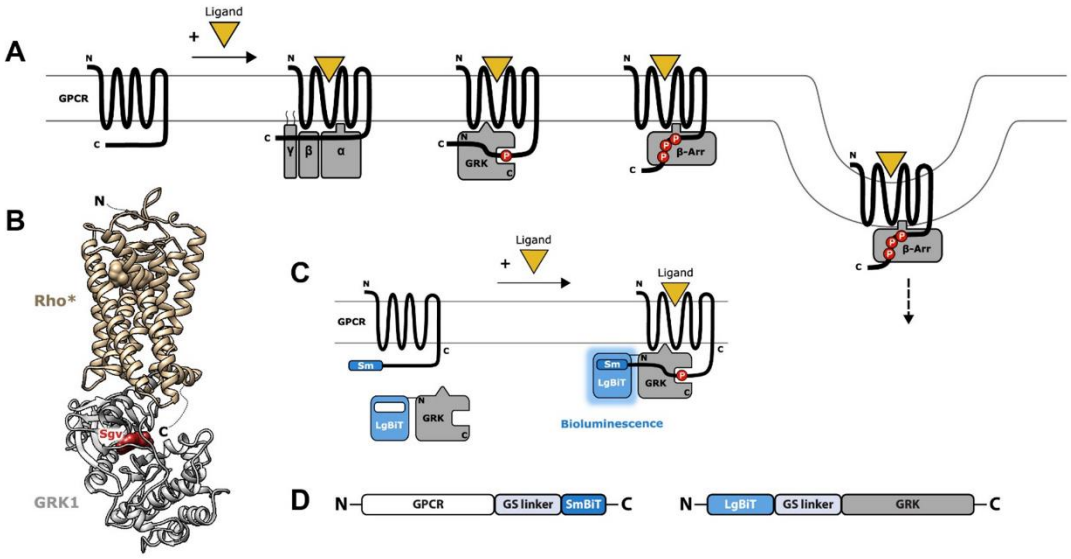

Figure 1. GRK interaction and NanoBiT assay. (A) Schematic representation of the steps following agonist-induced activation of a GPCR. The heterotrimeric G protein dissociates, allowing GRK-mediated phosphorylation, which subsequently leads to β-arrestin recruitment and receptor internalization. **(B)** Cryo-EM-resolved structure of the GRK1/Rhodopsin receptor complex (adapted from [19], PDB ID: 7MTA). Sgv, an adenosine analogue is indicated in red. **(C)** Schematic representation of the NanoBiT system applied to a GPCR and GRK pairing. **(D)** Representation of the GPCR and GRK constructs with the associated SmBiT and LgBiT tags.

The structure of several GRKs has been resolved by crystallography and cryogenic electron microscopy (cryo-EM) in complex with different interacting partners, including $G_{\beta\gamma}$ with GRK2 [16] and more recently Rhodopsin with GKR1 (**Fig. 1B**)[19]. The interaction of β2-adrenoceptor (β2AR) with GRK5 were also modelled[20]. However, little is still known about the GPCR–GRK interaction selectivity. While many techniques have been put forward to monitor and characterize the interaction of GPCRs with effectors and regulators, including G proteins[21] and arrestins[22], this has not yet been established as systematically for GRKs.

Recently, a panel of eleven edited cell-lines was described, with individual or combined knock-outs (KO) for GRK2/3/5/6[23]. This remarkable new tool opens up the investigation of the contribution of single or pairs of GRKs using β-arrestin recruitment as a readout. However, there still remains a need for a standardized approach to directly and systematically elucidate the GPCR–GRK interaction pattern. In the last decades, protein fragment complementation assays have shown significant potential in overcoming the pitfalls associated with other tagging techniques. For instance, while split fluorophores may be used to monitor protein–protein interactions (PPIs), they are not suitable for immediate measurements due to the delay associated with chromophore maturation[24]. Instead, split enzymes such as beta-galactosidase or Nanoluciferase (NLuc) recombine quickly and, when supplemented with the corresponding substrate, readily produce a strong luminescent signal[25,26]. Similar to the Nanoluc Binary Technology (NanoBiT) assay described to measure β-arrestin recruitment or G protein interactions[21,22], an assay was devised to assess and profile the immediate interaction of GRKs with GPCRs.

In the described assay, the GRK is N-terminally tagged with a fragment of the Nanoluciferase (NLuc) enzyme, coined the LgBiT fragment (18 kDa), while the GPCR of interest is C-terminally tagged with the complementary 11-residues fragment, namely the SmBiT tag (**Fig. 1D**). Upon ligand binding and G protein dissociation, the active conformation of the receptor is able to accommodate specific GRKs allowing NLuc complementation (**Fig. 1C**). Cells supplemented with the NLuc substrate produce strong bioluminescence, which can then easily be measured. While different orientations have been tested, in our hands, LgBiT-N-GRK/GPCR-C-SmBiT is the combination which yields the highest specific signal to background readouts.

This technique allows to systematically profile the selectivity of a single GPCR for the seven GRKs (**Fig. 2** and **Fig. 3A**) as well as to characterize the potency, relative efficacy and

kinetics (**Fig. 3**) of different ligands towards GRK recruitment. Moreover, it can easily be extended to screen the interactions of a single GRK with many GPCRs regardless of their G-protein coupling (**Fig. 2**). While PPIs can be assessed using other techniques such as FRET or BRET (fluorescence or bioluminescence resonance energy transfer [27-31]), the ease of implementation, sensitivity and specificity of NanoBiT make this approach ideally suited for medium to high-throughput screenings and profiling.

Here, we detail the systematic profiling of eight GPCRs, namely the μ-opioid receptor (MOR), the serotonin receptor 1A (5-HT_{1A}), the β_2-adrenoceptor (B2AR), the chemokine receptor CXCR3, the vasopressin receptor 2 (AVPR2), two intrinsically β-arrestin-biased receptors, the atypical chemokine receptors 2 and 3 (ACKR2 and ACKR3[27,32-35]) , and the calcitonin gene-related peptide (CGRP) receptor with the seven GRKs (GRK1-7) and highlight different receptor-GRK recruitment profiles. In keeping with a recent review[36], we also expand on the characterization of MOR, a crucial target in the development of analgesics, and describe its GRK profile, the kinetics and the pharmacology of several of its reported ligands.

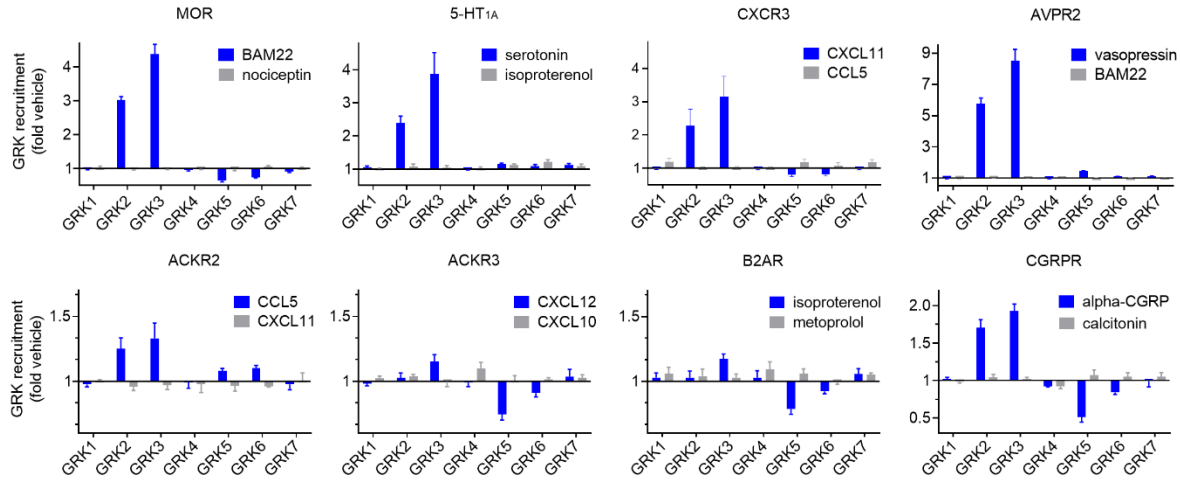

Figure 2. GRK profiling for representative GPCRs. Ligand-induced GRK profiling for MOR, 5-HT_{1A}, CXCR3, AVPR2, ACKR2, ACKR3, B2AR, CGRP using NanoBIT assay in HEK293T cells. The following ligand pairs were respectively used as agonists and negative controls: BAM22 and nociceptin (100 nM) for MOR, serotonin and isoproterenol (10 μM) for 5-HT_{1A}, CXCL11 and CCL5 (100 nM) for CXCR3, vasopressin and BAM22 (100 nM) for AVPR2, CCL5 and CXCL11 (100 nM) for ACKR2, CXCL12 and CXCL10 (100 nM) for ACKR3, isoproterenol and metoprolol (10 μM) for β_2AR, α-CGRP and calcitonin (100 nM) for CGRP receptor. Results are expressed as fold change over vehicle-treated cells and represent the mean ± S.E.M of three independent experiments (n = 3).

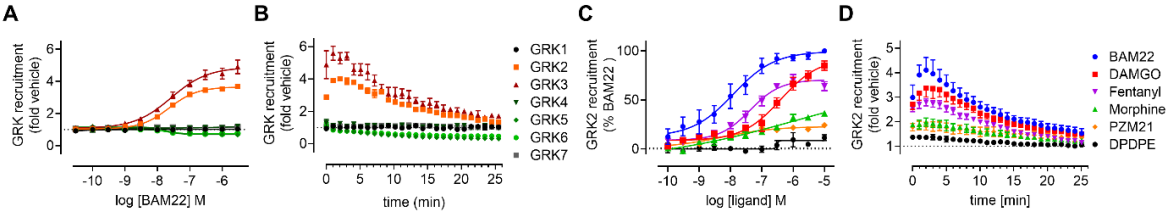

Figure 3. Assessment of MOR/GRKs interactions, kinetics and modulation by agonists. (**A-B**) GRK1-7 recruitment to MOR following stimulation with BAM22 as (**A**) concentration–response curves where data are expressed as fold vehicle or as (**B**) kinetic profiles. (**C-D**) GRK2 recruitment to MOR following stimulation with different agonists and partial agonists as (**C**) Concentration–response curves where data are expressed as percentage of BAM22 (10 µM) response or as (**D**) kinetic profiles for each ligand (10 µM) over 25 minutes expressed as fold vehicle. Results represent the mean ± S.E.M of three independent experiments (n = 3).

2. Materials

2.1. Cell culture and transfection

1. Human embryonic kidney 293T (HEK293T) cells (ATCC, USA)
2. Cell culture flasks (Greiner Bio-One, Germany)
3. Dulbecco's modified Eagle medium (DMEM, GIBCO, USA) supplemented with 0.04 mM phenol red, 1 mM sodium pyruvate, 4 mM L-glutamine, 4.5 g/L glucose, supplemented with 10 % fetal bovine serum (FBS, Merck, Germany) and 100 units/ mL of penicillin and 100 µg/mL of streptomycin (GIBCO, USA)
4. 6-well cell culture plates (Corning, USA)
5. Opti-MEM, Reduced Serum Medium (GIBCO, USA)
6. Polyethylenimine (PEI, 1 mg/mL, Merck, Germany)
7. GPCR-C-SmBiT and LgBiT-N-GRK expression constructs

2.2. Sample preparation and reading for NLuc complementation assay

1. Phosphate-buffered saline (PBS, GIBCO, USA)
2. Versene (GIBCO, USA)
3. 15-mL tube (Greiner Bio-One, Germany)
4. White flat-bottom LUMITRAC 96-well plates, medium binding (Greiner Bio-One, Germany)
5. Transparent U-bottom 96-well plates (Greiner Bio-One, Germany)
6. 12-channel pipets: 2–20 µL range and 20–200 µL range (Mettler Toledo, Germany)
7. CASY Automated cell counter (OMNI Life Science, Germany)
8. NanoLuc substrate: Furimazine (Nano-Glo® Luciferase Assay Substrate, Promega, USA) or coelenterazine H (Regis Technologies, USA)
9. Luminescence plate reader: Mithras LB940 (Berthold Technologies, Germany)

2.3. Sequences of tagged protein partners

2.3.1. GPCR-C-SmBiT

SmBiT (VTGYRLFEEIL) inserted with a flexible linker (GSSGGGGSGGGGSSG) at the C terminus of the receptor *(see Notes 1,2 and 9).*

2.3.2. LgBiT-N-GRK

LgBiT

(MVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSVTPIQRIVRSGEN
ALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGTLVIDGVTPN
MLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTIN)
inserted with a flexible linker (GSSGGGGSGGGGSSG) at the N terminus of the human GRK1-7 *(see Notes 1, 2 and 9).*

3. Methods

3.1. Cell culture and transfection

The protocol described below should be performed in sterile conditions under a laminar flow hood.

1. Maintain HEK293T cells in culture medium at 37°C with 5% CO_2.
2. Seed cells 100 000 cells/cm^2 in a 6-well cell culture dish with 2 mL of culture medium (see note 3).
3. Incubate the cells at 37°C with 5 % CO2 for 24 h.
4. Transfect the cells with the plasmids coding for GPCR-C-SmBiT and LgBiT-N-GRK expression constructs (*see Note 4*). Dilute 4.5 µL of polyethylenimine (PEI) in 150 µL of Opti-MEM. In a separate Eppendorf tube, dilute 1.5 µg of DNA to 150 µL of Opti-MEM. Mix thoroughly, then gently combine the PEI-Opti-MEM mix with the DNA by pipetting up and down.
5. Incubate the PEI-DNA mix for 20 min at RT.
6. Remove and add fresh pre-warmed medium to the 6-well plate.
7. Add the transfection mix to the cells in a dropwise manner and swirl the plate to ensure even distribution over the entire surface.
8. Incubate the cells at 37°C with 5 % CO_2 for 24 h.

3.2. Sample preparation and reading for NLuc complementation assay

1. Aspirate the culture medium and wash the cells with 2 mL of PBS.
2. Add 250 µL of Versene to each well and incubate at 37°C for 5 min.
3. Collect the cells in 4 mL of Opti-MEM by pipetting up and down and transfer the cells to a 15-mL conical tube.
4. Centrifuge the cells for 5 min at 300 x *g* at room temperature.
5. Discard the supernatant and resuspend the cells in Opti-MEM at a concentration of $1.1x10^6$ cells/mL.
6. Add the Nanoluciferase substrate: furimazine, prepared according to the manufacturer's instructions (final dilution of 1:100). Alternatively, coelenterazine H at 10 nM may be used (*see Note 5*).
7. Incubate the cells at 37°C for 20 min.
8. In a transparent U-bottom 96-well plate, prepare the ligands and dilutions to be tested at a concentration of 10X in Opti-MEM.
9. Distribute 90 µL of the cell suspension per well (100 000 cells/well) in a white flat-bottom 96-well plate (*see Note 6*).
10. Add 10 µL of the 10X concentration ligands manually with a multichannel pipet and measure directly the luminescence with a plate reader for 25 cycles, over 25 min (*see Note 7*).

11. Analyze the results (*see Note 8 and 9*).

4. Notes

Note 1: All vectors described in this chapter are available upon request (andy.chevigne@lih.lu). The seven GRKs tagged with LgBiT in both N and C orientations are available. In our hands, the best orientation pair was GPCR-C-SmBiT and LgBiT-N-GRK. However, this may be optimized specifically according to the receptor or the GRK of interest[26,37].

Note 2: In this chapter, we use plasmids under a CMV promoter. Considering the strength of both the promoter and the NLuc signal, diluting the DNA leads to more physiological receptor and effector expression. CRISPR/Cas9 technology can also be used to assess interactions between endogenously expressed partners, without the possible artifacts derived from overexpression[38,39]. NanoBiT plasmids encoding NLuc and fragments under a weaker promoter HSV-TK, linkers and multiple cloning sites are commercially available from Promega.

Note 3: Cell density was optimized for HEK293T but other cell lines as well as primary cells can be used. Other cell culture vessels may be used with adapted medium volumes, transfection mixes and the DNA:PEI ratio of 3:1 (w/w) maintained. We have found that 1.5 µg of DNA and 4.5 µg of PEI in 300 µl Opti-MEM are optimal for transfection of cells in 6 well-plates.

Note 4: We used a ratio of 1:10 GPCR-C-SmBiT and 1:100 LgBiT-N-GRK for the transfection of HEK293T cells (150 ng GPCR and 15 ng GRK) topped up to 1.5 µg with irrelevant DNA. These ratios should be treated as starting points and may be optimized for different promoters, receptors and cellular backgrounds. Of note, due to the brightness of the NLuc signal, we have found these dilutions to provide a compromise between physiologically representative expression levels and a consistent, reproducible bioluminescent output.

Note 5:Other substrates may be used to replace furimazine (e.g. coelenterazine H). However, it should be considered that coelenterazine H is prone to chemical instability primarily through auto-oxidation, generating a higher auto-luminescence background, which may decrease the sensitivity of the assays.

Note 6: Flat-bottom white plates are recommended as they reflect luminescence and hence yield the maximum light output to be measured by the plate reader.

Note 7: Kinetics measurements may be optimized using injectors coordinated with reading times (not applied here), to avoid the delay associated with manual ligand administration and subsequent reading.

Note 8: The results shown here were analyzed systematically averaging out the peak signals for each condition, and normalizing this value to the vehicle condition. Other analysis (e.g. area under the curve (AUC)) or normalization (e.g. % full agonist) methods may be applied.

Note 9: Overall, the analysis of the GRK recruitment profiles assessed by NanoBiT in this chapter revealed a predominant recruitment of GRK2 and GRK3, resulting in positive signals for most of the GPCRs exemplified, including the intrinsically β-arrestin-biased receptors ACKR2 and ACKR3. GRK3 consistently yields the largest measurement windows. However, as indicated in the note 1, we strongly recommend to systematically test different receptor and GRK tagging combinations and different time points following ligand stimulation, as important interactions may be missed. Indeed, interactions between B2AR or ACKR3 and GRK2 have been reported but were not detected using the conditions described above. No significant modulation of GRK1, GRK4 and GRK7 was monitored for any of the receptors tested, which is expected from previous reports on the GPCRs tested. Interestingly, the different receptors showed varying patterns for GRK5 and GRK6 recruitment profiles. The molecular basis and interaction events leading to these different behaviors, especially signal reduction, are still unclear and may be related to the membrane-anchored nature of GRK5 and GRK6, constitutive interaction and competition from other effectors or regulators (e.g. other GRKs or β-arrestins) [29,40-42]. More insight may be gained by focusing on the kinetics data of GRK recruitment (**Fig. 3D**); in our assay for instance, GRK5 and GRK6 recruitment seems much slower than GRK2 and GRK3, and decreases over time [28].

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Chapter II

CXCR3-B and its atypical features

My contribution to this Chapter:

The research article presented in this chapter reflects a major part of my PhD work. I contributed to the experimental design, conceptualization and data production of this study. I was involved in the generation, analysis and interpretation of several experiments included in this article (Figure 1, panels A, B, C, E; Figure 2; Figure 4; Figure 6; Supplementary Figure 2, panels A, B, C; Supplementary Figure 3, panels A, B, D; Supplementary Figure 4, panel B; Supplementary Figure 5, panel A; Supplementary tables 1, 2, 3). I also contributed to the analysis and interpretation of other data of this manuscript (Figure 1, panel F; Figure 3; Figure 5, Supplementary Figure 4, panel A; Supplementary Figure 5, panel B). In addition, I helped with the generation of the molecular tools necessary for cellular assays (Figure 1, panels C and E; Figure 2, panel C; Figure 6). I contributed to the writing of the initial draft of the manuscript, took care of the revisions and edited the final manuscript.

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Highlights & aims

The gene encoding for the chemokine receptor CXCR3 is subjected to alternative splicing, leading to the generation of two major protein isoforms, CXCR3-A and CXCR3-B. The CXCR3-B isoform is enigmatic with largely unknown signaling properties and roles.

In this article, we have carried out an in-depth comparative molecular analysis of the two isoforms. The assessment of their activities reveals profound impairment of G protein coupling and downstream signalling for CXCR3-B. This feature is reminiscent of atypical chemokine receptors. Despite its inability to trigger G protein activation, CXCR3-B retains the capacity of recruiting β-arrestins in response to chemokine stimulation. By flow cytometry and confocal microscopy, we also demonstrate a predominantly intracellular localization and efficient chemokine uptake capacity for CXCR3-B. We could attribute these features to the extended CXCR3-B N terminus by its progressive truncations. This work highlights the atypical chemokine receptor properties of CXCR3-B, suggesting that this isoform could regulate the availability of CXCR3 ligands in the extracellular space and represent an additional element of chemokine receptor modulation.

The extended N-terminal domain confers atypical chemokine receptor properties to CXCR3-B.

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Short title: Atypical chemokine receptor features of CXCR3-B

Keywords: CXCR3b, ACKR2, ACKR3, CXCL11, CXCL10, CXCL9, scavenger, isoform

Abstract

The chemokine receptor CXCR3 plays a critical role in immune cell recruitment and activation. CXCR3 exists as two main isoforms, CXCR3-A and CXCR3-B, resulting from alternative splicing. Although the two isoforms differ only by the presence of an N-terminal extension in CXCR3-B, they have been attributed divergent functional effects on cell migration and proliferation. CXCR3-B is the more enigmatic isoform and the mechanisms underlying its function and signaling remain elusive. We therefore undertook an in-depth cellular and molecular comparative study of CXCR3-A and CXCR3-B, investigating their activation at different levels of the signaling cascades, including G protein coupling, β-arrestin recruitment and modulation of secondary messengers as well as their downstream gene response elements. We also compared the subcellular localization of the two isoforms and 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 its 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 G protein coupling. Our study clarifies the molecular basis underlying the divergent effects of CXCR3 isoforms, and emphasizes the β-arrestin-bias and the atypical nature of CXCR3-B.

1. 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 can usually bind to many receptors, and a receptor recognizes several chemokines[1,2].

Over the last decades, a new subfamily of chemokine receptors, referred to as ‘atypical’ chemokine receptors (ACKRs) and presently comprising four members (ACKR1–4), has emerged as important regulators of the chemokine network[2,3]. ACKRs 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

chemokines. The molecular basis of the lack of G protein coupling 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[2,4]. Despite the absence of G protein signaling upon activation, ACKRs modulate cell migration and physiological processes by regulating the availability of chemokines for the classical receptors, among others through their internalization. This activity was previously considered to mainly rely on β-arrestins[4-8] but recent studies showed that alternative mechanisms may also drive the regulatory functions of ACKRs[9-12]. Other distinctive properties of ACKRs are their unconventional cellular localization, trafficking and expression profile. ACKRs are indeed predominantly found in endosomal vesicles and are generally expressed on endothelial cells 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[13,14].

The chemokine receptor CXCR3 is mainly present on activated immune cells and mediates their migration towards sites of inflammation but it is also expressed on barrier cells as well as on cancer cells and within the tumor microenvironment[15-17]. In humans, the gene encoding for CXCR3 can be transcribed to three alternative splice variants: CXCR3-A, CXCR3-B, and CXCR3-Alt, which give rise to structurally distinct proteins. CXCR3-B bears an extended N terminus wherein a 51-amino-acid stretch replaces the first four residues of CXCR3-A, while CXCR3-Alt lacks two transmembrane regions and shows a modified C terminus (Supplementary Fig. 1)[18,19]. The three isoforms 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 G_i protein activation nor β-arrestin recruitment could be observed [20]. More efforts have been put in investigating the two other isoforms, which have been attributed opposing cellular functions. Indeed, CXCR3-A, mainly present on leukocytes, mediates cell migration and proliferation through activation of G_i and calcium signaling[20-26]. In contrast, CXCR3-B is reported to be abundantly expressed on barrier cells[27], to inhibit cell migration and proliferation, and to induce apoptosis upon ligand stimulation[18,28-30]. However, although a β-arrestin bias was proposed for CXCR3-B[20,21], the molecular mechanism underlying the receptor functions remain unclear.

The divergent cellular effects and expression patterns of the two CXCR3 isoforms led us to hypothesize that CXCR3-B could act as an ACKR. Thus, we investigated the profile of

CXCR3-B with regard to the established features of ACKRs, namely the absence of G protein coupling, the predominant intracellular location and the scavenging properties. Using a large panel of live cell-based assays to monitor G protein and β-arrestin transducers, we showed that CXCR3-B does not signal via G proteins, while it maintains its ability to recruit β-arrestins. Furthermore, we observed important intracellular pools of CXCR3-B, which could be mobilized upon chemokine stimulation. Finally, we demonstrated herein that the N-terminal extension of CXCR3-B considerably alters its subcellular distribution and signaling capacity without changing the binding mode and selectivity of its chemokine ligands. Hence, we propose that the two CXCR3 isoforms could be regarded as distinct effectors in analogy to classical and atypical chemokine receptors.

2. Material and Methods

Chemokines and antibodies

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

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

Custom CXCL11 chemokines: The N-terminally truncated, P2G-mutated and N-loop chimeras were produced as previously described[2,31-33]. In brief, cells were grown in Terrific Broth and production of modified CXCL11, cloned into pQE30 vectors, was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cell pellets were then lysed, centrifuged at 12 000 x g for 20 minutes and the supernatant and solubilized inclusion body pellets were added to nitrilotriacetic acid resin for 1 hour. Bound proteins were eluted with 6 M guanidinium chloride, 50 mM Na_2PO_4 (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 (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: Phycoerythrin-conjugated anti-CXCR3 (1C6) and anti-CXCR4 (12G5) mAbs were purchased from BD Biosciences. Allophycocyanin-conjugated anti-ACKR2 (196124) was from R&D Systems and anti-ACKR3 (8F11-M16) from BioLegend.

Cell culture

HEK293T and U-87 MG 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 isoforms 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[34] and HEK293T-ACKR2[35] 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.

Recruitment assays

NanoBiT assay: miniG protein (mG, engineered GTPase domain of Gα subunit), β-arrestin-1 and β-arrestin-2 recruitment to WT and modified receptors (CXCR3-A, CXCR3-B, CXCR3-B N-terminally truncated isoforms, sp-IL6-CXCR3-B, where CXCR3-B sequence was preceded by Interleukin-6-derived signal peptide MNSFSTSAFGPVAFSLG-LLLVLPAAFPAP, CXCR4, and extCXCR4), was monitored using a nanoluciferase complementation-based assay (NanoBiT, Promega)[36-38]. 4 x 10^6 HEK293T cells or 1.5 x 10^6 U87 cells were plated in 10-cm dishes and cultured for 24 or 48 hours, respectively, before transfection with vectors encoding for miniG proteins or human β-arrestins N-terminally fused with LgBiT and the chemokine receptor isoforms C-terminally fused with SmBiT. 48 hours after transfection, cells were harvested, incubated for 20 minutes at 37°C with coelenterazine H in Opti-MEM, and distributed into white 96-well plates (1.5 x 10^5 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)[35,39]. 4 x 10^6 HEK293T cells were plated in 10-cm dishes and cultured for 24 hours before transfection with vectors encoding for the human β-arrestin-1 or β-arrestin-2 N-terminally fused with

nanoluciferase and the chemokine receptor isoforms C-terminally fused with mNeonGreen. 48 hours after transfection, cells were harvested, incubated with coelenterazine H in Opti-MEM and immediately distributed into black 96-well plates (1.5 x 10^5 cells/well). Upon addition of chemokines at the indicated concentrations, BRET signal was measured with a GloMax plate reader (Promega) equipped with 450/10 filter for donor luminescence emission and 530 LP filter for acceptor fluorescence emission. BRET signal is defined as acceptor/donor ratio. Ligand-induced changes in BRET ratio were expressed as ΔBRET, which was calculated as follows: $[(Ratio_{stimulated} - Ratio_{basal})/ Ratio_{basal}] \times 100)$.

Ligand-mediated G protein dissociation was monitored using BRET-based G protein activity sensors[40]. These biosensors are based on a tricistronic plasmid which encodes nanoluciferase-tagged G_α subunits together with the related G_β and circularly permutated Venus tagged G_γ. G protein activity is monitored through the reduction of BRET signal upon G protein subunit dissociation. 4 x 10^6 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 coelenterazine H in Opti-MEM and immediately distributed into black 96-well plates (10^5 cells/well). Upon addition of chemokines at the indicated concentrations, BRET signal was measured with a GloMax plate reader (Promega) equipped with 450/10 filter for donor luminescence emission and 530 LP filter for acceptor fluorescence emission. BRET signal is defined as acceptor/donor ratio. Ligand-induced changes in BRET ratio were expressed as ΔBRET, which was calculated as follows: $[(Ratio_{stim} - Ratio_{basal})/ Ratio_{basal}] \times 100)$.

cAMP measurements

cAMP measurements upon chemokine stimulation were performed using a luminescence GloSensor cAMP reporter assay (Promega)[41]. 4 x 10^6 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 1 hour at 37°C with the firefly luciferase substrate and IBMX (300 µM) in HBSS (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 10 mM HEPES, pH 7.4, 10 mM glucose) and distributed into white 96-well plates (1 x 10^5 cells per well). cAMP-dependent changes in luminescence in response to chemokines at the indicated concentrations was measured with a Mithras LB940 luminometer (Berthold Technologies).

Intracellular calcium mobilization

CXCR3-driven chemokine-induced calcium flux was assessed using an assay based on nanoluciferase complementation (NanoBiT) and Ca^{2+}-dependent calmodulin–MYLK2S protein association[42]. HEK293T cells were plated in a 6-well plate (1 x 10^6 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 $CaCl_2$ and 0.5 mM $MgCl_2$ for 10 minutes at 37°C. Coelenterazine H was then added, cells distributed in a 96-well plate (10^5 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 $CaCl_2$ and 0.5 mM $MgCl_2$ for 1 hour at 37°C. Cells were pelleted, resuspended in PBS/$CaCl_2$/ $MgCl_2$, distributed in a 96-well plate (10^5 cells per well) and incubated for 30 minutes. 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 minutes. 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.

Transcriptional nanoluciferase reporter assays

Activation of the MAPK/ERK-, RhoA-, prolonged calcium- and cAMP-dependent signaling pathways was evaluated using a serum response element (SRE), Serum Response Factor Response Element (SRF-RE), Nuclear Factor of Activated T-cell Response Element (NFAT-RE) and a cAMP response element (CRE) nanoluciferase reporter assay, respectively (Promega). 1 x 10^6 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, 5 x 10^4 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) for SRE, 10% FBS for CRE, 1 µM ionomycin for NFAT, 10% FBS for SRF) were then added and incubated for 6 hours. Coelenterazine H was then added and luminescence was read over 20 minutes on a Mithras LB940 plate reader (Berthold Technologies).

Receptor cellular distribution assays

Fluorescent microscopy: 3 x 10^4 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 then 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 acquiring images on a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss).

Flow cytometry: To determine chemokine receptor subcellular distribution, HEK293T cells transiently transfected with plasmids encoding CXCR3-A or CXCR3-B C-terminally fused with mNeonGreen or with an empty vector were used. 48 hours after transfection 1.5 x 10^5 cells were seeded in a 96-well plate and incubated with anti-CXCR3 (1C6) mAb or isotype control for 45 minutes at 4°C, washed twice with FACS buffer (PBS, 0.1% sodium azide, 1% BSA) and then incubated for 20 minutes at 4°C with Zombie NIR viability dye, before measuring the fluorescence on a Quanteon Flow Cytometer (NovoCyte).

To monitor receptor cycling, 1.5 x 10^5 HEK293T.CXCR3-A, HEK293T.CXCR3-B, HEK293T.ACKR3[34] or HEK293T.ACKR2[35] cells were seeded in a 96-well plate and incubated for 3 hours with 0.1 mg/mL proteinase K to remove 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 subcellular distribution after chemokine stimulation, 1.5 x 10^5 HEK293T.CXCR3-A, HEK293T.CXCR3-B, HEK293T.ACKR3 or HEK293T.ACKR2 cells were seeded in a 96-well plate, incubated or not with the V ATPase inhibitor, bafilomycin A1 (1.5 µM), 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 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 surface expression was measured on a Quanteon Flow Cytometer (NovoCyte).

Surface nanoluciferase complementation (HiBiT): 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 10^6 HEK293T cells were plated in 10-cm dishes and cultured for 24 hours before transfection with pHiBiT vectors encoding for CXCR3 isoforms N-terminally fused to HiBiT. 48 hours later, cells were distributed in white 96-well plates (5 x 10^4 cells per well) and stimulated with chemokines (100 nM) for 5, 10, 20, 40 minutes at 37°C. After addition of soluble LgBiT protein, luminescence was recorded over 30 minutes with a GloMax plate reader (Promega). In unstimulated 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.

Chemokine uptake and binding

Flow cytometry: To monitor chemokine uptake and binding, 1.5 x 10^5 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 33 nM Alexa Fluor 647-labeled chemokines (Protein Foundry). Cells were washed twice with FACS buffer and afterwards 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 incubated for 20 minutes at 4°C with Zombie NIR viability dye (BioLegend). After two PBS washes, the fluorescent chemokine uptake was quantified using a Quanteon Flow Cytometer (NovoCyte).

Confocal microscopy: 3 x 10^4 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 1 hour at 37°C with 100 nM of Alexa Fluor 647-labeled 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 with PBS. Nuclear staining was performed with Hoechst 33342 dye (1 µg/mL) for 20 minutes at RT and cells were washed three times with PBS. Images were acquired with a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss).

Data and statistical analysis

Concentration-response curves were fitted to the three-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 9.3.0) to provide EC_{50}, % maximum 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. ordinary or repeated measures one way- and two-way ANOVA, unpaired t-tests, Kruskal-Wallis and Mann-Whitney tests were performed with GraphPad Prism 9.3.0.

3. Results

The CXCR3-B isoform is not coupled to G proteins but maintains β-arrestin recruitment capacity

To evaluate and compare the functionality and signaling capacity of CXCR3-A and CXCR3-B isoforms, 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 $miniG_i$ and $miniG_q$ proteins after stimulation with all three chemokines. In stark contrast, CXCR3-B showed no $miniG_i$ recruitment while $miniG_q$ recruitment was nearly abolished (Fig. 1A). Both CXCR3-A and CXCR3-B failed to recruit $miniG_s$ and $miniG_{12/13}$ proteins (Supplementary Fig. 2). Next, we monitored the recruitment of β-arrestins towards both CXCR3 isoforms upon chemokine stimulation. CXCL11 or CXCL10 induced β-arrestin-1 and β-arrestin-2 recruitment to CXCR3-A, with CXCL11 having stronger potency and efficacy compared to CXCL10 (Fig. 1B and Supplementary Table 1). Interestingly, although G protein interaction was severely impaired, CXCR3-B efficiently interacted with β-arrestin-1 and β-arrestin-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 isoforms at the highest concentration tested (Fig. 1B). These results were confirmed using the same assays in U-87 MG cellular background as well as using NanoBRET-based approaches (Supplementary Fig. 2).

The lack of CXCR3-B coupling to G proteins was further investigated using a NanoBRET assay monitoring heterotrimeric G protein dissociation following chemokine-induced receptor activation. CXCR3-A stimulation by its ligands triggered the dissociation of $G_{\alpha i/o}$ and $G_{\alpha q}$ proteins from the $G_{\beta\gamma}$ dimer, albeit a weaker effect was observed for the latter. In contrast, CXCR3-B displayed no G_i and G_q activation and a severe impairment of G_o for CXCL10 and CXCL9. CXCL11 triggered slight activation of $G_{i/o}$ protein at high chemokine concentration (Fig. 1C). No other G protein subtype activation could be measured for either CXCR3 isoform (Supplementary Fig. 3). These results support the data obtained for miniG protein recruitment and further confirm the impairment of CXCR3-B coupling to G proteins.

To corroborate these observations, we investigated the modulation of two downstream G protein signaling secondary messengers: cAMP and calcium. cAMP modulation was monitored using a firefly luciferase-based Glo biosensor. Upon activation with CXCL11, CXCL10, CXCL9, we detected a concentration-dependent decrease in cAMP for CXCR3-A, confirming $G_{i/o}$ 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. 3). The positive control CXCL12 was able to reduce cAMP levels, by acting on endogenously expressed CXCR4, attesting to the assay functionality. Intracellular calcium mobilization was investigated using a NanoBiT-based calmodulin–MYLK2S assay to further characterize signaling abilities of the receptors. CXCR3-A induced a calcium flux in response to all three of its cognate chemokines. 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 using the ratiometric fluorescent calcium indicator Indo-1 (Supplementary Fig. 3). These experiments confirmed CXCR3-B's inability to trigger efficient downstream G protein signaling (Fig. 1D and E), which is in agreement with the impaired $G_{i/o}$ 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 led to an

increase in the activation of MAPK/ERK-dependent response element SRE, pointing to the involvement of ERK signaling, as opposed to CXCR3-B that showed no modulation of this pathway (Fig. 1F). The specific activation of G_i or G_s and subsequent cAMP modulation was monitored by CRE inhibition. 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 but statistically not significant decrease (Fig. 1F). We also examined prolonged increase of intracellular calcium ions and $G_{12/13}$ protein-specific RhoA-mediated signaling using NFAT and SRF response elements, respectively, which showed no differences in the signal measured in response to CXCR3 chemokines for both isoforms compared to the negative control CCL5 (Fig. 1F).

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

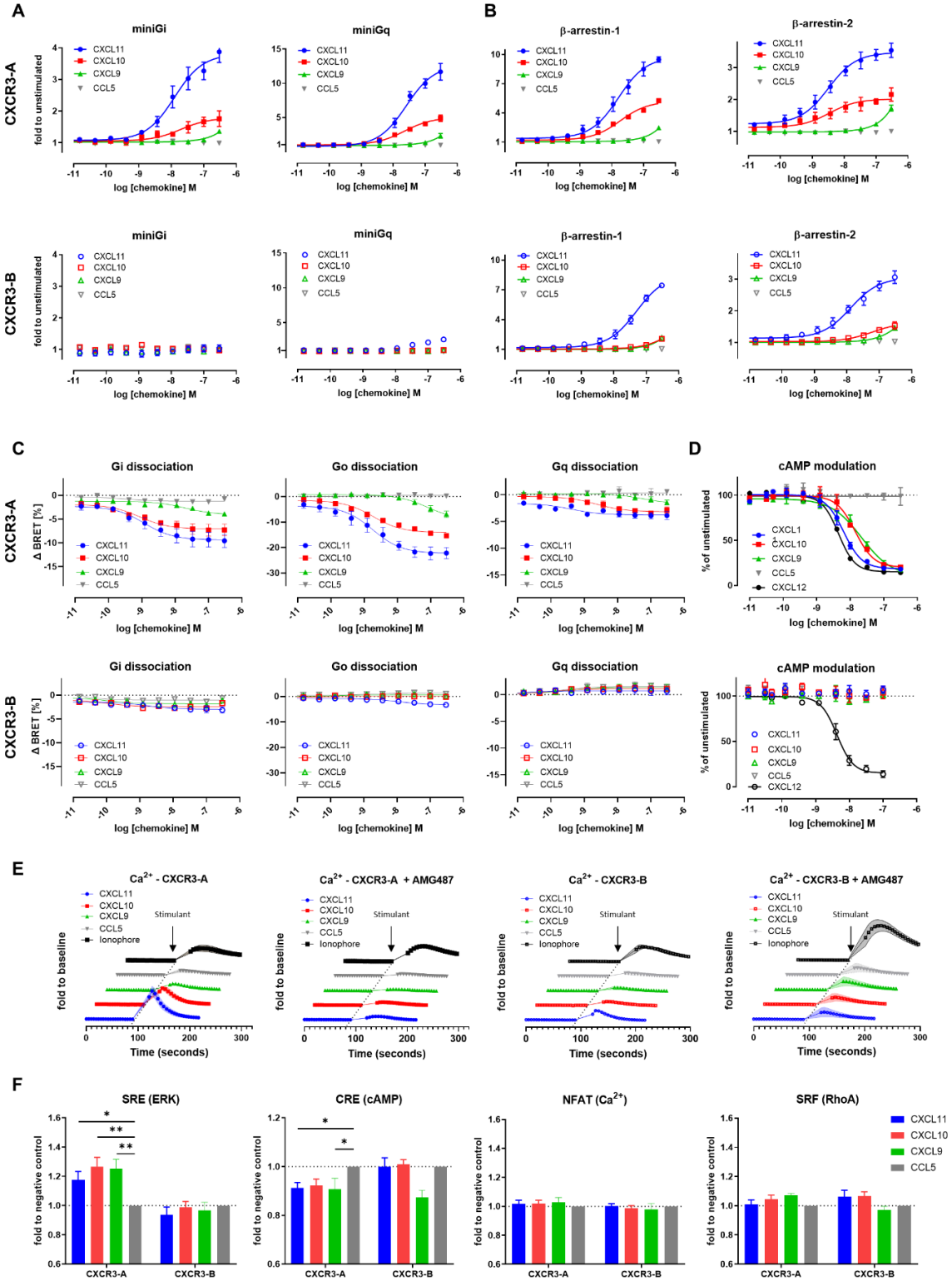


Figure 1. The CXCR3-B isoform does not induce G protein-mediated signaling. (**A**) miniG protein or (**B**) β-arrestin recruitment towards CXCR3-A and CXCR3-B in response to CXCL11, CXCL10, CXCL9 and the negative control CCL5 monitored by NanoBiT-based assay. (**C**) Heterotrimeric G protein dissociation upon CXCR3-A or CXCR3-B stimulation with CXCL11, CXCL10, CXCL9 and CCL5 monitored by NanoBRET. Concentration-response relationships for the alpha subunits G_{i2}, G_o and G_q are expressed as ΔBRET. (**D**) CXCR3-A- and CXCR3-B-driven

reduction of intracellular cAMP levels, in response to chemokine ligands. CXCL12-induced effect mediated by the endogenously expressed CXCR4 was used as positive control. (**E**) CXCR3-A- and CXCR3-B-mediated intracellular calcium mobilization in response to chemokines in the absence or presence of CXCR3 antagonist AMG487 (1 µM) monitored by NanoBiT assay. Calcium ionophore A23187 (1 µM) was used as receptor-independent positive control. (**F**) Comparison of CXCR3 isoform-mediated downstream signaling events using the nanoluciferase-dependent response elements SRE, CRE, NFAT and SRF in response to the chemokines CXCL11, CXCL10, CXCL9 and CCL5 (100 nM). All assays were conducted in HEK293T cells. Data points represent mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ by Kruskal-Wallis with two-sided Dunn's test.

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

Based on the observations that CXCR3-B efficiently recruits 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 isoforms 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[13].

The cellular distribution of CXCR3 isoforms, 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[18], CXCR3-A was mainly present at the cell surface as revealed by the strong co-localization of mNeonGreen and the CXCR3-specific antibody (Fig. 2A). 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 ACKR3 and ACKR2 (Fig. 2A). The differences in cellular distribution of the two CXCR3 isoforms were further confirmed by flow cytometry. Indeed, despite a similar total expression level of the two isoforms, CXCR3-A showed greater surface expression compared to CXCR3-B (Fig. 2B). Consistently, results obtained with the high-affinity nanoluciferase complementation-based HiBiT assay revealed that only 45 % of total CXCR3-B was found at the cell surface under basal conditions, contrasting with the almost exclusive surface localization of CXCR3-A (Supplementary Fig. 4).

These observations prompted us to investigate further the accountability of the different surface expression levels of the two isoforms in their abilities to recruit cellular effectors. To do so, we adopted two strategies. We first generated a CXCR3-B variant preceded by the signal peptide of human interleukin-6 (sp-IL6) known to facilitate receptor export to the plasma membrane. Despite the similar cell surface expression of the two variants (Fig. 2C, left panel), only low levels of miniGi or miniGq recruitment could be observed for sp-IL6-

CXCR3-B, while its ability to recruit β-arrestin-1 was comparable to CXCR3-A (Fig. 2C). In the second opposite approach, equivalent surface expression levels for the two isoforms were achieved by reducing four times the amount of CXCR3-A-encoding DNA used in transfection (Fig. 2D, left panel). In these conditions, CXCR3-B still showed drastically impaired G protein coupling compared to only slightly diminished CXCR3-A activation (Fig. 2D). These results show that although β-arrestin interaction and receptor-mediated G-protein activation are to a certain extent influenced by receptor surface expression, the different subcellular localization is not sufficient to fully explain the divergent properties of CXCR3-A and CXCR3-B.

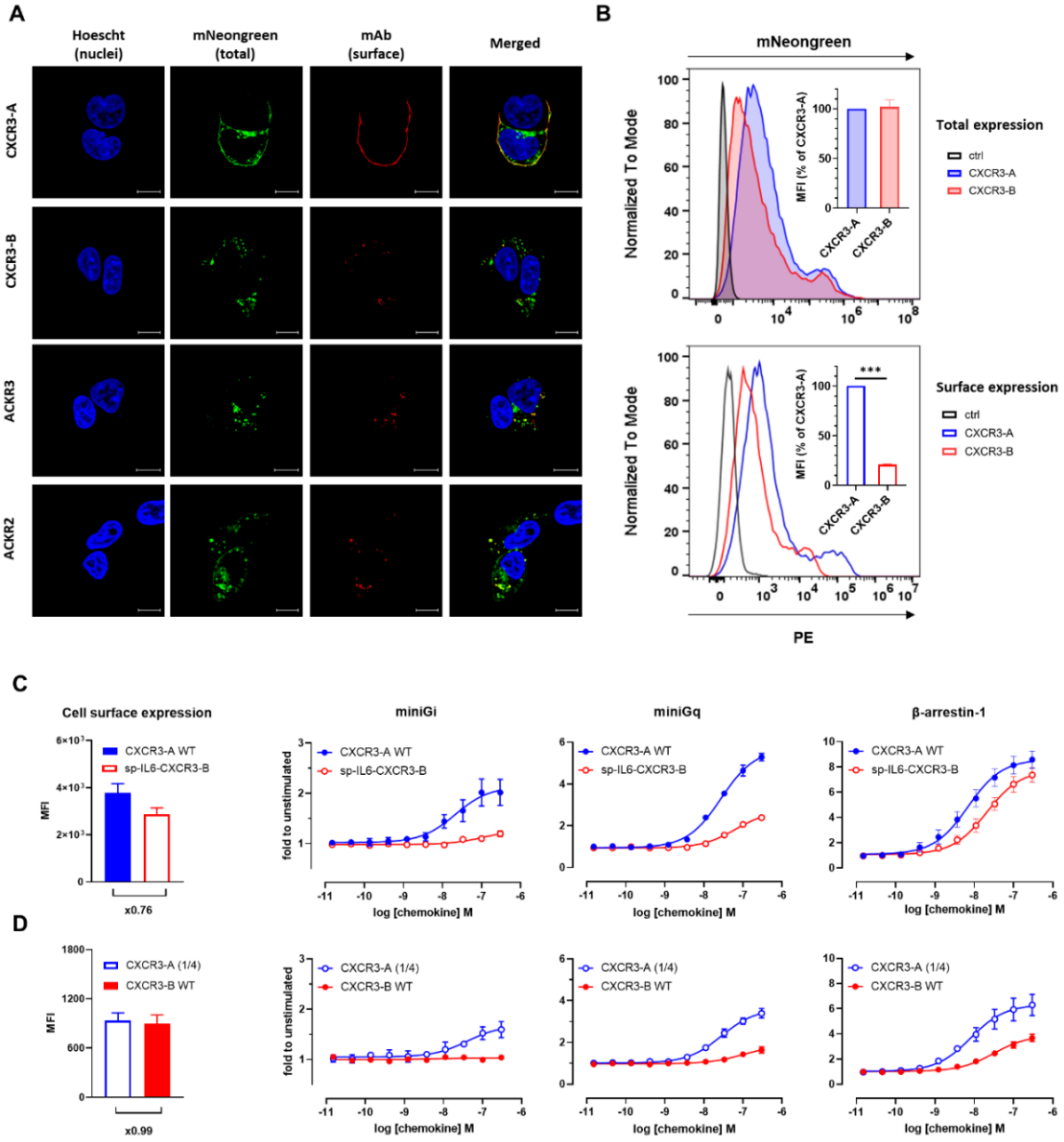


Figure 2. CXCR3-B shows intracellular localization and does not cycle in basal conditions but is mobilized to the cell membrane upon chemokine stimulation. (**A**) Receptor cellular distribution in basal conditions visualised by confocal fluorescent microscopy using mNeonGreen-fused CXCR3-A, CXCR3-B, ACKR3 and ACKR2 for total expression and receptor-specific mAbs (clones 1C6, 8F11-M16 and 196124, respectively) for surface expression. Pictures are representative of 12 acquired images from three independent experiments. (**B**) Receptor expression monitored by flow cytometry in cells transiently transfected with vectors encoding CXCR3-A or CXCR3-B C-terminally tagged with mNeonGreen or empty vectors (ctrl). Total expression was evaluated as mNeonGreen (upper panel) and surface expression by CXCR3-specific mAb (clone 1C6). Both are quantified as mean fluorescent intensity (MFI) and expressed as percentage of CXCR3-A (inset). Data shown are representative of three independent experiments and for inset, mean ± SEM of three independent experiments. ***p <0.0001 by unpaired t test. (**C-D)** miniG protein or β-arrestin recruitment towards cell surface-controlled levels of CXCR3-A and CXCR3-B monitored by NanoBiT. Equivalent surface expression levels for the two isoforms were obtained by the addition of human interleukin-6-derived signal peptide to facilitate CXCR3-B export to the plasma membrane (sp-IL6-CXCR3-B) (**C**) or by reducing the amount of CXCR3-A-encoding vector used for transfection (CXCR3-A (1/4)) (**D**). Cell surface expression of the receptor variants was assessed by flow cytometry with CXCR3-specific mAb (clone 1C6). CXCL11-induced miniGi, miniGq or β-arrestin-1 recruitment to CXCR3-A WT and sp-IL6-CXCR3-B (**C**) or CXCR3-A (1/4) and CXCR3-B WT (**D**).

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

The atypical subcellular distribution of CXCR3-B is reminiscent of that of ACKRs, which play an important role in regulating the extracellular chemokine availability for classical receptors. Chemokine binding to ACKRs may induce their internalization, but some receptors, like ACKR3, were shown to continuously cycle between the intracellular compartments and the cell surface, independently of ligand stimulation[43,44].

We therefore investigated CXCR3 cycling in the absence and presence of chemokines. Basal receptor cycling was first evaluated in flow cytometry following extracellular epitope cleavage by proteinase K, by monitoring receptor replenishment at the 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 isoforms and ACKR2 showed an approximately 10%-increase of receptor cell surface expression, while ACKR3 demonstrated a more pronounced increase of 30% (Fig. 3B and Supplementary Fig. 4). 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 isoforms, first by flow cytometry (Fig. 3C and Supplementary Fig. 4). 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. 3C). In contrast, for CXCR3-B,

CXCL11 induced only about 20% net internalization, while CXCL10 and CXCL9 had no effect (Fig. 3C). Overall ACKR2 surface expression was not modified by chemokine stimulation, reminiscent of CXCR3-B behavior, 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. 3C). In addition, receptor surface expression was evaluated 40 minutes after chemokine removal. None of the CXCR3 isoforms nor ACKR2 recycled back to the cell surface in the presence or absence of the V-ATPase inhibitor bafilomycin A1, suggesting an absence of rapid recycling following ligand stimulations (Fig. 3C) in contrast to ACKR3, for which bafilomycin A1-sensitive recovery at the plasma membrane could be detected[34,45].

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 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 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 respective potencies in β-arrestin recruitment assays (Fig. 3B and Supplementary Table 1). 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 but not statistically significant 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 (Fig. 3A) as recently described for ACKR2 following ligand stimulation [35].

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[35].

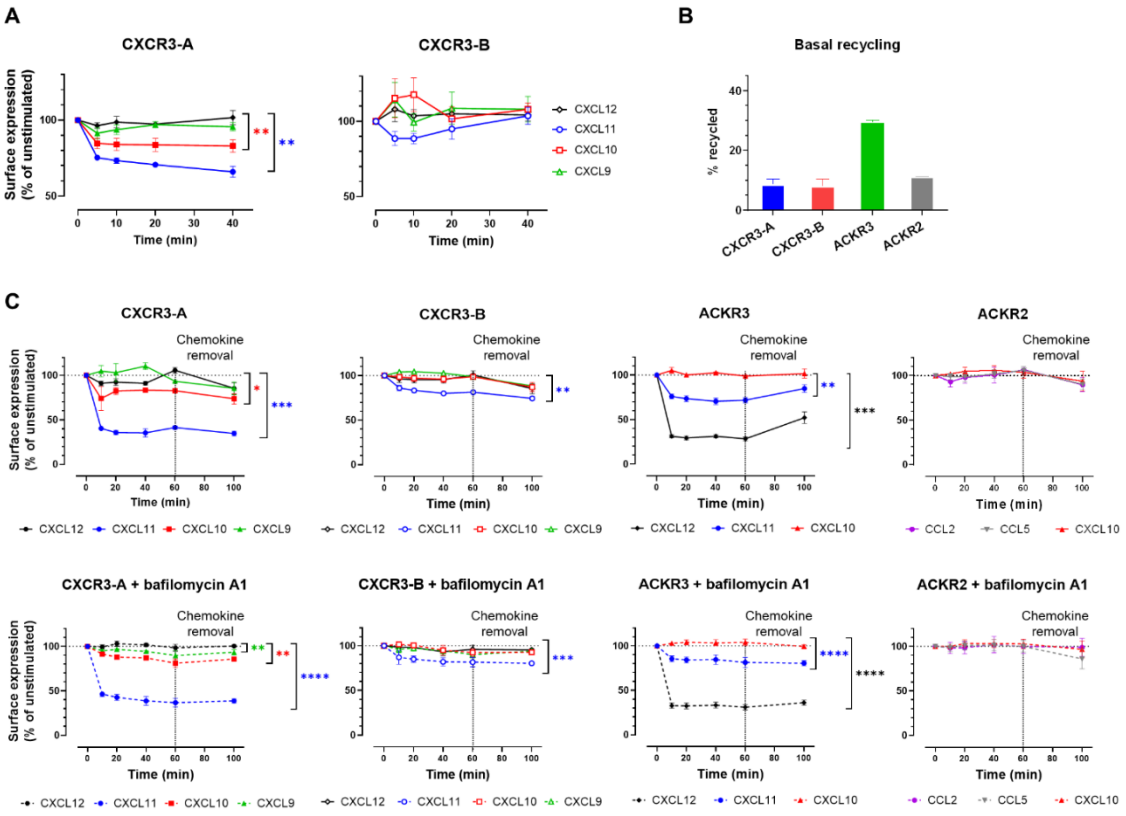


Figure 3. CXCR3-B is mobilized to the cell membrane upon chemokine stimulation but does not cycle in basal conditions. (**A, C**) Cell surface redistribution of CXCR3-A and CXCR3-B after stimulation with chemokines (100 nM) monitored by (**A**) surface nanoluciferase complementation (HiBiT) or (**C**) flow cytometry in the presence or absence of bafilomycin A1. All assays were conducted in HEK293T cells. Results are expressed as percentage of receptor surface expression in basal conditions (100%) and represent mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p<0.0001 by repeated measures one-way ANOVA with Bonferroni correction. (**B**) CXCR3-A, CXCR3-B, ACKR3 and ACKR2 basal cycling after extracellular epitope shaving by proteinase K. Results are expressed as percentage of cell surface expression after proteinase K treatment.

Both CXCR3 isoforms mediate efficient uptake of endogenous chemokines

As CXCR3-B is 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 isoforms, albeit with reduced intensities for CXCR3-B (Fig. 4A), which may reflect its lower expression level at the plasma membrane. No uptake was observed with the irrelevant chemokine CCL5 labeled 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 chemokines, as illustrated by the reduced fluorescence signal

in non-internalizing conditions (4°C) 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 (37°C) for the two CXCR3 isoforms, strongly pointing to chemokine uptake following receptor activation.

Receptor-dependent internalization of fluorescently labeled chemokines was then analyzed by confocal microscopy. Cells expressing CXCR3-A or CXCR3-B internalized their related chemokines (Fig. 4B) 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 despite its inability to trigger efficient G protein signaling.

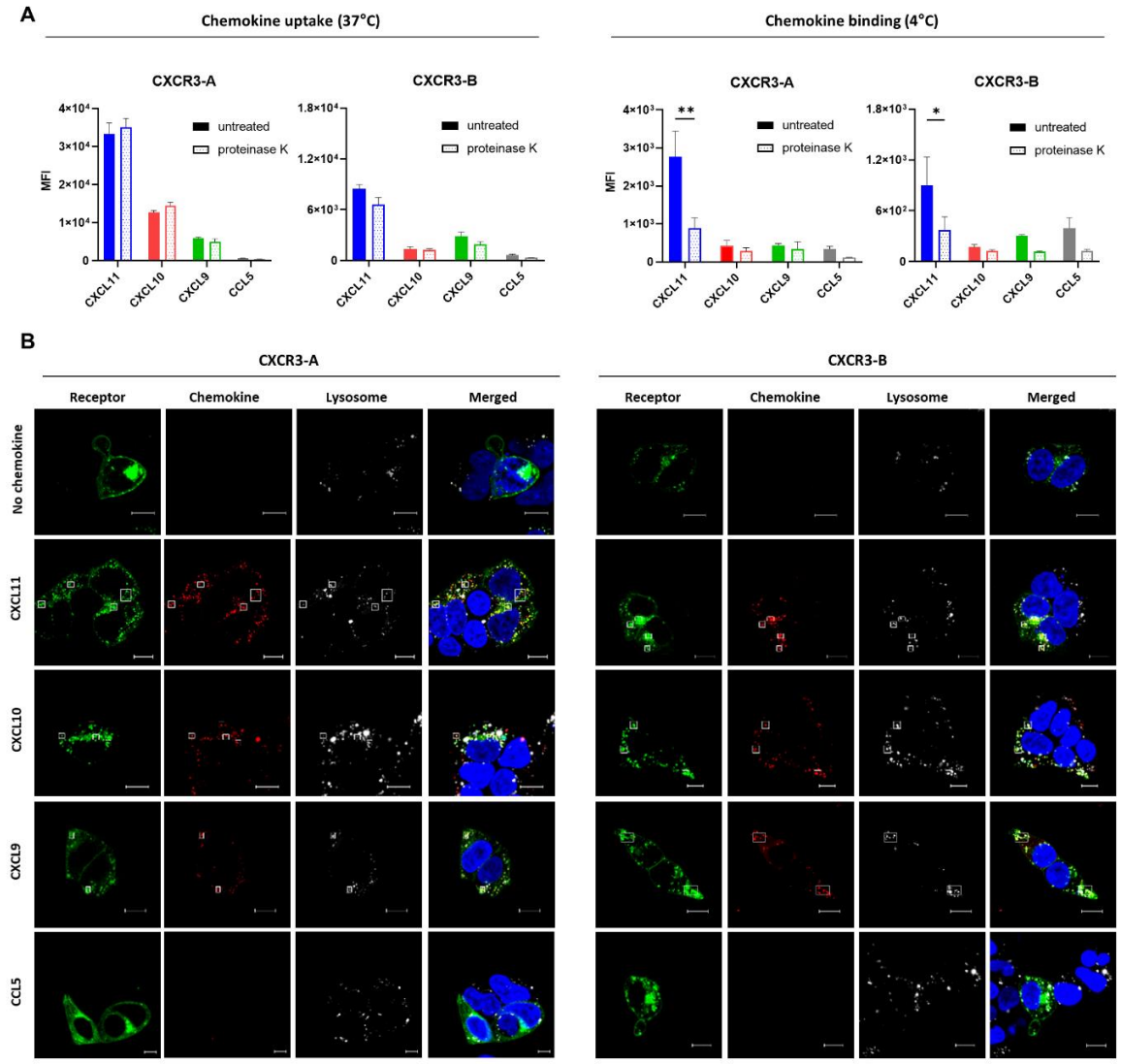


Figure 4. Both CXCR3 isoforms can mediate chemokine uptake. (**A**) Intracellular accumulation (37°C) and cell surface binding (4°C) of Alexa Fluor 647-labeled CXCL11, CXCL10, CXCL9 and CCL5 (33 nM) monitored by flow cytometry in CXCR3-A- and CXCR3-B-expressing cells. After 1-hour incubation cell surface-bound chemokines were removed with proteinase K treatment. Results represent mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ by two-way ANOVA with Tukey's post hoc analysis. (**B**) CXCR3-A- and CXCR3-B-driven uptake of Alexa Fluor 647-coupled chemokines visualized by confocal fluorescent microscopy. Cells transiently expressing CXCR3-A or CXCR3-B fused with mNeonGreen (green) were incubated for 2 hours with chemokines (100 nM) (red). 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: 10 µm. All assays were conducted in HEK293T cells.

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

The extracellular N-terminal domain of chemokine receptors plays an important role in chemokine binding and selectivity[46]. The presence of the unique extended CXCR3-B N

terminus and its impact on the ability of the receptor to efficiently couple to G proteins 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 isoforms aiming at evaluating the possible impact of the extended CXCR3-B N terminus on ligand selectivity. This systematic interaction assessment did not allow to identify new endogenous agonist chemokines for any of the two isoforms (Fig. 5A). Noteworthy, we could not confirm the activity of the orphan chemokine CXCL4 proposed in some studies as a CXCR3-B agonist[47]. Surprisingly, we observed that the viral chemokine vCCL2 showed antagonistic properties towards both CXCR3 isoforms, an interaction that has not been described previously (Supplementary Fig. 5)[48].

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 [49]. Therefore, we next investigated the ability of chemokines with N-terminal substitutions and progressive truncations or modified N loops to activate both CXCR3 isoforms. The removal of the first two residues of CXCL11, CXCL10, and CXCL9 by CD26 exopeptidase abolished their activity towards both CXCR3 isoforms (Fig. 5B). These results were confirmed with recombinant CXCL11 lacking the first two residues ($CXCL11_{3\text{-}73}$) or bearing further N-terminal truncations ($CXCL11_{5\text{-}73}$ and $CXCL11_{7\text{-}73}$) (Fig. 5C). Similarly, proline-to-glycine substitution at position 2 ($CXCL11_{P2G}$), known to improve CXCL11 potency towards ACKR3[31], had a negative impact on the chemokine's ability to activate the two CXCR3 isoforms. Finally, CXCL11 chimeras with CXCL12 or CXCL10 N loop substitutions ($CXCL11_{12Nloop}$ and $CXCL11_{10Nloop}$), had a reduced potency and efficacy towards both isoforms, $CXCL11_{12Nloop}$ being the most affected (Fig. 5D and Supplementary Table 2).

Taken together, these results demonstrate similar effect of chemokine modifications on receptor interactions for the two isoforms, suggesting a shared recognition and activation modes.

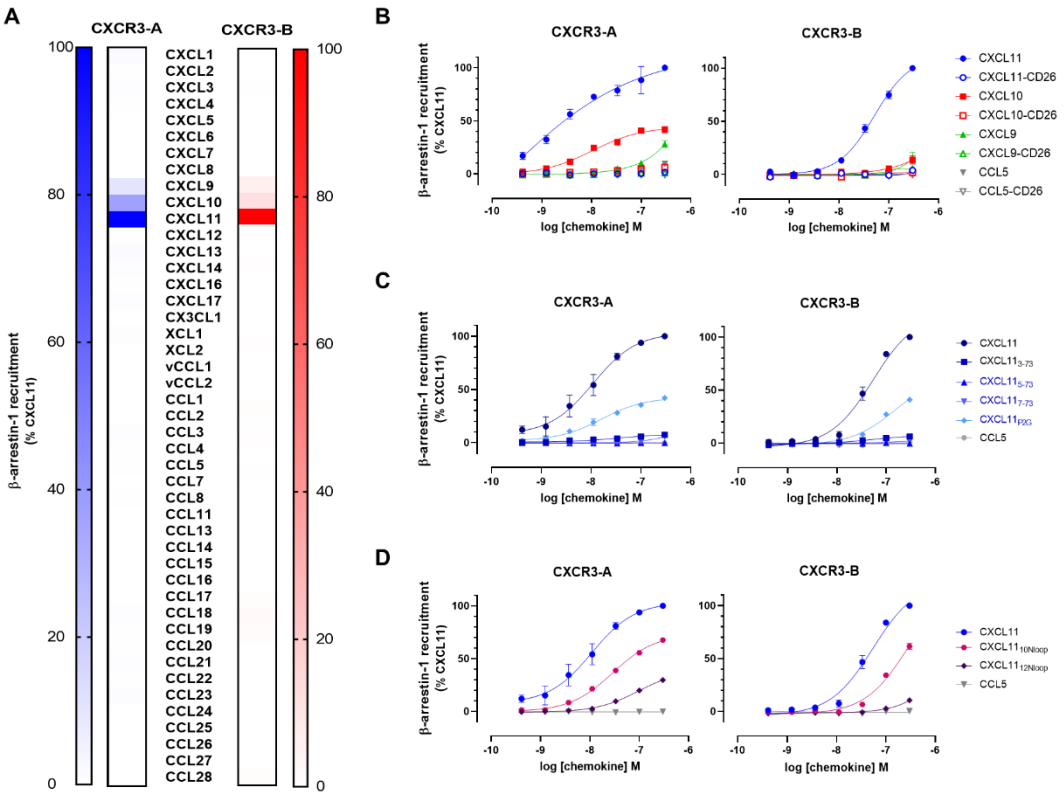


Figure 5. N-terminal extension of CXCR3-B does not change the receptor selectivity and chemokine binding mode. (**A**) β-arrestin-1 recruitment to CXCR3-A (blue color scale) and CXCR3-B (red color scale) in response to all known human and two viral chemokines (100 nM). (**B**) Impact of chemokine N-terminal processing by dipeptidyl peptidase 4 (DPP4/CD26) on the chemokine-induced β-arrestin-1 recruitment to CXCR3 isoforms. (**C**) Impact of N-terminal truncation and P2G-mutation on CXCL11-induced β-arrestin-1 recruitment to CXCR3-A and CXCR3-B. (**D**) Recruitment of β-arrestin-1 to CXCR3-A and CXCR3-B in response to CXCL11 Nloop chimeras. All data were generated using NanoBiT-based assays in HEK293T cells and are expressed as percentage relative to maximum of the full agonist CXCL11 at 100 nM (**A**) or 300 nM (**B**–**D**). Data represent mean ± SEM of three independent experiments.

The N-terminal extension of CXCR3 is responsible for its intracellular localization and associated lack of G protein coupling

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 truncations in the N terminus of CXCR3-B were introduced (Fig. 6A) and the membrane expression as well as the ability of the resulting receptors to interact with miniG proteins were evaluated. The N-terminal truncations resulted in a gradual increase of the receptor presence at the cell surface, the most significant increment being observed upon the removal of approximately half of the extension (CXCR3-B -30), reaching a plateau that was nevertheless lower than for the surface expression of CXCR3-A (Fig. 6B). A matching trend

in G protein coupling of the progressively truncated receptors was also observed (Fig. 6C and Supplementary Table 3).

Finally, we showed that a similar effect on G protein coupling can be achieved by inserting the CXCR3-B extension in another classical chemokine receptor, CXCR4. Indeed, the engraftment of the N-terminal extension of CXCR3-B to the N terminus of CXCR4 (extCXCR4) resulted in a loss of Gi/o protein coupling, while the ability to recruit β-arrestin-1 in response to CXCL12 was conserved (Fig. 6F). The flow cytometry and confocal microscopy analysis of surface expression and cellular distribution also demonstrated that just like for CXCR3 isoform, extCXCR4 resides more intracellularly, in dot like structures, than WT CXCR4 (Fig. 6D and 6E).

These observations demonstrate that the extended CXCR3-B N terminus impacts the classical receptor cellular localization and G protein coupling, while it has a limited effect on the its 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.

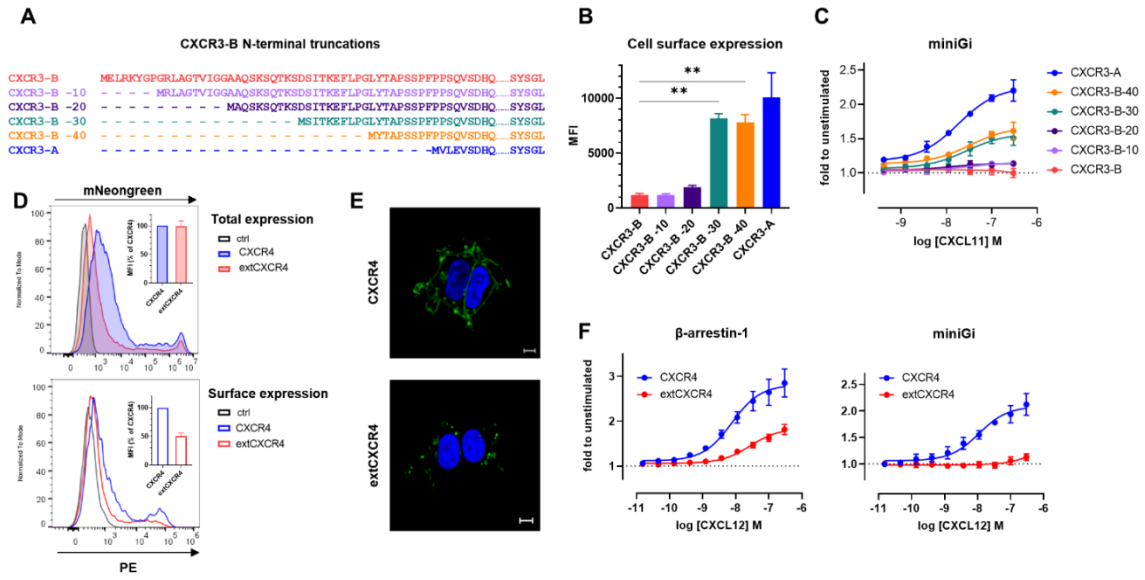


Figure 6. The N-terminal extension of CXCR3-B is responsible for its intracellular localization and lack of G protein coupling. (**A**) Amino acid sequence comparison of CXCR3-B variants with progressive N-terminal truncations (CXCR3-B -10, CXCR3B -20, CXCR3-B -30, CXCR3B -40). (**B**) Cell surface expression of CXCR3-B bearing progressive N-terminal truncations determined by flow cytometry using the CXCR3-specific mAb 1C6. (**C**) CXCL11-induced miniGi protein recruitment to N-terminally truncated CXCR3-B determined by NanoBiT-based assay. (**D**) Receptor expression monitored by flow cytometry in cells transiently transfected with vectors encoding CXCR4 or extCXCR4 C-terminally tagged with mNeonGreen or empty vectors (ctrl). Total expression was evaluated as mNeonGreen (upper panel) and surface expression by CXCR4-specific mAb (clone 12G5). Both are quantified as mean fluorescent intensity (MFI) and expressed as

percentage of CXCR4 (inset). (**E**) CXCR4 and extCXCR4 cellular distribution visualized by confocal fluorescent microscopy in cells transiently expressing mNeonGreen-fused CXCR4 or extCXCR4. HEK293T cells were used as cellular background for all the panels. Pictures are representative of 10 acquired images from two independent experiments. Scale bar: 10 μm. (**F**) CXCL12-induced β-arrestin-1 or miniGi recruitment to CXCR4 and the chimeric extCXCR4 monitored with NanoBiT-based assays. For (**A-D, F**) data represent mean ± SEM of three independent experiments. For (**B**) **p < 0.01 by ordinary one-way ANOVA with Tukey's post hoc analysis.

4. Discussion

CXCR3-B isoform is generated following alternative splicing of the *CXCR3* gene and presents an extended N terminus compared to CXCR3-A, the most studied isoform of the receptor.

CXCR3-B isoform is an elusive and enigmatic chemokine receptor for which divergent functional and signaling results exist [18,20,21,50]. CXCR3-B has been suggested to be biased towards β-arrestins [20,21] but the mechanisms for this bias and the atypical nature of the receptor have not been established. We therefore undertook an in-depth molecular characterization of CXCR3-B to provide signaling and mechanistic insights into its biology and function in comparison to CXCR3-A. Our study validates the β-arrestin bias of CXCR3-B and reveals that it shows several attributes characteristic of the atypical chemokine receptor family. This suggests that CXCR3-B may act as a scavenger for the CXCR3-binding chemokines, 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, most ACKRs recruit β-arrestins to mediate receptor internalization, although recent reports suggest that the presence of β-arrestins is not essential for this function[9-12]. Our study shows 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, CXCR3-B retained its ability to recruit β-arrestins and to mediate chemokine uptake. These observations are in line with other reports showing that the two CXCR3 isoforms differentially affect downstream pathways[20,21,51]

CXCR3-B was proposed to trigger cellular signaling such as ERK phosphorylation[20,21], although, it remains unclear by which effectors this pathway may be activated. Here, we show, using methods that allow to monitor directly protein-protein interactions, that no G

proteins, nor related signaling events, are activated by CXCR3-B, further underscoring its atypical nature.

We also show that the intracellular localization of CXCR3-B may be attributed to its N terminal extension. The N-terminal domain of chemokine receptors is known to play an important role in chemokine binding[46,52,53], and its elongation with various N-terminal tags or reporter proteins has already been shown to impact the receptor biology and pharmacology, requesting in some cases the addition of exogenous proximal signal peptide to ensure a proper export to the plasma membrane. Likewise the extension of CXCR3-B drastically changes the receptor cellular distribution, limiting its presence at the cell surface. Our data also show that under basal conditions, CXCR3-B does not cycle between the plasma membrane and intracellular compartments. In contrast, CXCR3-B intracellular pool is mobilized to the cell surface upon stimulation. Depending on the chemokine, different directionalities of CXCR3-B plasma membrane level modulation were observed, which may result from their respective potency to induce receptor internalization. Indeed, CXCL10 and CXCL9, known to be weak CXCR3-internalizing chemokines, induced a net increase of CXCR3-B at the plasma membrane, whereas the more efficacious receptor-internalizing chemokine, such as CXCL11, led to net reduction of the receptor present at the cell surface[54,55].

Uptake experiments using fluorescently labeled 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 isoforms, 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[11,12].

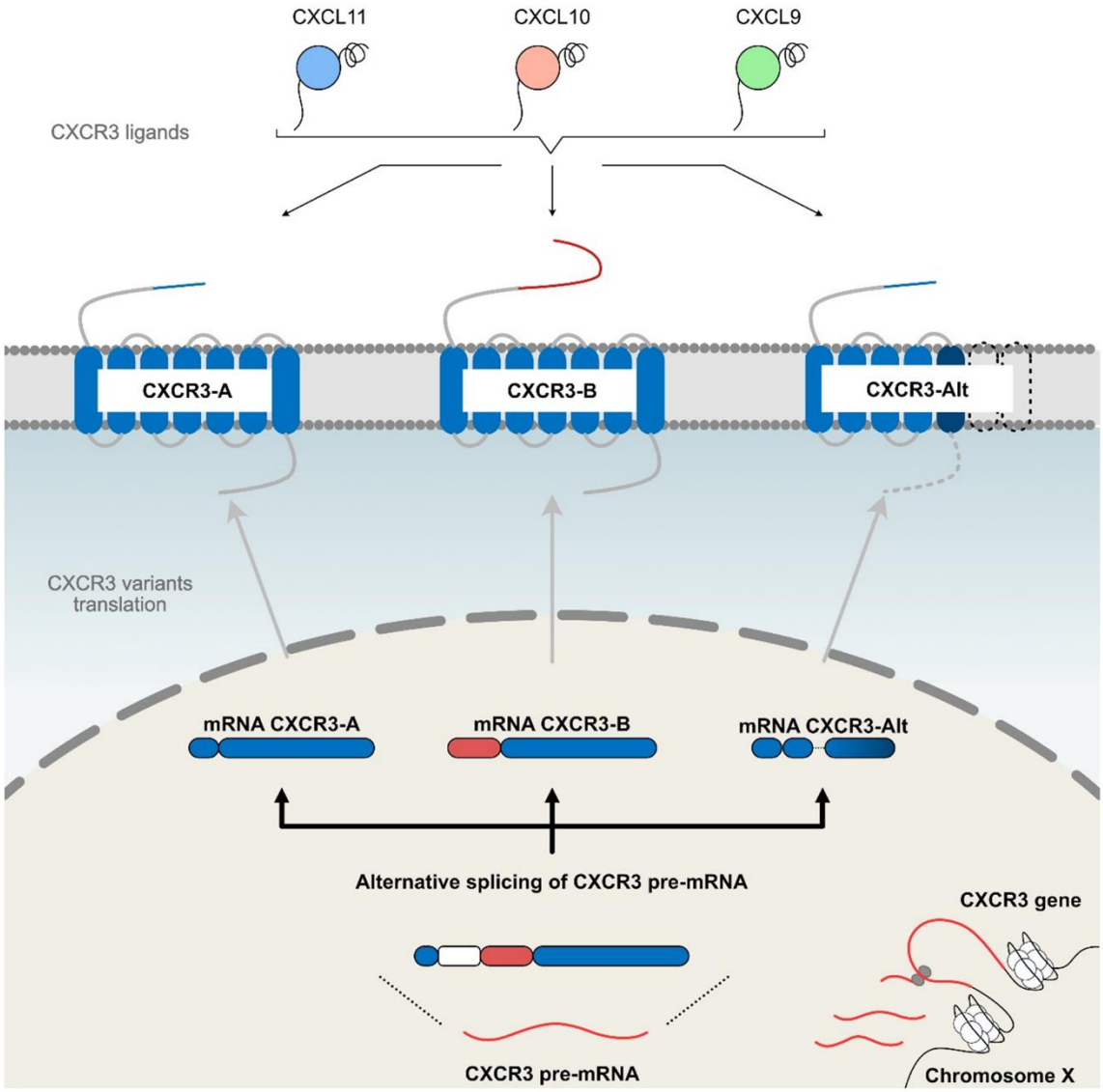
Although biophysical studies have suggested that small molecules can induce different conformational changes in CXCR3-A and CXCR3-B[56], our results generated using chimeric or truncated chemokines indicate that the two CXCR3 isoforms share the same chemokine binding and activation modes, suggesting 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 the N-terminal extension of CXCR3-B partially restored surface expression and the ability of the receptor to couple to G proteins. However, we showed that even when expressed at equivalent levels at the cell surface CXCR3-B still shows a reduced ability to interact with G protein. This suggests that the change in the cellular localization of the receptor and the impact of the N-terminal extension on the activated receptor conformation are the two main drivers of this shift of signaling properties, most probably limiting its ability to efficiently activate G proteins, while preserving its ability to cycle and internalize chemokines, consequently conferring to CXCR3-B attributes of atypical chemokine receptors.

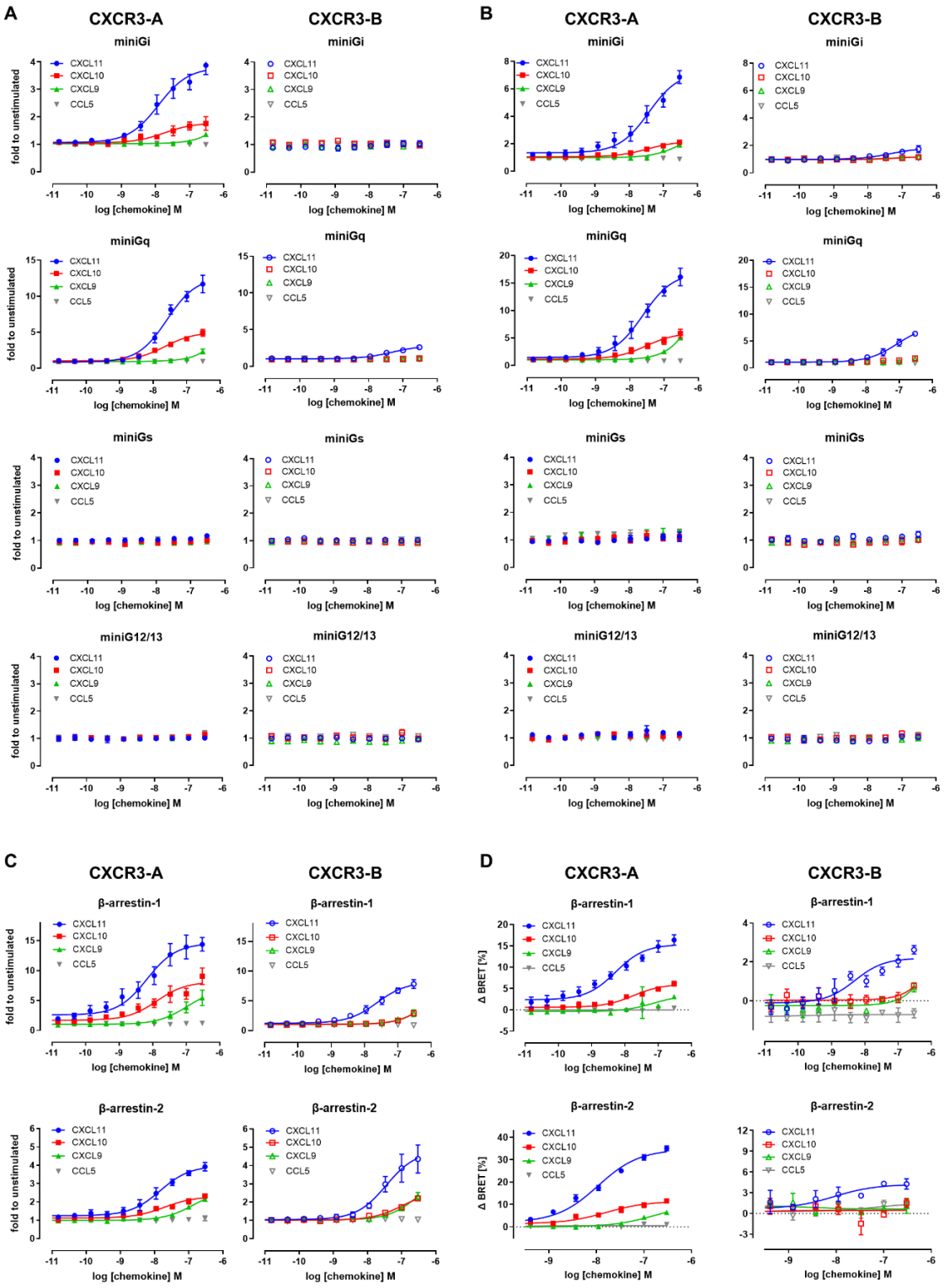
Indeed, the absence of G protein signaling, the predominantly intracellular localization and the ability to actively take up chemokines are three major attributes of atypical chemokine receptors[2]. We therefore propose that CXCR3-B could be regarded as an atypical chemokine receptor that modulates the 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 as signaling and scavenging receptors, respectively. Similar contrasting functions for CXCR3 have been documented in zebrafish where the gene is triplicated. In this organism, Cxcr3.2 and Cxcr3.3 copies, both expressed on macrophages, have been shown to coordinate their migration during bacterial infection by functioning antagonistically. Cxcr3.3 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 receptors share[57]. Of note, other natural human isoforms of chemokine receptors displaying N- or C-terminal extensions and showing altered biology have also been described for CXCR4, CCR9, CCR2, CX3CR1, but also for other GPCR families[58-61].

In conclusion, this study provides signaling and mechanistic insights into the differences of the CXCR3 isoforms that may explain their opposite effects. Our data indicate a strong atypical profile for CXCR3-B with features such as complete absence of G protein coupling, intracellular localization and chemokine uptake capacities, which can be attributed to its N-terminal extension. 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 CXCR3-B.

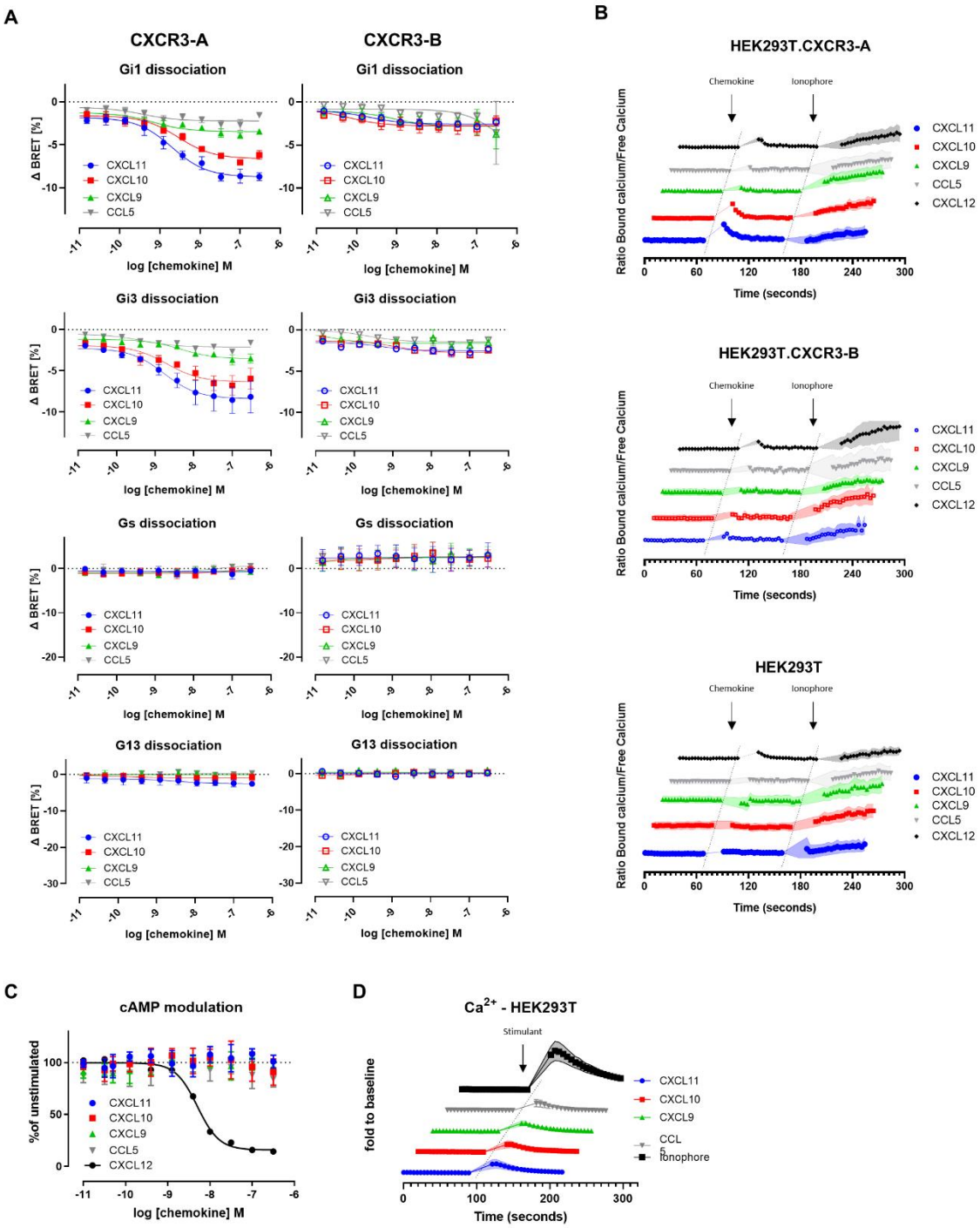
5. Supplementary material



Supplementary figure 1. Overview of the three CXCR3 isoforms. Due to alternative splicing of the pre-mRNA of the CXCR3 gene, located on chromosome X, three CXCR3 isoforms can be generated. The CXCR3-A isoform 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 isoform which has an N terminus longer by 47 amino acids 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 isoform 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 isoforms are able to bind three endogenous ligands CXCL9, CXCL10 and CXCL11, each with a different binding affinity and activation potential.

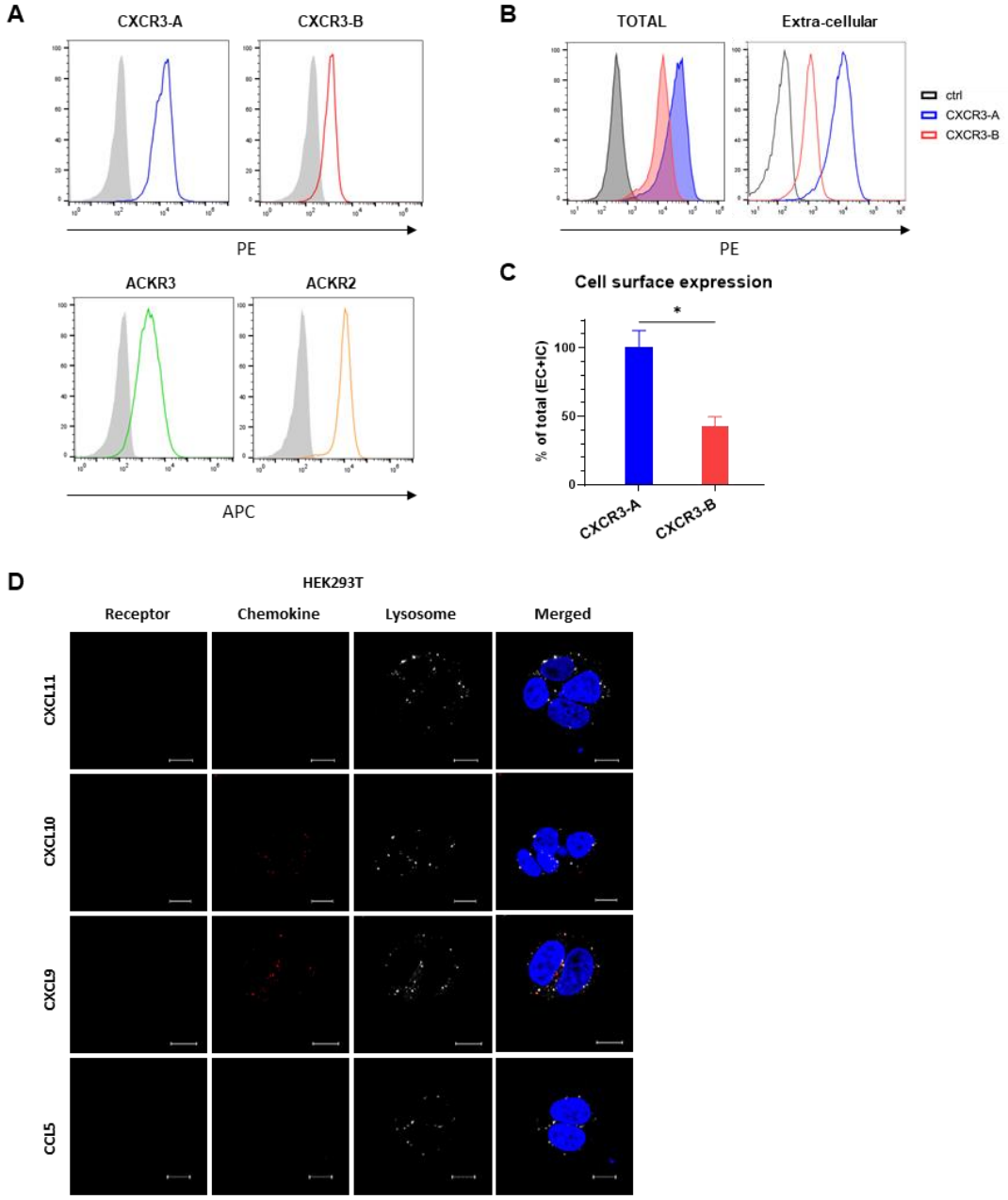


Supplementary figure 2. (**A**–**B**) Chemokine-induced recruitment of miniGi, miniGq, miniGs and miniG12/13 protein to CXCR3-A and CXCR3-B monitored by NanoBiT-based assay in HEK293T (**A**) or U-87 MG cells (**B**). (**C**) Chemokine-induced β-arrestin-1 and β-arrestin-2 recruitment to CXCR3-A and CXCR3-B after chemokine stimulation in U-87 MG cells using nanoluciferase complementation assay. (**D**) Chemokine-induced β-arrestin-1 and β-arrestin-2 recruitment to CXCR3-A or CXCR3-B monitored by NanoBRET assay in HEK293T cells. Data represent mean ± SEM of three independent experiments.



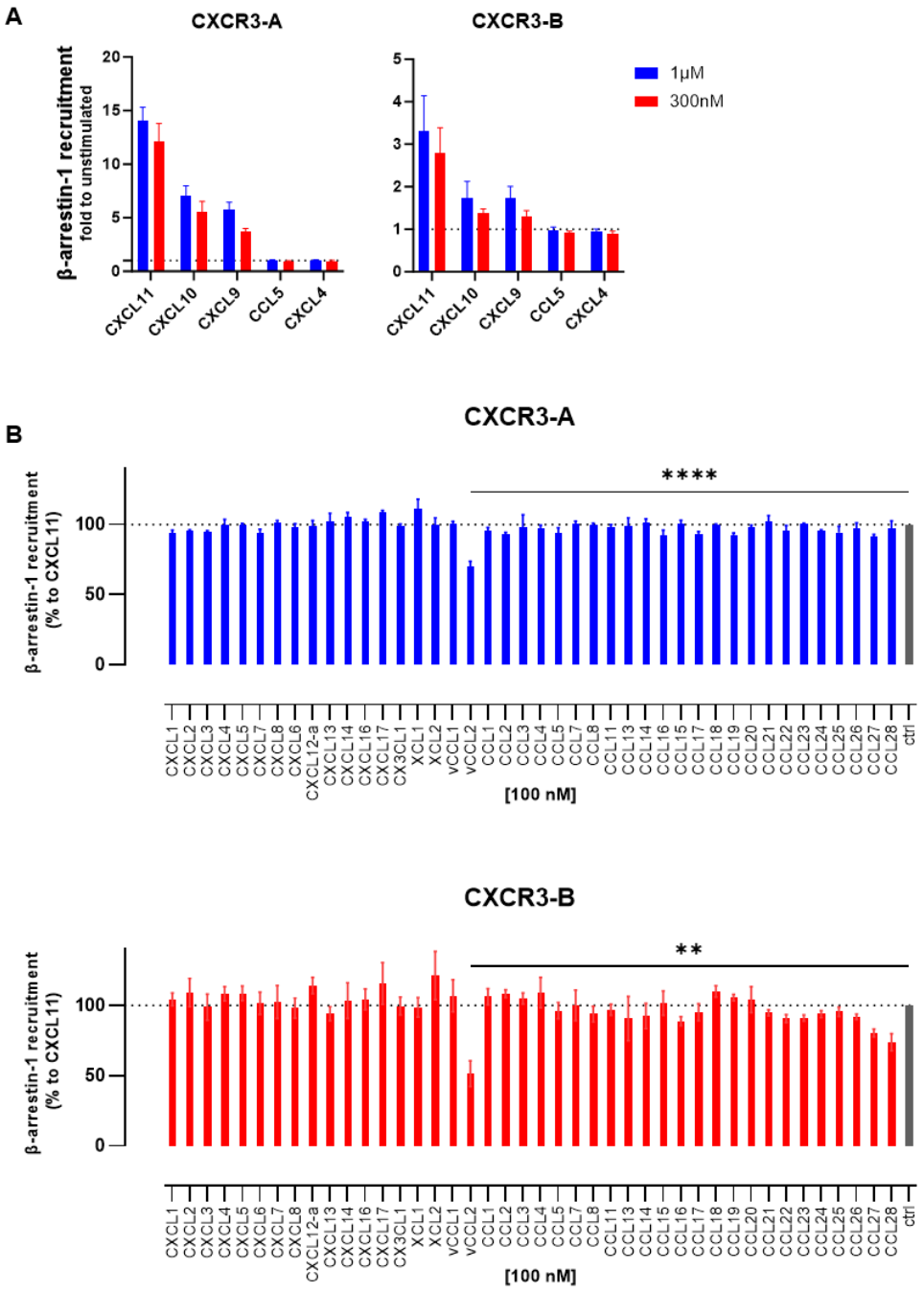
Supplementary figure 3. (**A**) Chemokine-induced heterotrimeric G protein dissociation profiles for Gi1, Gi3, Gs, G13 monitored in CXCR3-A- or CXCR3-B-expressing HEK293T cells by NanoBRET. (**B**) Mobilization of intracellular calcium in HEK293T cells stably expressing CXCR3-A or CXCR3-B or in naïve HEK293T cells in response to CXC or CC chemokines (100 nM) monitored with the ratiometric fluorescent indicator Indo-1 AM. Calcium ionophore A23187 (1 µM) was used as receptor-independent control. (**C–D**) Downstream G protein signaling in naïve HEK293T cells after treatment with CXCL12, CXCL11, CXCL10, CXCL9 or CCL5 by luminescence-based intracellular cAMP modulation (**C**) or NanoBiT-based intracellular calcium

release (**D**). CXCL12 or Ionophore A23187 were used as positive control to confirm assay functionality. For all panels, data represent mean ± SEM of three independent experiments.



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, ACKR3 or ACKR2, was evaluated with receptor-specific mAb (clones 1C6, 8F11-M16 and 196124, respectively) and the related isotype controls. **(B)** Flow cytometry comparison of total or extracellular expression of CXCR3-A and CXCR3-B HEK293T-derived stable cell lines evaluated with mAb 1C6 and related isotype control. (**C**) Cell surface expression of CXCR3-A and CXCR3-B quantified by HiBiT-mediated nanoluciferase complementation and expressed as percentage of total receptor expression (extracellular and intracellular). *$p < 0.05$ by Mann-Whitney two-tailed unpaired test. **D**) Confocal microscopy images of naïve HEK293T cells after 2-hour incubation with CXCR3-related chemokines or CCL5 (100 nM). Alexa Fluor 647-labeled chemokines are represented in red, lysosomes stained with LysoTracker™ Red DND-99, in white and nuclei stained with Hoechst

33342, in blue. Scale bar: 10 µm. Pictures are representative of 12 acquired images from three independent experiments.



Supplementary figure 5. (**A**) β-arrestin-1 recruitment towards CXCR3-A and CXCR3-B in response to 1 µM or 300 nM of CXCL11, CXCL10, CXCL9, CCL5 and CXCL4 monitored by NanoBiT-based assay in HEK293T cells. (**B**) Antagonistic activity of all non-agonist chemokines (100 nM) towards CXCR3-A or CXCR3-B evaluated following addition of CXCL11 (20 nM) by NanoBiT-based β-arrestin-1 recruitment assay in HEK293T cells. **p < 0.01, ****p < 0.0001 by ordinary one-way ANOVA with Bonferroni multiple comparison test was used. For all panels, data represent mean ± SEM of three independent experiments.

Table 1 - CXCR3 variants activation by CXCL11, CXCL10 and CXCL9

	CXCR3-A			CXCR3-B		
	CXCL11	**CXCL10**	**CXCL9**	**CXCL11**	**CXCL10**	**CXCL9**
miniGi	12.4 (7.2 - 21.6)	18.2 (3.9 - 74.0)	ND	NA	NA	NA
miniGq	25.9 (18.6 - 36.2)	22.2 (15.0 - 32.9)	ND	ND	NA	NA
β-arrestin-1	15.8 (10.6 - 23.5)	17.8 (12.9 - 24.8)	ND	49.4 (36.9 - 66.6)	ND	ND
β-arrestin-2	2.9 (1.8 - 4.8)	2.9 (0.8 - 10.2)	~ $1.6x10^4$	12.4 (7.3 - 21.2)	59.2 (23.5 - 159.7)	~ $1x10^5$
Gi2 dissociation	1.2 (0.4 - 3.9)	0.9 (0.3 - 2.7)	19.9 (5.9 - 54.4)	NA	NA	NA
Go1 dissociation	1.8 (0.8 - 4.2)	1.9 (1.1 - 3.1)	77.8 (34.1 - 201.5)	ND	NA	NA
Gq dissociation	0.4 (0.03 - 3.1)	2.3 (0.7 - 7.1)	~ 56	NA	NA	NA
cAMP	6. 3 (5.0 - 8.0)	14.1 (10.5 - 19.0)	21.9 (12.6 - 38.0)	NA	NA	NA

EC50 values are indicated in nanomolar (nM) with 95% confidence interval (CI).
ND: Not determinable since saturation was not reached.
NA: No activity or activity below 10% of positive control in the concentration range tested

Table 2 - N-terminally modified chemokines on CXCR3-A and CXCR3-B

	CXCR3-A		CXCR3-B	
Chemokines	**EC_{50} nM**	**Emax %**	**EC_{50} nM**	**Emax %**
CXCL11	9.6 (5.6 - 16.3)	100	51.6 (40.7 - 65.3)	100.0
CXCL11-CD26	NA	6.2	NA	3.9
$CXCL11_{3-73}$	NA	7.5	NA	6.3
$CXCL11_{5-73}$	NA	0	NA	0.0
$CXCL11_{7-73}$	NA	5.8	NA	1.5
$CXCL11_{P2G}$	14.8 (9.8 - 22.5)	42.1	152.7 (107.8 - 226.4)	40.9
$CXCL11_{10Nloop}$	28.8 (22.8 - 36.4)	67.8	269.7 (168.2 - 504.6)	61.6
$CXCL11_{12Nloop}$	99.2 (67.7 - 150.4)	29.8	ND	10.6
CXCL10	10.7 (6.8 - 16.5)	41.7	ND	13.7
CXCL10-CD26	NA	1.2	NA	1.8
CXCL9	ND	28.1	ND	15.3
CXCL9-CD26	NA	9.5	NA	5.2

EC50 values are indicated in nanomolar (nM) with 95% confidence interval (CI).
Emax %: maximum signal measured at 300 nM expressed as % of the full agonist CXCL11.
ND: Not determinable since saturation was not reached.
NA: No activity or activity below 10% of positive control in the concentration range tested.

Table 3 - CXCR3-B N-terminal extension

miniGi	EC_{50} nM	Emax %
CXCR3-A	18.2 (9.7 – 34.4)	100.0
CXCR3-B	NA	0.0
CXCR3-B -40	32.7 (11.4 - 97.2)	51.0
CXCR3-B -30	27.2 (6.5 - 128.0)	42.4
CXCR3-B -20	ND	11.6
CXCR3-B -10	ND	10.8
CXCR4	10.3 (4.6 - 23.2)	100.0
CXCR4-B	ND	11.6
β-arrestin-1	**EC_{50} nM**	**Emax %**
CXCR3-A	6.7 (2.9 – 14.8)	100.0
CXCR3-B	23.9 (14.4 – 39.6)	75.9
CXCR4	7.1 (3.8 - 13.3)	100.0
CXCR4-B	24.0 (12.6 - 46.1)	44.0

EC50 values are indicated in nanomolar (nM) with 95% confidence interval (CI).
Emax %: maximum signal measured at 300 nM expressed as % of the full agonist CXCL11.
ND: Not determinable since saturation was not reached.
NA: No activity or activity below 10% of positive control in the concentration range tested
CXCR3 artificial and natural isoforms were stimulated with CXCL11
CXCR4 artificial and natural isoforms were stimulated with CXCL12

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution statement

GDU, NR, JH, MS and AC designed the study and wrote the manuscript. GDU, NR, MM and DA performed the experiments. GDU, NR, MS and AC analyzed and interpreted the data. GDU, NR, MM, TU and MS generated molecular tools for cellular assays. BV contributed modified CXCL11 chemokines. TL, BJ, JH, MS and AC supervised the overall study. All authors approve the manuscript.

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Chapter III:

ACKR2 is a scavenger for CXCL10

My contribution to this Chapter:

I contributed with the confocal experiment to strengthen the chemokine internalization data. In particular, I took care of experimental design, sample preparation and optimization, image acquisition, analysis and interpretation (Figure 2, panel E). These data were important to confirm ACKR2-mediated uptake of CXCL10 in a visual manner. It is also possible to appreciate the co-localization of ACKR2 ligands with intracellular vesicles. I contributed to the writing of the manuscript by describing the experimental procedure related to Figure 2, panel E.

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Highlights & aims

In this original research article, we identify and characterize the inflammatory chemokine CXCL10 as a new strong agonist ligand for ACKR2. This atypical chemokine receptor has long been regarded as a scavenger of inflammatory chemokines exclusively from the CC family. With the use of highly sensitive assays based on split Nanoluciferase complementation (NanoBiT) and Nanoluciferase bioluminescence resonance energy transfer (NanoBRET) (methodologies detailed in chapter I.i) the first ligand belonging to the CXC chemokine family was shown to bind to ACKR2 and subsequently induce β-arrestin recruitment and be internalized. In contrast to CC chemokines, CXCL10 activity towards ACKR2 is drastically reduced by the CD26-mediated N-terminal processing.

To date, CXCL10, has been described to only interact with CXCR3. This study constitutes the first report highlighting the presence of a scavenger for this inflammatory chemokine. In this thesis, other receptors, notably CXCR3-B (Chapter II) and GPR182 (Chapter IV) are proposed as receptors able to internalize this chemokine without induction of the classical G protein signaling, therefore expanding the current understanding of the already intricate chemokine network.

CXCL10 is an agonist of the CC family chemokine scavenger receptor ACKR2/D6

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Simple Summary

The atypical chemokine receptor ACKR2 plays an important role in the tumour microenvirnoment. 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 on the complexity of the chemokine network and the potential role of CXCL10 regulation by ACKR2 in tumour immunology.

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 β-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.

1. 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 β-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 β-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.

2. Material and Methods

Cells and proteins

HEK-ACKR2 cell line stably expressing human or mouse ACKR2 were established by transfection of HEK293T cells (ATCC) with pIRES-puro vector (Addgene) 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) or polyclonal mACKR2-specific antibody (ab1656, Abcam). The absence of CXCR3 at the cell surface was confirmed using mAb clone 1C6 and the corresponding isotype control (Biologend). 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). Alexa Fluor 647-labelled CCL2 (CCL2-AF647) was purchased from Almac.

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 pH7.5 + 1 mM EDTA for 1 hour at 37 °C in the presence or absence of the sitagliptin (10 µM) (Sigma

Aldrich). The efficiency of processing was verified by MALDI-TOF analysis using a RapifleX, Bruker Daltonics instrument in positive ion mode and in reflectron mode.

Chemokine-induced β-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 minutes 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). Each point corresponds to average values acquired for 20 minutes, 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 β-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.

Chemokine binding

HEK293T and HEK-ACKR2 cells were incubated with CXCL10-Cy5 at indicated concentrations for 45 minutes at 37 °C, then washed twice with FACS buffer (PBS, 1 % BSA, 0.1 % NaN_3). 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).

Chemokine-induced receptor mobilisation to the plasma membrane

Ligand-induced receptor mobilisation to the plasma membrane was monitored by NanoBRET. 5×10^6 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 (1×10^5 cells per well) and treated with chemokines (100 nM). After 45-minute 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).

Chemokine-induced receptor-arrestin delivery to endosomes

Ligand-induced receptor-arrestin delivery to early endosomes was monitored by NanoBRET. In brief, 5×10^6 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 (1×10^5 cells per well) and treated with full-length or processed chemokines. After 2-hour 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).

Chemokine scavenging

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

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 minutes 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 minutes at 37 °C. Cells were washed twice with FACS buffer. Dead cells were excluded using Zombie Green viability dye (BioLegend). Images of 1×10^4 in-focus living single cells were acquired with an ImageStream MKII imaging flow cytometer (Amnis) 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, 4×10^4 HEK-ACKR2 cells/well were seeded on poly-L-lysine coated 8-well chamber slides (µ-Slide 8 well, Ibidi). After 36 hours, cells were incubated 2 hours 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). Cells were then washed twice with PBS, fixed with 3.5 % (w/v) paraformaldehyde for 20 minutes at room temperature and washed again twice with PBS. Nuclear staining was performed with Hoechst 33342 dye (1 µg/mL) for 20 minutes at room temperature and cells were washed 3 times with PBS. Images were acquired on a Zeiss LSM880 confocal microscope using a 63x oil-immersion objective and Zen Black 2.3 SP1 software (Zeiss). Representative cells from 12 image acquisitions of three independent experiments are shown.

Inhibition of chemokine uptake by anti-mACKR2 antibodies

HEK-mACKR2 or B16-F10 cells were incubated 45 minutes at 37 °C with Cy5-labeled 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). 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.

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 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. 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: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p<0.0001$.

3. Results

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 (Fig. 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] (Fig 1B, E, H and I). Given this limited activity, they were not further investigated. CXCL10 however, showed a strong potency towards ACKR2 (EC_{50} = 8.2 nM, pEC_{50} = 8.08 ± 0.14) and induced approximately half of the maximal response compared to the full agonist CCL5 (Fig. 1B). This partial agonist behaviour of CXCL10 was reminiscent of the activity towards its long-established signalling receptor CXCR3 relative to the full agonist CXCL11 (Fig. 1C, F and G)[57,58]. The potency of CXCL10 towards ACKR2 appears approximately 3 times stronger than towards CXCR3 (EC_{50}= 24.9 nM, pEC_{50} = 7.60 ± 0.12), consistent with a potential scavenging role of ACKR2. In NanoBRET, the potency of CXCL10 towards ACKR2 (EC_{50} = 5.1 nM, pEC_{50} = 8.29 ± 0.11) was close to that of CCL2 and approximately 20 fold stronger than towards CXCR3. The efficacy of CXCL10 in this assay reached approximately 70% of the maximal signal measured with CCL5 (Fig. 1E and F). Similar observations were made for the recruitment

of β-arrestin-2 (Fig. 1H) and were further confirmed in a different cellular background (Fig. 1I). . Moreover, the screening of CXCL10 on 23 chemokine receptors showed that CXCR3 and ACKR2 are the only human receptors activated by CXCL10 (Fig. 1D). Fluorescently labelled CXCL10 also strongly and specifically bound to HEK293T cells expressing ACKR2 (IC_{50} = 5.4, pIC_{50}= 8.27 ± 0.09) (Fig. 1J and K) and was only displaced by ACKR2-related chemokines CCL5, CCL2 and by CXCL10 itself (Fig. 1K inset). Inversely, binding competition studies showed that CXCL10 was able to fully displace fluorescently labelled CCL2 from the receptor with an IC_{50} of 2.1 nM (pIC_{50} = 8.68 ± 0.03) (Fig. 1L).

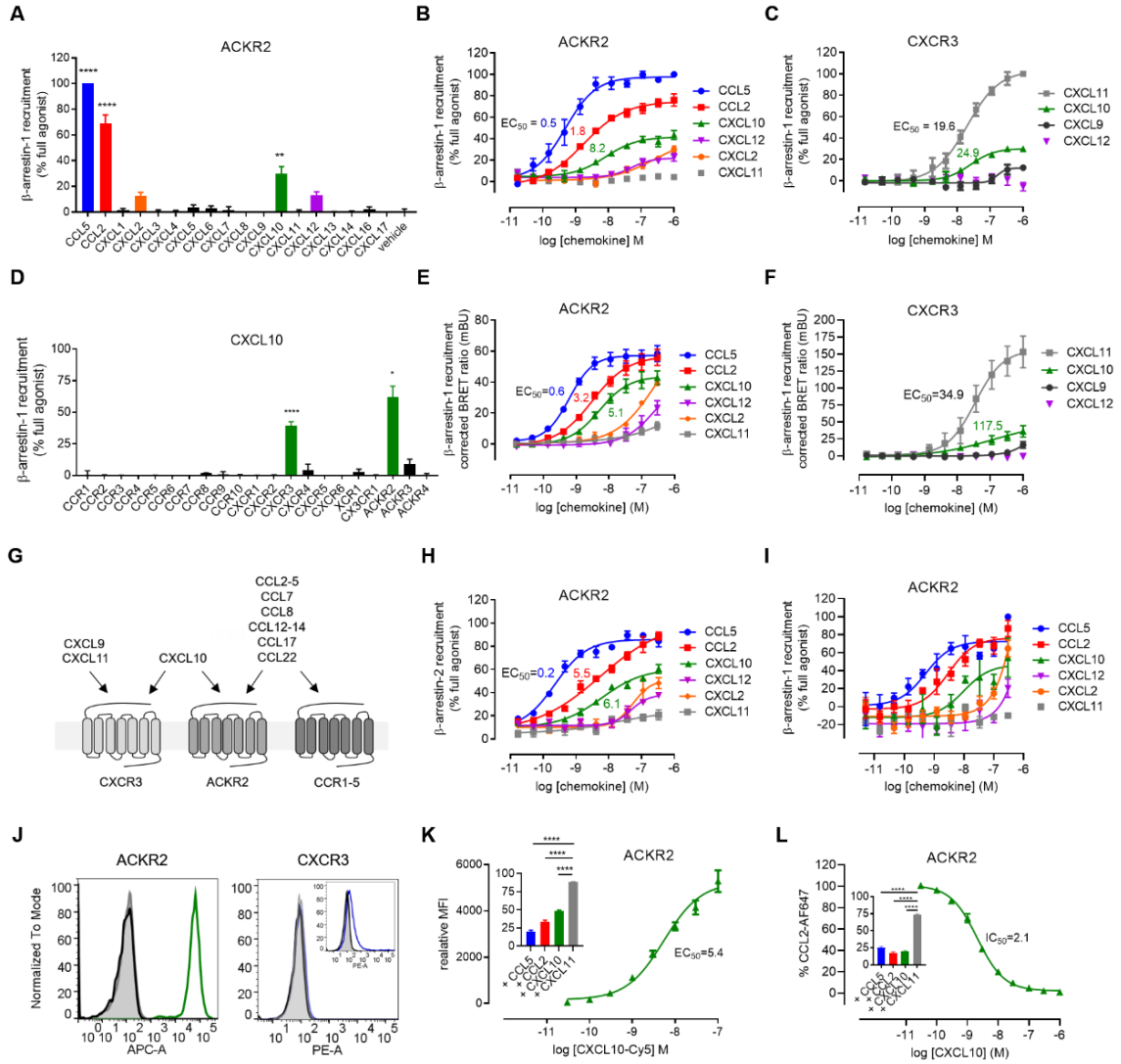

Figure 1. ACKR2 activation by CXCL10. (**A**) β-arrestin-1 recruitment to ACKR2 in response to all known human CXC chemokines (100 nM) monitored by NanoBiT-based assay. CCL2 and CCL5 were used as positive control chemokines. (**B)** β-arrestin-1 recruitment to ACKR2 by the CXC chemokines CXCL2, CXCL10 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 (grey filled 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). EC_{50} and IC_{50} 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 < 0.001, ****p<0.0001 by one-way ANOVA with Dunnett (A and D) and Bonferroni (K and L) post hoc test.

The ability of ACKR2 to mediate CXCL10 scavenging and control its extracellular concentration was then analysed. CXCL10 stimulation resulted in rapid mobilisation of intracellular ACKR2 to the plasma membrane reminiscent of the activity of CC chemokines [59,60] (Fig. 2A). The CXCL10-induced receptor mobilisation was followed by its delivery to the endosomes with an EC_{50} of 6.0 nM (pEC_{50} = 8.22 ± 0.06) (Fig. 2B and C). Imaging flow cytometry also revealed specific and efficient uptake of labelled CXCL10 by ACKR2-expressing cells. A notably higher number of distinguishable intracellular vesicle-like structures and mean fluorescent intensity were observed compared to HEK293T cells or HEK-ACKR2 cells pre-treated with CCL5 (Fig. 2D and 2F). Confocal microscopy further confirmed CXCL10 uptake and in addition showed its distribution within acidic intracellular vesicles (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 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 (Fig. 2K-L) and subsequent receptor targeting to the early endosomes (Fig. 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, similarly 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 behaviour 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].

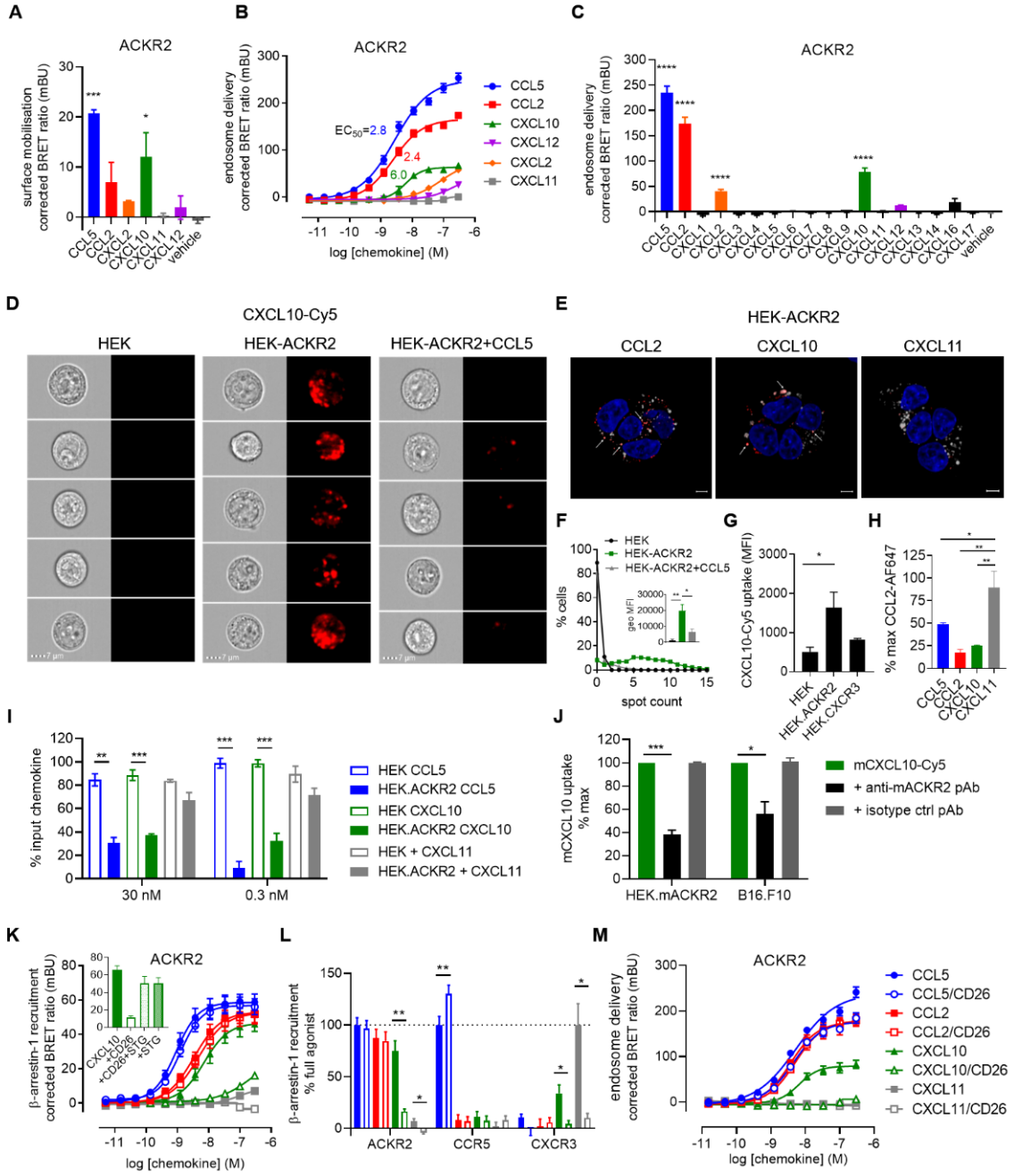

Figure 2. CXCL10 scavenging by ACKR2. (**A**) ACKR2 mobilisation to the plasma membrane in response to chemokines (100 nM) monitored by NanoBRET-based assay (**B-C**) β-arrestin-1/ACKR2 complex delivery to the early endosomes in response to the CXC chemokines CXCL2, CXCL10 and CXCL12 (B) or the 16 human CXC chemokines (100 nM) (C) monitored by NanoBRET-based assay. CCL2 and CCL5 were used as positive control chemokines. (**D-F**) Uptake of fluorescently labelled CXCL10 by ACKR2-expressing cells visualized by imaging flow cytometry (D and 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 minutes 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 hours 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 µm. 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-hour 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 minutes 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 µM) 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. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with Dunnet (A and C) and Bonferroni (H) post hoc test or repeated measures one-way ANOVA with Bonferroni post hoc test (J) and two-tailed unpaired Student's t-test (I).

4. 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 to re-examine previous studies taking into account CXCL10-ACKR2 interactions (Fig. 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.

Conflict of Interest

A patent application has been filed on "Specific ACKR2 modulators for use in therapy" (Applicant: Luxembourg Institute of Health).

Authors contribution statement

AC and MS designed the study. AC, BJ, MZN and MS supervised the study. AC, BJ, MM, NR, GD'U, TU, MX, Y-JK, MO, GB, MZN and MS performed the experiments, analyzed and interpreted the data. AC and MS wrote the manuscript. AC, BJ, MM, NR, MZN and MS reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Chapter IV:

New pairings among ACKRs

My contribution to this Chapter:

This minireview represents an important section of my thesis. It emphasizes the importance of better characterizing the crosstalk between the chemokines and their receptors and in particular summarizes and highlights novel findings on the interaction network within the atypical family of receptors. This publication contextualizes some results I contributed to during my PhD studies (Chapter III) and during my previous research activity on ACKR4 in Bellinzona at the Institute for Research in Biomedicine (IRB).

I focused on the section titled "*Deorphanization of GPR182/ACKR5 as a promiscuous scavenger receptor for both CC and CXC chemokines"*, performed literature search to detail the current knowledge about this recently deorphanized receptor. I took care of the writing of this section and contributed to general writing of the initial draft of the manuscript and reviewing. I also contributed to the generation of Figure 2.

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Highlights & aims

ACKRs play a crucial regulatory role in chemokine biology by capturing, scavenging, transporting or presenting chemokines, thereby regulating their availability and signaling through classical chemokine receptors. Targeted approaches and screening programs started reassessing chemokine activity towards ACKRs and identified new pairings for the four established atypical chemokine receptors (ACKR1, ACKR2, ACKR3 and ACKR4). Moreover, GPR182 (ACKR5) has been recently suggested as additional atypical chemokine receptor able to scavenge several chemokines, including CXCL9, CXCL10, CXCL12 and CXCL13. These findings reveal additional complexity of the chemokine–chemokine receptor network and expand the set of ligands regulated by atypical chemokine receptors.

New pairings and deorphanization among the atypical chemokine receptor family — physiological and clinical relevance

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Abstract

Atypical chemokine receptors (ACKRs) form a small subfamily of receptors (ACKR1–4) unable to trigger G protein-dependent signaling in response to their ligands. They do, however, play a crucial regulatory role in chemokine biology by capturing, scavenging or transporting chemokines, thereby regulating their availability and signaling through classical chemokine receptors. ACKRs add thus another layer of complexity to the intricate chemokine–receptor interaction network. Recently, targeted approaches and screening programs aiming at reassessing chemokine activity towards ACKRs identified several new pairings such as the dimeric CXCL12 with ACKR1, CXCL2, CXCL10 and CCL26 with ACKR2, the viral broad-spectrum chemokine vCCL2/vMIP-II, a range of opioid peptides and PAMP-12 with ACKR3 as well as CCL20 and CCL22 with ACKR4. Moreover, GPR182 (ACKR5) has been lately proposed as a new promiscuous atypical chemokine receptor with scavenging activity notably towards CXCL9, CXCL10, CXCL12 and CXCL13. Altogether, these findings reveal new degrees of complexity of the chemokine network and expand the panel of ACKR ligands and regulatory functions. In this minireview, we present and discuss these new pairings, their physiological and clinical relevance as well as the opportunities they open for targeting ACKRs in innovative therapeutic strategies.

1. Introduction

Chemokines (or chemotactic cytokines) are small soluble proteins (8–14 kDa) that guide cell migration and orchestrate several vital processes, including leukocyte recruitment during immunosurveillance. They are also involved in numerous inflammatory diseases and the development and spread of many cancers[1]. They act through classical chemokine receptors (CKRs) that belong to the seven-transmembrane domain G protein-coupled receptor (GPCR) superfamily. Functionally, chemokines can be categorized as homeostatic or inflammatory according to their properties. Structurally, based on specific cysteine motifs in their N termini they are classified as CC, CXC, XC and CX3C chemokines and their receptors are consequently named CCR, CXCR, XCR and CX3CR[2].

Over the past years, an important subfamily of chemokine receptors has emerged as key regulators of chemokine functions. Formerly named chemokine-binding proteins, decoys, scavengers or interceptors, the standard nomenclature for this membrane protein family is now atypical chemokine receptors (ACKRs)[3,4] (Figure 1). ACKRs are generally expressed

on lymphatic and vascular endothelium, the epithelium of barrier organs and to a lesser extent on circulating leukocytes, in contrast to the classical chemokine receptors that are mainly found on hematopoietic and immune cells[5,6]. Although ACKRs form a rather diverse group and do not cluster phylogenetically, they do share several characteristics. Among their main common features is the inability to trigger the canonical G protein-mediated signaling or to directly induce cell migration in response to chemokines. Despite this atypicality, ACKRs fulfill essential regulatory functions in the chemokine–receptor network. Their well-established role is the tight regulation of chemokine concentration, for instance in inflammatory processes, and the formation of chemokine gradients for the signaling chemokine receptors, which is accomplished by the capture, transport or internalization of chemokines into degradative compartments or their presentation on cells[4,7,8]. Other distinctive properties of ACKRs are their unconventional cellular localization, trafficking and expression profile. Indeed, most ACKRs are predominantly found in endosomal vesicles and several can cycle constitutively between the plasma membrane and the intracellular compartments, efficiently scavenging the bound chemokines[3,7,9-11]. Although these functions were previously considered to mainly rely on β-arrestins, recent reports showed that they are not indispensable[12-17]. Dimerization with canonical receptors and consequent alteration of expression and signaling properties is another characteristic of ACKRs that allows modulation of the chemokine network[8,18,19].

To date, out of the 23 chemokine receptors recognized by International Union of Basic and Clinical Pharmacology (IUPHAR), four are members of the ACKR family (ACKR1–4)[20]. This group of atypical receptors will presumably increase in the near future, both in terms of number and relevance. Indeed, for each of the ACKRs, recent pairings with chemokines or, as in the case of ACKR3 non-chemokine ligands, have been reported, and it is to expect that new members, such as the recently deorphanized promiscuous chemokine scavenger GPR182 (ACKR5), will further enlarge this family.

In this minireview, we present and discuss these new pairings, their physiological and clinical relevance but also the growing number of properties that unify this somewhat heterogeneous receptor subfamily.

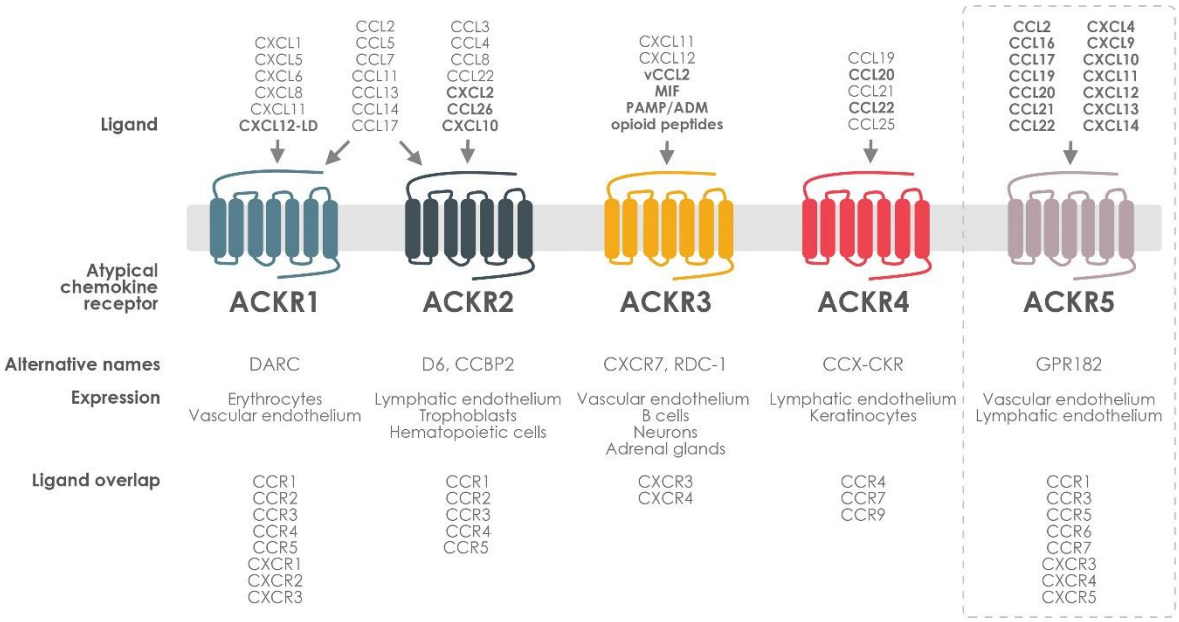


Figure 1. ACKR expression, ligand selectivity and crosstalk with classical chemokine receptors. Atypical chemokine receptors are expressed on different types of endothelial or immune cells. ACKR1 and ACKR2 bind a broad spectrum of inflammatory chemokines that they share with CXCR1-3 and CCR1-5. ACKR3 binds the homeostatic chemokine CXCL12, which it shares with CXCR4, and the inflammatory CXCL11, shared with CXCR3. ACKR3 also binds MIF and small non-chemokine peptides such as the proadrenomedullin-derived peptides, ADM and PAMP as well as several opioid peptides. ACKR4 interacts with a limited number of mainly homeostatic chemokines that it shares with CCR4, CCR7 and CCR9. ACKR5 binds a wide range of both CC and CXC chemokines shared with CCR1, CCR3, CCR5-7 and CXCR3-5 and is still awaiting official IUPHAR recognition as an atypical chemokine receptor (dashed rectangle). Newly identified pairings are indicated in bold. CXCL12-LD: CXCL12 locked dimer.

Pairing of dimeric CXCL12 with ACKR1

ACKR1 (formerly DARC for Duffy Antigen Receptor for Chemokines) is the oldest known chemokine receptor. It is barely recognizable as one from its primary amino acid sequence and its phylogenetic association[21,22] and was initially described as blood group antigen and as a receptor for the Duffy Binding Proteins (DBP) from *Plasmodium knowlesi* and *Plasmodium vivax* malaria parasites[23-25]. ACKR1 is prominently expressed on erythrocytes and venular endothelial cells, but not on capillaries or arteries[26-28]. ACKR1 owes its distinctive regulatory function to its ability to internalize chemokines in polarized cells, mediating their transcytosis and increasing their bioavailability by presenting bound chemokines to other chemokine receptors in a spatiotemporally well-defined manner[29]. Although ACKR1 is unable to promote the degradation of its ligands, it can compete with classical receptors for chemokine binding or reduce their availability in defined regions via internalization. By this mechanism, ACKR1 was proposed to play a role in impairing chemokine-induced angiogenesis[30,31]. On erythrocytes, ACKR1 binds circulating inflammatory chemokines with high affinity and can act as a "sink" or as a "buffer". Indeed,

a number of studies showed that ACKR1 modulates inflammatory responses by depleting its ligands[32,33].

ACKR1 is the most promiscuous chemokine receptor with over ten chemokine ligands from the CC and CXC chemokine families[34-36]. Studies carried out in the 1990s identified several chemokine ligands for ACKR1, which included CXCL1, CXCL4, CXCL7, CXCL8, CCL5, and CCL2[34,37]. Since then, many more have been discovered with a broad range of affinities. Among the additional chemokines, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL11, CCL7, CCL11, CCL13, CCL14 and CCL17 exhibit strong binding to ACKR1 [36]. Most of ACKR1 ligands are classified as inflammatory chemokines, with the receptor exhibiting no preference for either CC or CXC chemokines[36]. In contrast, the majority of homeostatic and angiostatic ELR-chemokines show weak or no binding [36,38,39].

Recently, using biophysical analysis and immunofluorescence microscopy, ACKR1 was shown to bind with the dimeric form of CXCL12[40]. CXCL12 plays an important part in tissue development, vascular integrity, hematopoiesis, and immunity. Its effects through the interaction with the classical receptor CXCR4 and the atypical receptor ACKR3 have been studied extensively[41-43]. It has now been suggested that ACKR1 promotes CXCL12 dimerization, which could potentially interfere with its monomeric signaling[44]. The interaction between the CXCL12 dimer and ACKR1 suggests a potential new function for ACKR1 to modify the chemokine's monomer–dimer equilibrium, further deepening the complexity of the functional regulation of CXCL12[40].

Pairing of CXC and CC chemokines with the promiscuous CC chemokine scavenger ACKR2

ACKR2 (formerly D6 or CCBP2), identified in 1997, was until recently reported to exclusively bind inflammatory CC chemokines[45]. The main ACKR2 ligands include CCL2-8, CCL11-14, CCL17 and CCL22, which are shared with the classical inflammatory receptors CCR1-5[46-49]. By scavenging these chemokines, ACKR2 is proposed to drive the resolution phase of inflammation and prevent exacerbated immune responses[50-55].

The pairing of ACKR2 with CC chemokines dates from when many chemokines, especially from the CXC class, were not yet known or readily available[45,46,49]. A recent effort to systematically evaluate the activity of a full array of human and viral chemokines on ACKR2, by examining their ability to induce β-arrestin recruitment, revealed at least one

more CC, CCL26, and two CXC chemokines, namely CXCL2 and CXCL10 as ligands of ACKR2[56] with different potencies and efficacies (Supplementary Figure 1).

CCL26 was identified as a low-potency partial agonist of ACKR2, able to compete with other partial agonists for the binding and uptake by the receptor. CCL26 was previously demonstrated to bind and activate CCR3, although it has also been proposed as a ligand of CX3CR1[57,58]. Though the functional relevance of the interaction between ACKR2 and CCL26 remains largely unknown, this chemokine–receptor pair may play a major role in a range of immune-mediated diseases. For instance, in persistent asthma, CCL26 was shown as the most effective inducer of eosinophil migration[59], while ACKR2, which is constitutively expressed in the lung, was shown to reduce airway reactivity by scavenging chemokines[60]. Furthermore, considering ACKR2 was described to prevent spread of psoriasiform inflammation[61] and high serum levels of CCL26 were correlated with atopic dermatitis severity[62], it is possible that this new pairing will shed light on mechanisms of autoimmune inflammation. CXCL10, previously known to bind exclusively to CXCR3, is the strongest CXC chemokine identified activating ACKR2. CXCL10 was shown to act as a partial agonist of ACKR2 with potency in the low nanomolar range, inducing approximately half of the maximal response measured with its known full agonist CCL5. This partial agonist behavior was reminiscent of the activity towards its long-established signaling receptor CXCR3 relative to the full agonist CXCL11[63,64]. Moreover, the potency of CXCL10 towards ACKR2 was approximately three times stronger than towards CXCR3. The rapid mobilization of ACKR2 to the plasma membrane induced by CXCL10 was similar to that observed in the presence of CC chemokines[65,66], while imaging flow cytometry revealed specific and efficient uptake of labelled CXCL10 by ACKR2-expressing cells. Importantly, the ACKR2-driven intracellular accumulation of CXCL10 was also associated with a reduction of its availability in the extracellular space, pointing towards a regulatory role of ACKR2 for this CXC chemokine. Of note, CXCL10 is a pivotal inflammatory CXC chemokine in many physiological and pathological processes, including angiogenesis, chronic inflammation, immune dysfunction, tumor development and dissemination[67,68], in which ACKR2 was also shown to be involved[6].

Noteworthy, CXCL2 also showed activity towards ACKR2, although it was weak in comparison to CXCL10 or to the activity it displays towards its classical receptor, CXCR2[69-72]. CXCL2 has no scavenger reported and is an important inflammatory chemokine and a powerful neutrophil chemoattractant. Interestingly, it has recently been reported that

ACKR2-deficient mice show increased neutrophil infiltration in different tissues [73] and a higher anti-metastatic activity of neutrophils than normal mice[74]. It remains to be investigated whether the enhancement of these neutrophil-related processes results from the suppression of CXCL2 regulation by ACKR2.

Pairing of a CC chemokine and non-chemokine endogenous peptides with ACKR3

ACKR3 (CXCR7 or RDC-1) is the second to last deorphanized chemokine receptor. It was initially shown to bind and be activated only by CXC chemokines, namely CXCL12 and CXCL11, which are also ligands for CXCR4 and CXCR3, respectively[41,75]. ACKR3 is expressed by endothelial cells, mesenchymal cells, B cells[76-78], in diverse regions of the central nervous system and in the adrenal glands[79-81]. ACKR3-deficient mice die perinatally due to semilunar heart valve malformation and ventricular septal defects and show disrupted lymphangiogenesis and cardiomyocyte hyperplasia, despite no alterations in hematopoiesis [82,83]. Similarly to other scavenging receptors, ACKR3 is generally present intracellularly, and cycles continuously between the plasma membrane and the endosomal compartments[84-86]. The scavenging function of ACKR3 was convincingly illustrated in studies using zebrafish embryos, where it shaped CXCL12 gradient during development[42,87].

In 2018, a study demonstrated that the broad-spectrum antagonist CC chemokine vMIP-II/vCCL2 encoded by the sarcoma-associated herpesvirus (HHV-8) can bind and activate ACKR3 with potency somewhat lower than the endogenous CXC chemokines[88]. ACKR3 scavenging of vCCL2 was proposed to impact the life cycle and immune escape of HHV-8 by controlling the availability of this important chemokine and its activity on both viral and host receptors. The identification of vCCL2 as a third chemokine ligand for ACKR3 and the first CC chemokine was also particularly valuable in the understanding of the activation mechanism and function of this atypical receptor[70].

ACKR3 was also shown to be the receptor for the pseudo-chemokine macrophage migration-inhibitory factor (MIF)[89]. MIF is an inflammatory cytokine that functions as a chemoattractant and participates in innate and adaptive immune responses by promoting macrophage activation and B-cell survival[90-92]. MIF is also a mediator in numerous inflammatory conditions and cancers[91,93]. MIF binding to ACKR3 was shown to promote receptor internalization and to contribute to cell signaling and B-cell chemotaxis[89]. Moreover, MIF-induced ACKR3 signaling in platelets was described to modulate cell survival and thrombus formation[94].

Besides chemokines and pseudo-chemokines, ACKR3 was shown to bind several small peptide ligands. ACKR3 was proposed as a scavenger receptor for the two pro-angiogenic peptides adrenomedullin (ADM) and proadrenomedullin N-terminal 20 peptide (PAMP)[95] both encoded by the *Adm* gene, regulating their activity for the cognate receptors CLR/RAMPs and MgRX2, respectively[96,97]. These findings were in line with the observation that *Ackr3* knockout recapitulates the *Adm* overexpression phenotype and that silencing *Adm* expression counterweighs lymphatic and cardiac aberrations observed in *Ackr3* knockout mice[96]. Nevertheless, the respective contribution of the two Adm-encoded peptides in the phenotype observed requires further investigation as ADM binds to ACKR3 at high micromolar concentrations whereas processed forms of PAMP have potencies in the nanomolar range[95].ACKR3 was also shown to be a high-affinity scavenger for a broad spectrum of opioid peptides, especially enkephalins and dynorphins, binding and internalizing them, reducing thus their availability in important opioid centers in the central nervous system, where ACKR3 is co-expressed with the classical opioid receptors. Modulation of the negative regulatory function of ACKR3 by molecules such as LIH383 or conolidine, an analgesic alkaloid used in traditional Chinese medicine, was shown to potentiate the activity of endogenous opioid peptides towards classical receptors, possibly opening alternative therapeutic avenues for opioid-related disorders[13,98-101].

Pairing of the CC chemokines CCL20 and CCL22 with ACKR4

ACKR4 was deorphanized in 2000[102]. It was proposed to bind CCL19, CCL21, CCL25 and CXCL13, which are the ligands for CCR7, CCR9 and CXCR5, respectively[12,102,103]. By scavenging these chemokines, ACKR4 was shown to regulate the trafficking and positioning of T cells and dendritic cells[104,105]. ACKR4 is best known for its role in shaping the gradient of CCL19 and CCL21 for CCR7-expressing dendritic cells in the subcapsular sinuses of the lymph nodes in the initial phase of the adaptive immune response[106,107].

In a recent study, CCL20, previously known to bind exclusively CCR6, was identified as a novel ligand for ACKR4[108]. The authors predicted this chemokine–receptor pairing based on CCL20 sequence and expression similarities with CCL19 and CCL21. They demonstrated that CCL20 triggers β-arrestin recruitment to ACKR4, and is efficiently scavenged by ACKR4-expressing cells, both *in vitro* and *in vivo*. They proposed that by scavenging CCL20, ACKR4 regulates its availability for the classical receptor CCR6 and

thereby plays a role in the positioning of CCR6-positive leukocytes within secondary lymphoid tissues for effective humoral and memory immune responses[108].

A parallel systematic pairing analysis using β-arrestin recruitment as readout confirmed CCL20 as a new full agonist ligand for ACKR4 with nanomolar potency[109]. This study also found that CCL22 acts as a potent partial agonist of ACKR4. CCL22, which is a key player in both homeostasis and resolution of inflammatory responses was until then known for its ability to interact with CCR4 and ACKR2. Interestingly, in line with a previous report[110] this study also disproved the agonist activity of CXCL13 towards ACKR4[109].

Deorphanization of GPR182/ACKR5 as a promiscuous scavenger receptor for both CC and CXC chemokines

Until very recently the G protein-coupled receptor 182 (GPR182), formerly known as ADMR) was classified as a class A orphan GPCR. Phylogenetically, it clusters within the chemokine receptor family owing to its 40% sequence similarity to ACKR3[111]. GPR182 was previously suggested as a receptor for adrenomedullin[112], which was later not confirmed[113]. It was initially described to be present in several organs[80,111], further studies identified its prevalent expression in endothelial cells in mouse and zebrafish[114], where it was proposed as a regulator of hematopoiesis.

In 2021, GPR182 was deorphanized and proposed as a new atypical chemokine receptor for CXCL10, CXCL12 and CXCL13[115]. The study confirmed the GPR182 expression in the endothelial compartment by using a transgenic mouse model expressing mCherry fluorescent protein under the control of mouse *Gpr182* promoter. GPR182 was detected in vascular endothelium of lungs, bone marrow, lymph nodes, Peyer's Patches, liver and spleen but not in the vascular endothelium of conductive arterial vessel. It was also detected in lymphatic vessels from skin, intestine and lymph nodes. As its closest paralogue ACKR3, GPR182 was shown to bind CXCL12 with nanomolar affinity. CXCL10 was also a strong ligand for GPR182 and several other binders could be identified from a large set of human chemokines screened in binding competition studies with fluorescently labelled CXCL10, including CXCL13, CCL19 and CCL16.

More recently, a study highlighted GPR182 expression in lymphatic endothelial cells in human melanoma[116]. In accordance with the first report, GPR182 was suggested as a novel atypical chemokine receptor for an extended spectrum of chemokines of different families and was tentatively named ACKR5. The authors primarily identified the CXCR3 ligand

CXCL9 as able to bind GPR182. Competition binding studies with a set of 35 chemokines revealed the ability of GPR182 to interact also with the other CXCR3 ligands, CXCL10 and CXCL11 as well as promiscuous binding for chemokines belonging to the four different classes (CCL, CXCL, CX3CL and XCL). The authors suggested that GPR182 might be able to recognize GAG-binding motif, which is critical region for chemokines to adhere to the endothelium. Different GAG-binding peptides were able to disrupt CXCL9–GPR182 interaction, which led the authors to consider the GAG-binding motif as determinant for chemokine interaction.

Interestingly, both studies demonstrated the absence of G-protein signaling in response to chemokine binding to GPR182[115,116], which is a common feature in the atypical chemokine receptor family[3,4]. Of note, a strong constitutive interaction with β-arrestin-2 was observed but no ligand-induced β-arrestin recruitment could be detected[115,116]. However, β-arrestins were suggested to be responsible for the rapid and spontaneous receptor internalization[115]. One important feature of atypical chemokine receptor is chemokine scavenging, this ability was highlighted by rapid uptake of labelled chemokine in GPR182-expressing cells and the increased plasma levels of CXCL10, CXCL12 and CXCL13 in both full- and endothelial compartment GPR182 knockout mice[115]. These mice also showed alteration in hematopoiesis, which is consistent with GPR182 scavenging of CXCL12[115], a chemokine notably involved in this process[115,117]. Absence of GPR182 also determined increased intratumoral concentration of different chemokines (CCL2, CCL22, CXCL1, CXCL9 and CXCL10)[116], which was suggested to contribute to an increased recruitment of tumor infiltrating lymphocytes and, therefore, hypothesized as potential target for improved immunotherapy[116].

Further studies are needed to validate GPR182 ligand specificity, as this aspect is not entirely consistent between the two studies. Both studies do however propose GPR182 as a broad-spectrum atypical chemokine receptor. This is particularly interesting as it would represent the only scavenger receptors identified so far for chemokines like CXCL9, CXCL13, CCL16 and CCL28. In the absence of detectable ligand-induced GPR182 signaling, it is challenging to determine precisely the receptor selectivity as well as its molecular characterization. It renders the official inclusion of GPR182 in the atypical chemokine receptor family by the IUPHAR, particularly complex.

Discussion

Significant progress has been made over the last decade towards a better comprehension of the functional and molecular aspects underlying the activity of ACKRs in health and disease. They have been gaining continuous consideration and are presently regarded as one of the most important receptor family standing at the forefront of the chemokine research and holding great therapeutic potential[6,118-120].

The unifying characteristic of ACKRs and unique integration criteria is so far their inability to trigger G protein signaling in response to chemokine binding. However, ACKRs often share other properties, such as the predominant intracellular localization or the ability to constitutively cycle between the plasma membrane and the intracellular compartments. Furthermore, early and more recent pairings suggest that ACKRs are commonly responsive to chemokines from different families. Indeed, the ability to bind and respond to both CC and CXC chemokines was historically described for ACKR1 [121] and — although it was subsequently challenged[109,110] — for ACKR4[102]. This cross-family selectivity has now been extended to ACKR2[56], ACKR3[88] and ACKR5[115,116] and therefore appears to represent an additional functional characteristic of ACKRs[3] that is not observed among the classical chemokine receptors.

Despite the many similarities, each ACKR presents its own distinct particularities in terms of expression pattern, ligand selectivity, function and mode of action. For instance, while most ACKRs interact with β-arrestins, ACKR1 seems to be an exception. ACKR3 also stands out in its atypicality as it is highly prone to activation[70] and can act as a receptor also for non-chemokine small peptide ligands[13,95,98]. Whether these two properties are linked and exclusive to ACKR3 or shared with other ACKRs remains to be investigated. Finally, GPR182 (ACKR5) seems to be a highly promiscuous receptor continuously scavenging chemokines with high basal β-arrestin association[115,116].

While it may seem surprising that several chemokine–ACKR pairings have been identified only recently, it was made possible thanks to different technological and scientific advances. For the long-established ACKRs, the better understanding of their function, mode of action and the commercial availability of chemokines as recombinant proteins have facilitated the recent pairings. Most importantly, the development of various sensitive assays allowing to accurately detect the activity of chemokines on the receptors independently of G protein signaling, e.g. via the induction of β-arrestin recruitment or the modification of the receptor

trafficking or localization, have been instrumental to identifying new ligand–receptor interactions[122]. In case of GPR182, which shows high level of basal cycling activity and β-arrestin interactions, a combination of experimental approaches allowed for its deorphanization. Receptor sequence comparison, precise determination of the expression profile and the use of binding competition studies confirmed by increased chemokine plasma concentration in knockout mice, were required to circumvent the problems related to the absence of direct chemokines-induced effects on the receptor[115,116]. For this receptor, additional independent investigations are now needed to precisely define the panel of chemokines it can scavenge and obtain an official inclusion by the IUPHAR in the ACKR family as ACKR5.

The chemokine–receptor network is well recognized for its highly intricate interactions where a chemokine may interact with several receptors, while a chemokine receptor has usually multiple ligands (Figure 2). On the other hand, some chemokines may be exclusive of a single classical receptor. However, the recent pairings described above identified at least one ACKR for a number of these chemokines, such as CCL20 (CCR6), CCL25 (CCR9), CXCL2 (CXCR2), CXCL9 and CXCL10 (CXCR3), CXCL13 (CXCR5) expanding the panel of ACKR ligands and functions. To date, out of the 45 human chemokines, several of them binding to XCR1 (XCL1 and XCL2), CCR8 (CCL1 and CCL18), CCR10 (CCL27 and CCL28), CCR3 (CCL15 and CCL24), CCR1 (CCL23), CXCR1 (CXCL6), CXCR6 (CXCL16), CX3CR1 (CXC3L1) and the orphan chemokine CXCL17 have not been paired with an ACKR (Figure 2).

The recent new pairings suggest that a systematic reassessment of chemokine–receptor interactions for ACKRs but also long-established classical chemokine receptors may still be necessary. Indeed, owing to the functional selectivity and biased signaling reported for some chemokines and receptors the attempts to uncover new pairings should not be limited to monitoring G protein signaling or β-arrestin recruitment. Other approaches such as measuring fluorescent ligand uptake, receptor trafficking or chemokine degradation in both agonist and antagonist modes should also be considered, as important crosstalks may remain unexplored.

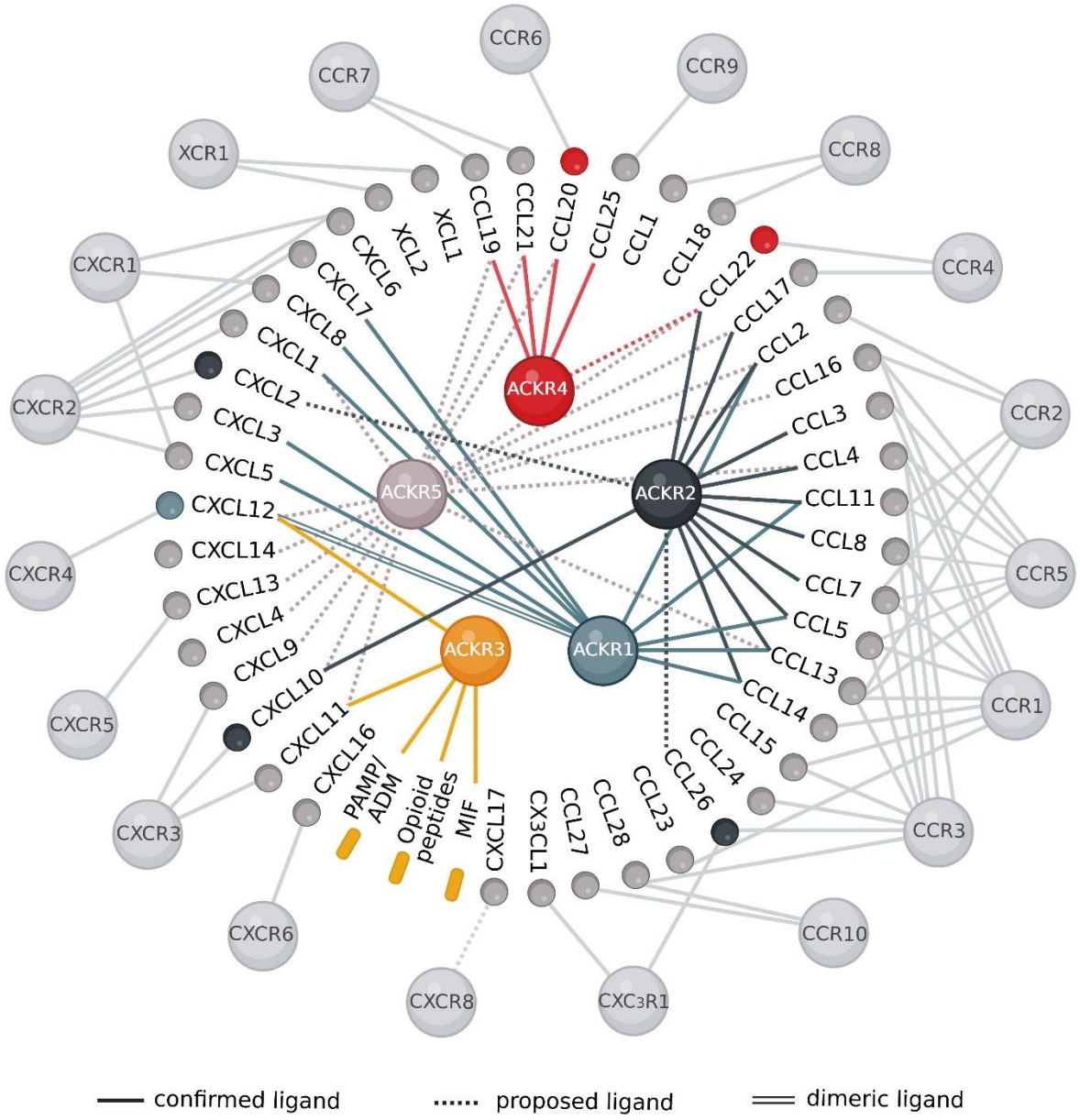


Figure 2. Overview of the chemokine interaction network with classical and atypical receptors. The interactions between different chemokines and their signaling and regulatory receptors are highly promiscuous. Most chemokines can bind several receptors and the majority of the receptors have multiple ligands. Receptors and chemokines are represented as spheres, while non-chemokine ligands are represented as rounded rectangles. There are 45 chemokines, 19 classical chemokine receptors (light grey) and 5 atypical chemokine receptors: ACKR1 (light blue), ACKR2 (dark blue), ACKR3 (yellow), ACKR4 (red) and the newly proposed ACKR5/GPR182 (light grey). Colored chemokines and non-chemokine ligands represent recently identified pairings, dashed lines indicate proposed ligands and double lines designate the binding of the dimeric ligand to the receptor. Created with BioRender.com

The novel pairings among ACKRs add an unforeseen level of complexity to their functions and regulatory roles for chemokines and non-chemokine ligands, while they also open interesting therapeutic opportunities, notably for cancer and chronic pain. For instance, the identification of ACKR2 and GPR182 as scavenger receptors for CXCL10 and/or CXCL9,

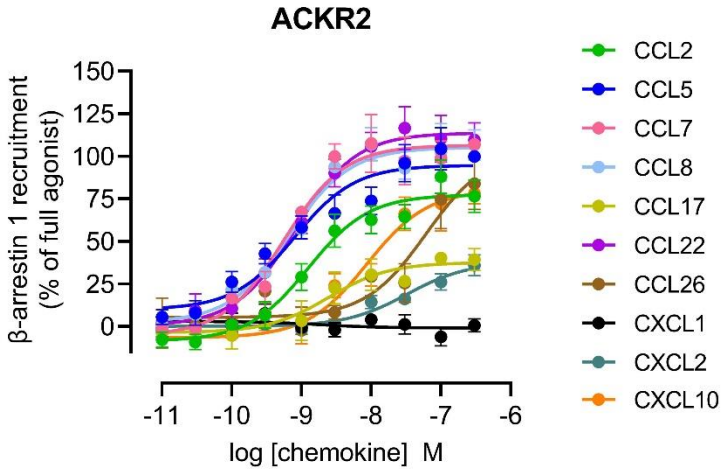
in addition to their well-established inflammatory CC chemokine ligands such as CCL2, CCL4 and CCL5, may be exploited in approaches seeking to turn cold tumors to hot tumors to improve the effectiveness of immunotherapies. Indeed, these newly identified chemokines for ACKR2 and GPR182 are key players in driving NK cells and CD8+ T cells into the tumor bed[123-126]. Therefore targeting their receptors may consequently increase the chemokine levels in the tumor microenvironment and subsequently sensitize them to immunotherapy[56,118]. On the other hand, targeting ACKR3 and blocking its proposed opioid peptide scavenging function was proposed as a new avenue to develop safer drugs with less side effects, which is critically needed to treat chronic pain[100,101].

However, considering the importance and multiplicity of their functions, the constantly growing number of ligands identified, the complexity of their biology and the interconnectivity with multiple systems, the targeting of ACKRs remains a great challenge. So far, only small molecules, peptides, modified chemokines and antibody fragments targeting ACKR3 have been reported, partly owing to the long-established importance of the CXCR4–CXCL12 axis in cancer, autoimmune and cardiovascular diseases[13,70,119,120,127-131]. Nevertheless, the increasing number of studies showing implication of other ACKRs, including ACKR5, in cancer development, progression but also protection together with the increasing availability of screening assays specific for each ACKRs will likely favor in the new future the development of modulators for other members of the family[100,122].

In the coming years, the ACKR family may be further enlarged[132]. Indeed, CXCR3B, the extended isoform of CXCR3, was recently proposed to display attributes of ACKRs[133], while CCRL2 and PITPNM3 await validation with regard to chemokine binding and direct regulatory functions[134 135,136]. Additional studies will reveal whether the latter two share common functional properties with the established and newly deorphanized atypical chemokine receptors.

In summary, investigations on ACKR are still in a highly dynamic phase and the recent identification of new pairings for established members of the family and of GPR182 as new member will certainly reinforce the interest of the community for this fascinating class of receptors. A better understanding of their functional complexity and heterogeneity is still needed in light of the extended panel of ligands they regulate and the therapeutic potential they seem to hold.

Supplementary material



Supplementary Figure 1. ACKR2 activation by different ligands. β-arrestin-1 recruitment to ACKR2 induced by known and the newly proposed ligands such as CCL26, CXCL2 and CXCL10 monitored by Nanoluciferase complementation assay (NanoBiT). CXCL1 was used as negative control. The assays were conducted in HEK293T cells. Data points represent mean ± SEM of three independent experiments.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution statement

MS and AC designed the manuscript. MS, GDU, RL, AAB and AC wrote the manuscript. MS, GDU, RL, MT, DFL and AC revised the manuscript. All authors approved the final manuscript.

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Chapter V – Brain malignancies

i) Chemokine receptors in glioma

My contribution to this Chapter:

This study represents a major work of my PhD project and I was involved in its general conceptualization. I explored different publicly accessible datasets containing glioma patients' transcriptomic data and contributed to their extraction. Additionally, I performed literature analysis with particular focus on the current understanding of chemokine receptors involvement in glioma and GBM, described by single-cell RNA sequencing-based studies based on glioma patients. I took care of the writing of the section "*Chemokine Receptors in Diverse GBM Cell Subtypes"* and contributed to the writing of the original draft of the manuscript and its reviewing.

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Highlights & aims

The involvement of chemokine receptors in tumoral processes, including glioma development and progression, has become more and more evident.

In this study, we evaluated and discussed the presence and relevance of chemokine receptors in glioma. CCR1, CCR5, CCR6, CCR10, CX3CR1, CXCR2, CXCR4, ACKR1, ACKR2, and ACKR3 emerge as chemokine receptors most abundantly expressed in glioma patients based on the analysis of publicly available clinical datasets. For a comprehensive understanding of their roles and implication in glioma and GBM, their expression has been assessed taking into account various glioma molecular groups, brain region distribution, emphasizing tissue-specific receptor functions, and cell type enrichment.

This clinically relevant and patient-oriented guide recapitulates the expression profile and the complex roles of chemokine receptors within the highly diversified glioma landscape. Moreover, it may pave the way for the improvement of targeted therapies and identification of new potential targets.

Patient-Oriented Perspective on Chemokine Receptor Expression and Function in Glioma.

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Simple summary

Chemokines and their receptors have been pointed as key actors in a variety of human cancers, playing pivotal roles in multiples processes and pathways. The present study aims at deciphering the function(s) of several chemokine receptors in gliomas, starting from publicly available patient-derived transcriptomic data with support from the current literature in the field, and sheds light on the clinical relevance of chemokine receptors in targeted therapeutic approaches for glioma patients.

Abstract

Gliomas are severe brain malignancies with glioblastoma (GBM) being the most aggressive one. Despite continuous efforts for improvement of existing therapies, the overall survival remains poor. Over the last years, the implication of chemokines and their receptors in GBM development and progression has become more evident. Recently, large amount of clinical data has been made available, prompting us to investigate chemokine receptors in GBM from a still-unexplored patient-oriented perspective. This study aims to highlight and discuss the involvement of chemokine receptors—CCR1, CCR5, CCR6, CCR10, CX3CR1, CXCR2, CXCR4, ACKR1, ACKR2, and ACKR3—most abundantly expressed in glioma patients based on the analysis of publicly available clinical datasets. Given the strong intratumoral heterogeneity characterizing gliomas and especially GBM, receptor expression was investigated by glioma molecular groups, by brain region distribution, emphasizing tissue-specific receptor functions, and by cell type enrichment. Our study constitutes a clinically relevant and patient-oriented guide that recapitulates the expression profile and the complex roles of chemokine receptors within the highly diversified glioma landscape. Additionally, it strengthens the importance of patient-derived material for development and precise amelioration of chemokine receptor-targeting therapies.

Introduction

Gliomas are glial tumors of the central nervous system (CNS), which are categorized into different subtypes and clinical grades based on their histological features as well as molecular markers (according to the World Health Organization (WHO)[1]. Adult-type diffuse gliomas represent the majority of primary brain tumors detected in adults, glioblastoma (GBM) being the most malignant subtype[1]. It accounts for 48,3% of malignant tumors of the adult central nervous system[2] and systematically results in fatal outcome for patients. For over 15 years, standard-of-care treatment has combined maximal safe surgical resection, radiotherapy and concurrent temozolomide-based chemotherapy[3]. In spite of extensive

preclinical and clinical research continuously aiming to improve therapeutic efficacy, GBM recurrence is commonplace and patient survival from the time of diagnosis remains very low[4]. Several mechanisms underlie tumor relapse: (1) the infiltrative nature of GBM that invades and disseminates through the whole brain tissue[5]; (2) the multilevel heterogeneity of GBM tumors, which exhibit inter-patient and intra-tumoral disparities[6], include diverse cell types and cellular states[7]; and (3) the ability of GBM cells to interact with and adapt to their microenvironment[8], to interconnect with neighboring tumor cells[9] or to harness healthy brain cells[10]. These devious mechanisms together support GBM to escape and resist treatment.

Chemokines are a subfamily of chemotactic cytokines secreted by a wide range of cell types in various tissues and are important regulators of developmental processes, immune responses and tissue repair[11]. Chemokines exert their effect by activating G protein-coupled receptors, which triggers downstream signaling pathways leading to cell migration, modulation of gene expression and cell phenotypes[12,13] They are classified in four subfamilies —CC, CXC, CX3C and XC—based on the arrangement of the cysteine motif in their N-terminal part, while their receptors are classified according to the type of chemokines they bind (CCR, CXCR, CX3CR and XCR). Recently, four chemokine receptors have been grouped in a subfamily of "atypical chemokine receptors" (ACKRs) owing to their inability to activate the classical ligand-induced G protein signaling cascades. They do however have an important regulatory role and can act as scavengers by reducing chemokine availability in the extracellular environment[14,15]. Chemokines and chemokine receptors have been proposed as key actors in cancer cell growth, migration, invasion, neovascularization, as well as in the fine-tuned interplay between tumor cells and tumor-associated immune cells[16,17]. The growing interest in chemokine receptor function in GBM is of complex nature. Not only are chemokines and chemokine receptors involved in GBM cell malignant phenotype, they also play an important part in the immune cell recruitment to the tumor. These molecules are therefore being increasingly considered as potential targets in immunotherapy approaches for GBM[18].

The last decade has witnessed an unprecedented effort in collecting samples and clinical data from patients suffering from solid cancers, including brain tumors. International consortia and multicenter projects (e.g. The Cancer Genome Atlas (TCGA)[19], Glioma Longitudinal AnalySiS (GLASS) consortium[20], Gliogene[21], etc) have gathered considerable patient cohorts that provided the neuro-oncology community with large multi-omics datasets,

offering invaluable information for the classification and grading of tumors as well as for the understanding of molecular mechanisms underlying glioma biology. Whereas multiple therapeutic strategies have so far failed to translate from the bench to the clinic because of limited research tools, the availability of patient data and biological material now facilitates clinically relevant research and fosters the development of personalized therapies[22,23].

Here, we aim to refine the current knowledge about the role of chemokine receptors in glioma from a patient-oriented perspective. We analyzed publicly available datasets and highlighted a subset of receptors that appear to be significant in GBM patients, for which we gather and discuss recent insight from the literature. The purpose of this study is to provide researchers in the field with a clinically-relevant, up-to-date practical resource that could orient the next steps towards chemokine receptor-based treatment for glioma patients. We voluntarily highlight the literature that describes data generated from patient material, and mention preclinical data on cellular and animal models when considered pertinent. We do not detail the mechanistic and functional aspects of each described receptor, which were exhaustively reviewed recently[24].

Material and Methods

We aimed to highlight putative variations in the expression of chemokine receptors in different types of gliomas, as well as in different tumor subregions and cellular subsets. To do so, we browsed four different glioma patient datasets using available online tools and exploited the data related to the information of interest (Table 1). We focused on gene expression data, generated by RNA sequencing of patient-derived residual tumor tissue, obtained after surgical resection. We analyzed the expression of 22 genes encoding for chemokine receptors, namely CC receptors 1 to 10 (CCR1-10), CX3C receptor 1 (CX3CR1), CXC receptors 1 to 6 (CXCR1-6), XC receptor 1 (XCR1) and the atypical chemokine receptors ACKR1 (or DARC), ACKR2 (or D6), ACKR3 (or CXCR7/RDC1) and ACKR4 (or CCRL1). The alternative gene names were used when required by the online platform. Original publications, online tools, RNA sequencing method as well as number of samples and patients included in the datasets are listed in Table 1. No recalculation nor modification of the existing data was performed. Figures included in this manuscript are either original heat maps displaying the unchanged data downloaded from the databases, or were directly generated on the online platforms (for scRNAseq data).

Table 1. General information about the datasets used in the review.

	1	2	3	4
Publication, project	**Ceccarelli et al, 2016, Cell**[25] ***The Cancer Genome Atlas (TCGA) Project***	**Puchalski et al, 2018, Science**[26] ***Ivy Glioblastoma Atlas Project***	**Darmanis et al, 2017, Cell Reports**[27]	**Neftel et al, 2019, Cell**[7]
Selected information	Gene expression in three glioma subgroups (correlated with severity)	Gene expression in five anatomical locations within GBM tumors	Gene expression in 4 different glioma-related cell subtypes	Gene expression in 7 different glioma-related cell subtypes
Online tool	http://gliovis.bioinfo.cnio.es[28]	https://glioblastoma.alleninstitute.org	http://gbmseq.org/	https://singlecell.broadinstitute.org/single_cell/study/SCP393/
Method	Bulk RNAseq (HiSeq)	Bulk RNAseq (HiSeq) after laser microdissection	Single cell RNAseq (NextSeq)	Single-cell RNAseq (SMART-Seq2)
Datasets, number of samples and patients	Brain lower grade glioma, LGG (513 patients) Glioblastoma, GBM 154 patients)	Glioblastoma (122 samples/10 patients)	Glioblastoma (3589 cells/4 patients)	Adult and pediatric glioblastoma (IDHwt) (7930 cells/28 patients)
Data expressed as	Log2 RSEM	Log2 RSEM	Log2 CPM	Log TPM

Legend: CPM: Counts per million; IDHwt: IDH wild-type; RNAseq: RNA sequencing; RSEM: RNA-Seq by Expectation Maximization; TPM: Transcripts per million.

Chemokine Receptor Expression in Gliomas

We first aimed to highlight which chemokine receptors are most abundantly expressed in gliomas. We unraveled the expression of 22 chemokine receptors in tumor tissue collected from newly diagnosed diffuse glioma patients using the TCGA LGG-GBM dataset (including 513 low-grade gliomas (LGG) and 154 GBM diagnosed patients with available RNAseq data). In an attempt to relate gene expression to glioma clinical subgroups associated with respective disease severity, we classified patients based on isocitrate deshydrogenase (IDH) mutation and 1p/19q co-deletion status, as these features were previously suggested to correlate with histological types and clinical grades. We therefore consider “IDH mutant 1p19q codel” gliomas as oligodendrogliomas, “IDH mutant 1p19q non codel” gliomas as enriched in low grade astrocytomas, and “IDH wt” gliomas as

enriched in high grade astrocytomas and glioblastomas[29,30] (**Figure 1**). Note that such enrichment does not imply the exclusivity of a group for a given histological assessment.

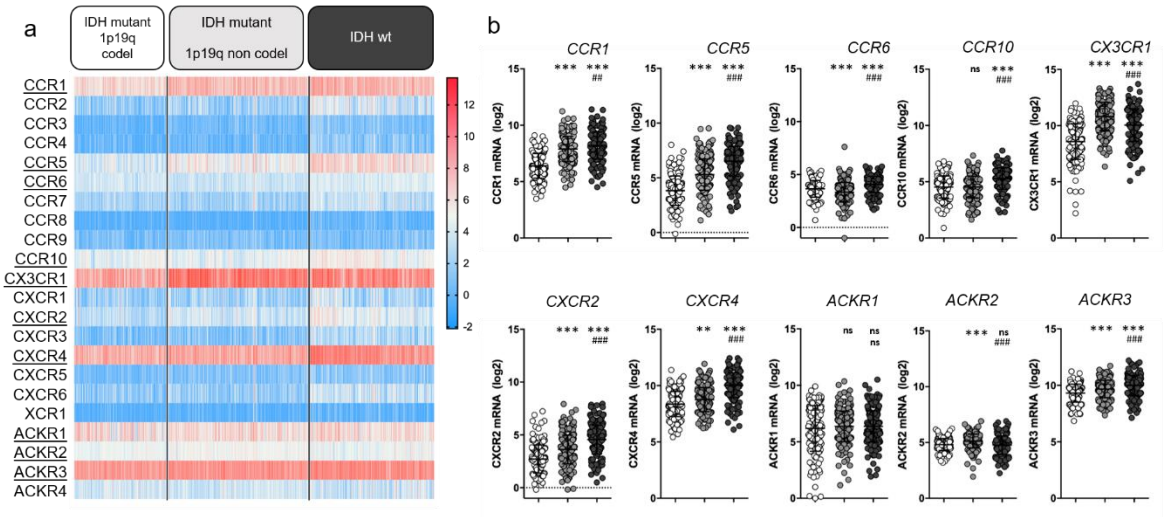

Figure 1. Chemokine receptor expression in glioma patients (TCGA LGG-GBM dataset[25], GlioVis platform[28]) **a)** Heatmap displaying log2 RSEM value for the 22 receptors of interest. Each cell represents one patient. The receptors that will be highlighted in this review are underlined. **b)** For every receptor, log2 RSEM values were grouped into 3 categories (based on glioma genomic features). Each dot represents one patient. Data is downloaded from http://gliovis.bioinfo.cnio.es, data are represented as Mean ± SD, and analyzed via one-way ANOVA (vs "IDHmut 1p19q codel": **p<0,01; ***p<0,001 and vs "IDHmut 1p19q non codel": $^{\#\#}$p<0,01; $^{\#\#\#}$p<0,001).

We here highlighted **CCR1, CCR5, CCR6, CCR10, CX3CR1, CXCR2, CXCR4, ACKR1, ACKR2** and **ACKR3** based on their average mRNA expression within at least one patient subgroup (threshold arbitrarily placed at average RSEM≥4). For most of the selected receptors, mRNA expression increases with glioma grade. Note that we do not rule out that unselected receptors may yet be of interest. We will therefore focus this manuscript on these ten chemokine receptors and unravel relevant literature data to further discuss their respective contribution to glioma biology.

In the last two decades[31], extensive evidence has proven **CXCR4** as significantly related to glioma malignancy[32-34]. Its crucial contribution to the disease is supported by the phase I/II clinical testing of CXCR4 inhibitors for GBM treatment (e.g. plerixafor)[35,36], as further discussed in section 4.1. The clinical relevance of the other selected chemokine receptors is supported by more or less abundant (pre)clinical data from the literature, which mostly analyzed mRNA/protein expression in glioma tissue sample cohorts (vs control tissue samples) and related these to tumor grade and patient survival. Among the receptors that appear highly expressed in all gliomas, **CX3CR1** is a macrophage associated receptor, whose expression has been shown similar in patient tissue from both low and high grade

gliomas[37,38], substantiating the TCGA data in Figure 1. Of note, a specific CX3CR1 defective polymorphism (V249I) correlates with increased patient survival in patients with GBM[39] and LGG[40], stressing its important role in tumor maintenance. **ACKR3**, formerly known as CXCR7/RDC1, has also been investigated in glioma patient tissue where its expression pattern appears quite inconstant: several studies highlight an increased mRNA expression in GBM tissue samples compared to non-malignant brain samples[41,42], while other studies do not[43]. Moreover, TCGA data show **CCR1** expression in glioma samples. Though this has not been exhaustively documented in patient tissue so far, insights in CCR1 activity in glioma are currently emerging (see section 4). In comparison to the above-cited receptors, **CCR5, CCR6, CCR10** and **CXCR2** all display moderate expression in glioma patients from TCGA database. Their expression in tumor tissue has been assessed in diverse studies and was found upregulated in glioma (compared to non-tumor samples), correlating with the tumor grade as well as with shorter disease-free and overall patient survival[44-47] Higher expression of CCR5 and CXCR2 has also been associated with recurrent tumors[48,49]. The roles of **ACKR1** and **ACKR2** in tumor growth have also been evaluated in other cancer types[50,51] where their expression has been correlated with a reduced tumor growth and survival benefit (reviewed in[15,52]). However, the role of these atypical receptors in gliomagenesis remains to be elucidated.

Chemokine Receptors in Glioma Malignant Processes

GBM is an extremely heterogeneous tumor, endowed with high invasive capacity, harboring hypoxic areas, necrotic and proangiogenic environments. Such heterogeneity complicates GBM treatment and constitutes an immense challenge for neuro-oncologists. The Ivy Glioblastoma Atlas Project (IvyGAP) has addressed this intra- and inter-tumoral heterogeneity by correlating anatomo-histological features with gene expression data in a panel of GBM patients[26]. In this study, five separate areas were analyzed after laser microdissection: (1) leading edge (LE), outermost boundary of the tumor; (2) infiltrating tumor compartment (IT), intermediate zone; (3) cellular tumor (CT), core part of the tumor with high ratio of tumor cells vs healthy cells; (4) pseudopalisading cells around necrosis (PAN), densely aligned tumor cells surrounding necrotic areas; (5) microvascular proliferation (MVP) marked by two or more blood vessels. This freely accessible anatomo-transcriptional atlas provides a valuable ground to interrogate gene function in GBM growth processes. Here, we utilize this IvyGAP resource to look at the expression of the selected

chemokine receptors in these five regions of interest and further decipher their activity in these specific regions (**Figure 2**).

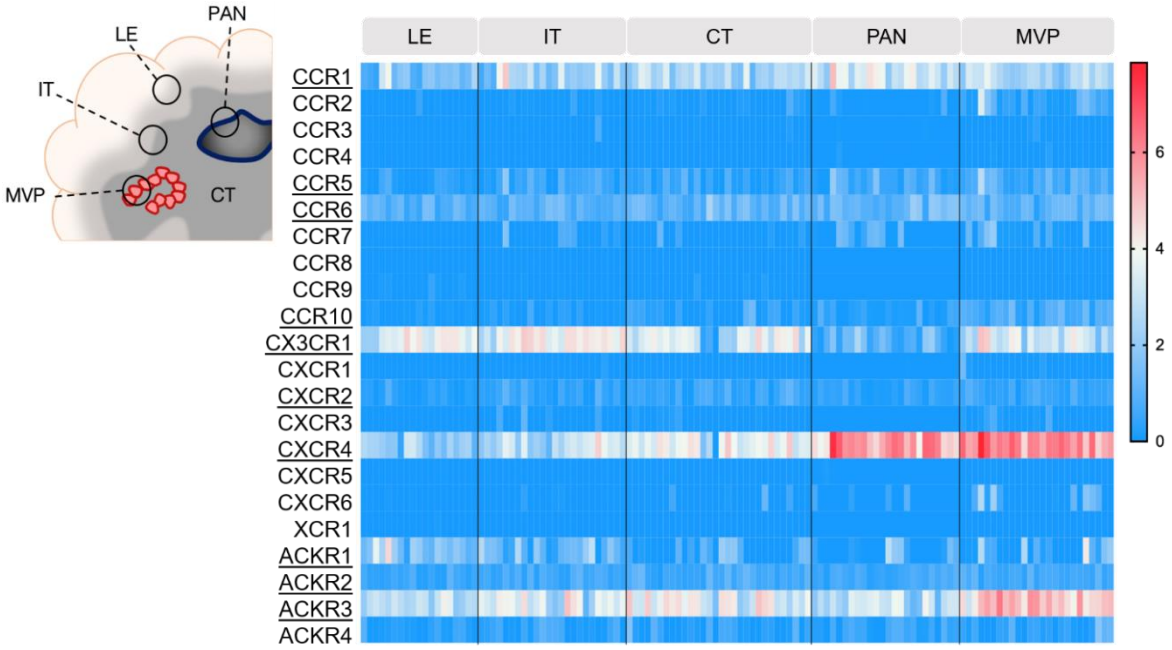

Figure 2. Chemokine receptor expression in various areas of GBM tumors (IvyGAP project). The heatmap displays log2 RSEM value for each receptor, in the various tumor subregions. Each cell represents one sample. Legend: LE: leading edge; IT: infiltrative tumor; CT: cellular tumor; PAN: pseudo-palisading cells around necrosis: MVP: microvascular proliferation. Data is downloaded from https://glioblastoma.alleninstitute.org.

CXCR4 once again stands out as highly expressed in the pseudopalisading cells around necrosis (PAN) and microvascular proliferation (MVP) regions, respectively described as related to hypoxia and angiogenesis/immune regulation[51]. **ACKR3** also appears associated with MVP regions. Additionally, these two receptors are detected in the central tumor (CT), infiltrative tumor (IT) and leading edge (LE) regions, where their contribution could be of variable nature (see sections 4.1, 4.2 and 4.3). **CX3CR1** and **CCR1** are also expressed and distributed in all tested regions. **CCR5, CCR6, CCR10, CXCR2, ACKR1** and **ACKR2** display moderate expression in GBM samples, regardless of the area, which is in line with the TCGA data from Figure 1.

Using a similar approach, another study described chemokine receptor profiling in different GBM subregions[53], which were isolated after 5-aminolevulenic acid (5-ALA) fluorescence-guided surgery of six newly diagnosed GBM patients[54]. GBM cells were isolated from the tumor core (strong fluorescence, ALA+), infiltrating area (pale fluorescence, ALA-PALE) and healthy tissue (no fluorescence, ALA-). **CXCR4** and **ACKR3** were found upregulated in tumor core GBM cells (without distinction of necrotic and/or angiogenic features)

compared to infiltrating area and healthy tissue, which is supportive of the IvyGAP data. Conversely, **CCR1** and **CCR10** were found upregulated in GBM infiltrating area compared to the tumor core, which suggests a role for these receptors at the margin of the tumor, probably linked to cell invasion or communication with the tumor microenvironment (TME).

Angiogenesis

Angiogenesis is an important feature of high-grade gliomas, supporting tumor cell survival and invasion[55]. In line with the IvyGAP analysis of chemokine receptor expression in MVP and PAN regions, studies have demonstrated that **CXCR4** is largely expressed in endothelial cells of the normal brain, as well as in GBM blood vessels and hypoxic areas of necrosis[42]. CXCR4 is enriched in highly vascularized GBM tissue[56] and its role in hypoxia-induced angiogenesis has been widely documented[31,57]. CXCR4 inhibition using plerixafor was therefore proposed in combination with bevacizumab (anti-vascular endothelial growth factor monoclonal antibody) for diminishing resistance to this anti-angiogenic therapy and has so far proven safe in patients with high-grade gliomas[36] **ACKR3** is also found in endothelial cells as well as in tumor cells and microglia in GBM patient tissue specimens[42,58]. Moreover, a role for ACKR3 in tumor neovascularization has been suggested using *in vitro* models of tube formation with glioma endothelial cells[59], breast cancer cells[60] or human umbilical vein endothelial cells[61]. In glioma cells, ACKR3 expression appears upregulated in hypoxic conditions[62]. Given the discernible expression of ACKR3 in MVP areas of GBM tumors, its contribution to the angiogenic mechanisms in glioma patients and its interplay with CXCR4 definitely warrant further investigation. Although less prominently expressed in the MVP region based on the IvyGAP data, **CXCR2** has also been associated with neovascularization. It colocalizes with blood vessels in GBM patient tissue and functionally helps GBM cells to transdifferentiate and acquire an endothelial-like phenotype, inducing vascular mimicry[47]. Finally, a co-culture model of glioma cells with normal astrocytes suggests that astrocyte-mediated production of CCL20 facilitates **CCR6**-expressing GBM cell adaptation to hypoxic TME via upregulation of hypoxia-inducible factor 1-alpha (HIF1-α). In particular, xenografts lacking CCR6 showed an impaired vascularization and reduced adaptability to hypoxic stress, supporting a role for this axis in GBM[63].

GBM Cell Migration and Invasion

Although not extremely prominent, several of the selected receptors are expressed at the invasive front of the tumor, which may suggest their involvement in GBM cell incursion

through the brain parenchyma. **CXCR4** expression has been associated with the extent of tumor cell dissemination within the patient brain (based on tumor imaging features)[34]. Preclinical models of gliomas highlighted its role in mediating cell migration and invasion[64,65], especially in the migration of specific "stem-like" cell subsets (see section 4.3). In contrast, the activity of **ACKR3** in glioma cell motility remains elusive and its function in the invasion of other cancer cell types is still a matter of debate. Indeed, in head and neck squamous cell carcinoma patients, ACKR3 expression has been associated with increased lymph node metastasis rate[66] and the relationship between CXCR4 and ACKR3 has also been linked to increased breast cancer metastasis in experimental models[67]. Contrasting results rather propose that CXCR4 and ACKR3 have distinct roles. CXCR4 seems to enhance cell invasiveness, while ACKR3 appears to be mainly associated with decreased invasive properties as well as inhibition of metastasis. ACKR3 is also suggested to promote tumor growth by stimulating angiogenesis[68].

Experimental data indicate that **CCR5** and **CXCR2** are involved in glioma cell invasion through tridimensional environments, when induced by co-cultured human mesenchymal stem cells[48,69] or endothelial cells[70] that were shown to secrete key chemokines. Hence, these receptors may play a role in GBM cell invasion through brain tissue.

GBM "stem" Cell Properties and Resistance to Treatment

GBM progenitor/initiating/stem cell phenotype characterizes the self-renewing and plastic cell population within the tumor that sustains tumor growth and promotes resistance to treatment. Hence significant efforts have been undertaken to specifically target these glioma stem cells (GSCs) (reviewed in[71]). GSCs have been associated with specific "vascular niches" within tumors[72], but also have been shown to be enriched in the cellular tumor (based on the IvyGAP data)[73]. **CXCR4** was detected in cells expressing stem cell-associated markers (e.g. SOX2, KLF4, OCT4, NANOG) in both primary and recurrent GBM patient tissue sections[74]. Additionally, CXCR4 expression was found in patient-derived GSC primary cultures *in vitro*, where the receptor was implicated in cell survival, self-renewal and invasion upon xenografting[75-77]. Specifically, we previously showed that after orthotopic implantation, GSCs migrated towards the subventricular zone in an oriented, CXCR4-mediated fashion[78], which was associated with GSC protection from radiation therapy[79]. In contrast, only a minor subset of stem-like cells were found positive for **ACKR3** in GBM patient tissue[74] and less information is available from *in vitro* patient-derived models. A study using selective ACKR3 modulators emphasized the involvement of ACKR3 in GSC

growth *in vitro* together with CXCR4, although this report revealed that GSC tumor formation *in vivo* was independent of CXCR4 or ACKR3 activity[80]. Aside from sustaining tumor initiation, GSCs were shown to determine GBM cell response to therapy, and were particularly suggested as crucial for the resistance to temozolomide (TMZ)[81]. A recent study has demonstrated that **CXCR2** expression increased in patient-derived GSCs (expressing CD133, another stem cell-associated marker) upon treatment with TMZ *in vitro*. Activation of CXCR2-related pathways was indeed associated with alterations in the epigenomic landscape of cells, which impact GBM cell plasticity and resistance to TMZ[82]. Furthermore, **CCR5** has been linked to TMZ resistance. Pericytes secrete CCL5 which activates CCR5 and downstream pathways in GBM cells. This leads to the activation of DNA damage response and thus reduces the efficiency of TMZ in killing GBM cells[83].

Chemokine Receptors in Diverse GBM Cell Subtypes

As mentioned above, the intratumoral heterogeneity of gliomas is largely accountable for therapeutic failure. Over the last years, the emergence of single-cell profiling technologies has deepened our understanding of glioma biology and the tumor heterogeneity that outreaches the anatomical level. Single-cell RNA sequencing is an advanced tool to decrypt the individual role of the different cell types forming the tumor, it allows to investigate the glioma heterogeneity at single-cell resolution. Several recent studies have shed light on the diverse malignant and non-malignant cell types that together compose gliomas and dictate their development, maintenance and response to therapy[6,7,27,84,85]. In high-grade gliomas, over a third of the tumor mass is constituted of non-malignant cells. The TME includes neuronal and glial cells, macrophage/microglial cells, representing the major immune cells component, endothelial cells and a low number of T cells[86].

Within the tumor, malignant/neoplastic cells were distinguished from non-malignant TME cell types using inferred copy-number alterations, and specific cell clusters were further categorized into TME subtypes based on their gene expression profile (for more detailed information, please refer to the original publications[7,27]). In the process of deciphering chemokine receptor function in gliomas, we explored two publicly available single-cell RNAseq datasets obtained from glioma patient tissue[7,27]. We looked into the expression of CCR1, CCR5, CCR6, CCR10, CX3CR1, CXCR2, CXCR4, ACKR1, ACKR2 and ACKR3 in the various cell type-related signatures that were reported (**Figure 3**).

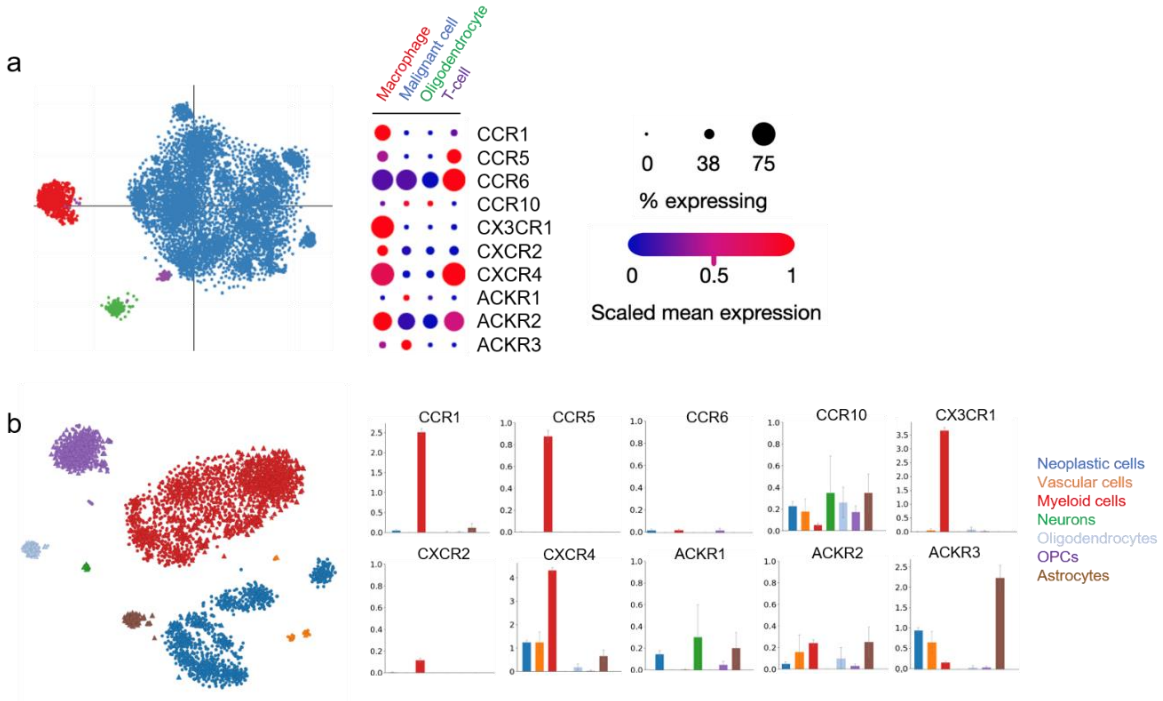

Figure 3. Chemokine receptor expression in various cell types within patient glioma samples. **a)** Single cell RNAseq data from Neftel et al (2019, Cell)[7] show the expression of the ten selected receptors in cells regrouped in four specific annotations (macrophage, malignant cell, oligodendrocyte and T-cell). The "% expressing" value indicates the proportion of cells in the signature that are positive for a given transcript, and the "scaled mean expression" is relative to each gene's expression level (logTPM) across all cells within the signature (https://singlecell.broadinstitute.org/single_cell/study/SCP393/). **b)** Single-cell RNAseq data from Darmanis et al (2017, Cell Reports)[27] show the expression of the ten receptors of interest in cells regrouped in seven specific annotations (neoplastic cells, vascular cells, myeloid cells, neurons, oligodendrocytes, oligodendrocyte precursor cells (OPCs) and astrocytes). Bar plots indicate log2CPM values (http://www.gbmseq.org/).

In both datasets, **CCR1** and **CX3CR1** expression strongly correlated with the "macrophage" and "myeloid cell" signatures, while the two receptors were virtually absent from other cell types, unsurprisingly pointing to their key role in immune cell recruitment and function in gliomas. A high expression of **CCR5** and moderate expression of **CXCR2** is found in the same groups. CCR5 expression is also detected in the minor population of "T-cells", as well as **CCR6. CXCR4** is abundantly present in cells assigned to the "macrophage" and "myeloid cell" signatures, as well as in "vascular cells" and "neoplastic cells", which is in line with the various roles of this receptor in diverse tumor-related processes. **ACKR3** is detected in "neoplastic cells" and "vascular cells", again supporting the data obtained from GBM patient tissue specimens[42,58]. Of note, this receptor is also abundantly expressed in "astrocytes" present in the tumor tissue. **CCR10, ACKR1** and **ACKR2** could be detected in different

cell types at low level. The current knowledge on the function of these receptors in the respective cell entities will be further discussed in the following sections.

Tumor-Associated Macrophages (TAMs)

We previously mentioned that glioma TME largely contributes to the tumor bulk and influences tumor cell maintenance and growth. Tumor-associated macrophages (TAMs) derive from bone marrow circulating monocytes or from resident microglial cells and affect glioma progression in diverse manners depending on their activation status, interaction with TME components, phenotype or location within the tumor (reviewed in[87]). So far, TAMs are generally considered as supportive of GBM growth. **CX3CR1**-mediated macrophage infiltration into gliomas has been confirmed in patient tissue[88]. In GBM and LGG patients carrying the defective CX3CR1 V249I polymorphism[39,40], such infiltration is reduced which is associated with better prognosis.

Recently, a study investigated the single-cell transcriptome of multi-sector biopsies from 13 glioma patients (with various WHO grades)[89]. These data were used to reconstruct a ligand–receptor interaction map describing the most relevant chemoattractant relationships existing between tumor cells and TAMs in glioma TME. Nine chemokine receptors were detected in the 13 tumors, including CCR5, CCR6, CX3CR1, CXCR2 and CXCR4. This study reported that glioma cells overexpress CX3CL1, which is responsible for the recruitment of **CX3CR1**-expressing microglia and macrophages. **CCR5** and **CXCR4** were found on TAMs as well.

Tumor-Infiltrating Lymphocytes (TIL) and Other Immune Types

Generally, gliomas are recognized as "cold" tumors endowed with poor immune response, where glioma cells expressing diverse immune checkpoint molecules (e.g. PD-L1) that hamper immune cell activation. Moreover, tumor infiltrating lymphocytes (TILs) poorly penetrate tumors, among which regulatory lymphocytes (T_{reg}) secrete immunosuppressive cytokines (IL10 and TGF-β) and cytotoxic T cells exhibit a specific exhaustion profile (expression of PD-1 and CTLA4). In addition to the extensive glioma heterogeneity, such immune suppressive environment makes glioma refractory to targeted immunotherapy[90]. Literature data suggest that the level of TILs varies between different glioma genomic subtypes, with high grade (IDHwt) gliomas showing the highest TIL amount and the worse prognosis[91,92]. This encourages to (1) consider genomic profiles for predicting response to immunotherapy and (2) better understand and modulate TIL function and access to the

tumor. To that purpose, regulating chemokine receptor function is of interest. The aforementioned report on GBM single-cell transcriptome confirmed that TILs express CCR6 (corroborating the data from Figure 3), as well as CCR5 and CXCR4, which all could contribute to lymphocyte recruitment towards the tumor[89].

Other immune-related cell types such as neutrophils, dendritic cells, myeloid progenitors and even hematopoietic stem cells could also be found in gliomas[86,93] and their roles in glioma development and response to therapy are still under investigation. The recent literature provides pieces of information regarding the activity of chemokine receptors in these subsets. Early studies of TME in mouse models allowed to identify immature and immune-suppressive myeloid cells within solid tumors, which were called myeloid-derived suppressor cells (MDSCs) (likely encompassing diverse cell entities). Although efforts are currently carried out to standardize nomenclature and characterization of these cells, the MDSC term is still often used. MDSCs isolated from glioma patient tissue could be classified in monocytic (M-MDSCs) and granulocytic subsets (G-MDSCs). G-MDSCs presented increased CXCR2 expression, but showed minor accumulation in the tumors compared to M-MDSCs[94]. Accordingly, CXCR2 was associated with neutrophils in the aforementioned single cell mapping of glioma TME components[89]. Of note, the degree of neutrophil infiltration has been positively correlated with glioma severity[95,96].

Efforts still have to be carried out to decipher the functional aspects of the complex glioma-associated immune orchestra to eventually shed new light on effective treatment options, which could rely on the modulation of chemokine-mediated immune cell recruitment to the tumors.

Vascular cells

Endothelial cells from brain capillaries, as well as contiguous pericytes and astrocytic feet, are key components of the blood-brain barrier (BBB), which constitutes a selective filter that tightly regulates brain penetration of a variety of molecules and compounds. The integrity of this BBB is compromised in brain tumors[97], and endothelial cells exhibit various molecular alterations that reflect on their dysfunction, anatomical location, and variable permeability[98]. The expression of chemokine receptors in endothelial cells from glioma tissue has been detailed in section 4.1 together with their role in angiogenesis. The implication of **CXCR4** and **ACKR3** in this process has particularly been documented. However, CXCR4 and ACKR3 expression is not specific to glioma-associated endothelial cells, and both receptors are also detected in endothelial cells from the developing brain[99] or

from the adult brain[100]. A recent study developed an ACKR3 knock-in mouse model and highlighted ACKR3 expression in the cerebral vasculature, distributed across various brain structures[101], thus stressing a role of this atypical chemokine receptor in brain physiology that deserves deeper investigation.

Pericytes also play a pivotal role in the BBB maintenance. In GBM, pericytes exhibit specific genetic alterations. They mostly derive from GBM stem cells, which are recruited to blood vessels via CXCL12/**CXCR4**-mediated axis and evolve towards pericytes that contribute to vascular niche remodeling[102] and modulate GBM cell activity. Pericytes secrete CCL5, which binds the CCR5 receptor expressed by GBM cells. This interaction triggers the activation the DNA damage response, thereby overcoming TMZ-induced cell death. Inhibiting CCL5/CCR5 signaling abrogates the protective effects of pericytes against GBM and improves the efficacy of TMZ[82,83].

Non-malignant Glial Cells and Neurons

As shown in Figure 3, **ACKR3** as well as **CXCR4** appear to be expressed also in non-malignant brain cells, notably in astrocytes. A study previously reported the presence of ACKR3 in adult rat astrocytes and further showed that its expression increases upon non-cancerous, neuroinflammatory conditions. ACKR3 is also detected in human astrocytes from the brain cortex and hippocampus and in oligodendrocytes and oligodendrocyte precursor cells (OPCs)[103]. In addition, preclinical models have shown the physiological role of ACKR3 in adult neuron physiology[104] and during development[105,106]. All in all, aside from the expression of ACKR3 and CXCR4 in glioma cells as well as in multiple cell subtypes from the TME, it appears that cell components of the neighboring healthy brain tissue require ACKR3 and CXCR4 for their maintenance and function, which could complexify their targeting in GBM therapy. Similarly, CCR10 and ACKR2 were also found to be expressed in astrocytes, albeit at lower level than ACKR3, while a small population of oligodendrocytes and OPCs express CCR6, CCR10 and ACKR2.

Conclusions

Gliomas are tumors of the central nervous system that remain associated with dismal prognosis in spite of innovative diagnostic strategies and modern therapies. Chemokines and their receptors play crucial roles in glioma development and progression and therefore constitute attractive candidates for targeted treatment. However, although their implication and targeting in *in vitro* and *in vivo* rodent models are well documented, especially for

CXCR4 and ACKR3, their clinical relevance requires confirmation with patient data and biological material. Further analysis in terms of brain region distribution and by cell type enrichment is also necessary to better understand the complex roles of chemokine receptors within the highly diversified glioma landscape.

	Role in	***CCR1***	***CCR5***	***CCR6***	***CCR10***	***CX3CR1***	***CXCR2***	***CXCR4***	***ACKR1***	***ACKR2***	***ACKR3***
Processes	*Angiogenesis*			IV			P	P			P
	Invasion		IV				IV	P			?
	Stem cell properties						IV	P			?
	Resistance		IV				IV	P			
Cell types	*Tumor cells*							P			P
	Vascular cells							P			P
	TAMs/Microglia		P			P		P			
	TILs		P	P				P			
	Neutrophils						P				
	Normal glial cells							P			P

Table2. Summary of chemokine receptor function in GBM tumorigenic processes and in GBM cell subtypes. Legend: TAMs: Tumor-associated macrophages; TILs: Tumor-infiltrating lymphocytes; IV: Evidence from *in vitro* experiments (GSCs & others); P: Evidence from patient tissue; ?: still debated.

We found an overall good coverage and concordance of the different datasets used for the present analysis and congruence with targeted reports from the literature describing patient-derived material (Table 2). In this study, we focused on CCR1, CCR5, CCR6, CCR10, CX3CR1, CXCR2, CXCR4, ACKR1, ACKR2 and ACKR3, whose expression is detected in patient glioma tissue and rather well correlated with disease severity. With the aim of shedding light on chemokine receptor function in glioma physiopathology, this analysis integrated data from (1) publicly available bulk and single-cell transcriptomic datasets providing various types of information together with (2) evidence from the literature.

CXCR4 emerged as the most prominent receptor expressed on many different cell types within the tumor and associated with various tumorigenic processes like angiogenesis, cancer cell invasion and resistance to treatment. This review also highlights ACKR3 as a multifaceted player in almost every of these glioma-related cellular processes and subtypes and prompts researchers in the field to further apprehend the subtleties of the

CXCR4/ACKR3/CXCL12 triad in glioma. The importance of the CXCL12/CXCR4/ /ACKR3 axis in GBM is emphasised by multiple efforts towards the clinical translation of related inhibitors largely validated at the preclinical level[32,36,107-109]. A phase I/II clinical trial (NCT04121455) is currently investigating the impact of the CXCL12 inhibitor olaptesed pegol or NOX-A12 as part of combination therapy with radiation therapy and bevacizumab. Another phase I/II trial (NCT01977677), aiming at studying the safety and efficacy of the CXCR4 inhibitor plerixafor, after chemo/radiotherapy with the chemotherapeutic agent temozolomide (TMZ), suggests CXCR4-targeting as beneficial for patient survival and local control of tumor recurrence[35]. Finally, a clinical study (NCT03746080) has been recently initiated to better characterize the use of plerixafor in combination with whole-brain radiation therapy and TMZ.

Other receptors such as CCR5, CCR6, CXCR2 and CX3CR1 were mostly identified for their expression and function in immune cells from the tumor microenvironment. Despite the lack of supporting data from the literature, CCR1, CCR10, ACKR1 and ACKR2 also appear as significantly expressed in glioma tissue and deserve thus deeper investigation.

Although our study focuses on human classical and atypical chemokine receptors, Herpesviridae-encoded G protein-coupled receptors (GPCRs), homologous to human chemokine receptors, were also proposed to be important players in GBM. For instance, HCMV encodes for four viral GPCRs (US27, US28, UL33 and UL78) among which the oncomodulatory activities of US28 and UL33 have been recently described in GBM models[110-112].

Overall, this review highlights the intricacies of chemokine receptor activity in glioma, from central roles in glioma cells to key functions in TME partners and tumor-associated vasculature. It also highlights the complex and sometimes opposing roles certain receptors may have in GBM and related TME, making their targeting challenging and the benefits thereof uncertain. Therefore, the present study constitutes a valuable tool to gain awareness on receptor expression and function in GBM, which is fundamental for the development of efficient therapeutic approaches that would have the chemokines-chemokine receptors axis as main target.

Conflict of Interest

The authors declare no conflict of interest.

Authors contribution

Conceptualization: D.I., G.D'U., A.C., V.N.; Investigation: D.I, G.d'U., M.W., V.N.; Writing—original draft preparation, D.I, G.D'U., M.W., V.N.; Writing—review and editing, B.R., A.L., A.C., M.S., V.N.; Supervision, B.R., A.L., A.C., M.S., V.N.; Funding acquisition, B.R., A.C., M.S., V.N. All authors have read and agreed to the published version of the manuscript.

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Chapter V – Brain malignancies

ii) Chemokines in glioma

My contribution to this Chapter:

This article represents an important outcome of my PhD thesis, it is a follow-up study to the chemokine receptor analysis (Chapter V.i). To expand our understanding of the involvement of the chemokine system in GBM, for this second study, we decided to focus on the chemokines instead of the receptors and to dissect and discuss the most abundant and relevant ligand-receptor interactions in such malignancies.

I contributed to the conceptualization of this study. I took care of the extraction of bulk RNA-seq data from different datasets and generated several figures (Figure 1; Figure 2; Figure 4; Supplementary Figure 1 and Supplementary Figure 2). I was involved in the definition and the design of the figures derived from single-cell based RNA sequencing resource (Figure 3, Figure 5 and Figure 6). I prepared the tables (main and Supplementary). I took care of the writing of initial draft, including the following sections: Abstract, Introduction, Material and Methods, and the Results sections: *Global profiling of chemokine expression in gliomas* and *Relevant chemokines – chemokine receptors axes in GBM Chemokines*. In addition, I contributed to the general manuscript writing and editing and coordinated the exchanges and discussions between the co-authors.

This Chapter is under review at *Cell communication and signaling.*

Highlights & aims

Transcriptomic datasets from patient tissue cohorts are crucial resources for clinically relevant research.

Chemokine receptors were shown to be largely present in GBM tissue (chapter V.i), but, for this study we decided to focus on their often overlooked partners, the chemokines. Among 43 chemokines, we found that 18 of them are abundantly expressed in glioblastoma patient tissue. Different subregions within GBM tumors were analyzed and we observed expression in several areas for some chemokines and specific tissue enrichment for some others, that could suggest a precise function (e.g. CXCL8, CXCL12 and CCL20). Single-cell approaches delineate chemokine expression profiles in cells from TME but it was crucial to shed light on the chemokine-receptor crosstalks among different cell subset of the GBM tumor mass. CCL5/CCR1, CXCL16/CXCR6, and CXCL12/CXCR4/ACKR3 pathways appear to drive putative cell-cell interactions in GBM.

This analysis has potential to serve as a handbook for those interested in the relevance of chemokines in the context of cancer in general and GBM in particular, and to address novel immunomodulatory and antitumor approaches.

Patient-based multilevel transcriptome exploration highlights relevant chemokines and chemokine receptor axes in glioblastoma.

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Keywords: glioblastoma; chemokine; tumor microenvironment, single cell RNAseq; CellChat

Abstract

Chemokines and their receptors form a complex interaction network, crucial for precise leukocyte positioning and trafficking. In cancer, they promote malignant cell proliferation and survival but are also critical for immune cell infiltration in the tumor microenvironment. Glioblastoma (GBM) is the most common and lethal brain tumor, characterized by an immunosuppressive TME, with restricted immune cell infiltration. A better understanding of chemokine-receptor interactions is therefore essential for improving tumor immunogenicity. In this study, we assessed the expression of all human chemokines in adult-type diffuse gliomas, with particular focus on GBM, based on patient-derived samples. Publicly available bulk RNA sequencing datasets allowed us to identify the chemokines most abundantly expressed in GBM, with regard to disease severity and across different tumor subregions. To gain insight into the chemokines–receptor network at the single cell resolution, we explored GBmap, a curated resource integrating multiple scRNAseq datasets from different published studies. Our study constitutes the first patient–based handbook highlighting the relevant chemokine–receptor crosstalks, which are of significant interest in the perspective of a therapeutic modulation of the TME in GBM.

1. Introduction

Gliomas are glial primary tumours of the central nervous system (CNS), which are classified according to the World Health Organization (WHO) into different grades and subtypes depending on their histological features and molecular profile[1]. The importance of integrating molecular profiling with the histological characteristics, which have been the longstanding criteria for diagnosis of specific glioma types, has been introduced in the 2016 CNS WHO classification and confirmed in the 2021 edition[1,2]. Critical molecular features include mutations in the isocitrate dehydrogenase 1 or 2 (IDH1/2 mut) and codeletion of the short arm of chromosome 1 and the long arm of chromosome 19 (1p/19q codel), which distinguish diffuse gliomas into WHO grade 2-3 oligodendrogliomas (IDHmut, 1p/19q codel) and low- to high-grade astrocytomas (IDHmut, 1p/19q intact). IDH wild-type tumours correspond to glioblastoma (GBM, IDHwt)[1,3,4], which is the most aggressive subtype that accounts for approximately 54.7% of adult gliomas[5] and is characterized by additional molecular features (e.g. chr7/chr10 copy number alterations, and/or TERT promoter mutations). With the standard-of-care therapy associating maximal safe resection and concomitant radio-chemotherapy with temozolomide (TMZ), the median survival after diagnosis is around 13 months[4,6,7]. The progression of the disease is characterized by

systematic recurrences, mostly explained by the infiltrating nature of GBM cells that penetrate through the surrounding brain tissue, hampering complete tumour resection[8,9]. Plasticity and heterogeneity of GBM tumours additionally lead to intra-tumoural and inter-patient variability with regard to tumour progression and response to treatment[10-14]. Finally, GBM tumours strongly rely on an immunosuppressive tumour microenvironment (TME) that supports their growth and resistance to therapy[13,15,16].

In different solid tumours, including GBM, chemokines and chemokine receptors have been identified as critical players in shaping of the TME[17-19]. Chemokines are small soluble chemotactic cytokines that are able to bind and activate the related classical as well as atypical chemokine receptors[20,21]. So far, 43 human chemokines have been described and based on the positions of the first cysteine residues, they are classified into four classes (CCL, CXCL, CX3CL and XCL)[20-22]. They can be categorized on a functional basis[23], as (i) homeostatic, showing expression in steady-state conditions; (ii) inflammatory, whose expression is tightly regulated, and rapidly increases during inflammatory processes to specifically recruit immune cells; or (iii) mixed-function chemokines[23-25]. Chemokines and their receptors have been shown crucial for cancer cell proliferation, migration and invasion, in addition to mediating the crosstalk between cancer cells and tumour-associated immune cells[26-28]. Based on publicly available datasets, we have recently identified and described the chemokine receptors most highly expressed in glioma patient tissue and their putative role in malignant processes. This study confirmed the implication of CXCR4 and ACKR3 in GBM, and revealed a potential involvement of other receptors, although their exact role in brain tumours remains to be characterized. This thorough receptor analysis did not, however, consider their ligands, chemokines, which all have their own unique expression profile and function[29]. Therefore, with the present study we aimed at providing a more exhaustive patient-based handbook, highlighting the impact of chemokine–receptor interactions in glioma processes. Using publicly available patient-related data, we explored the chemokines that are expressed in adult-type diffuse gliomas and elaborated on the important cellular crosstalks within the TME that rely on chemokines and their receptors.

2. Material and Methods

Analysis of The Cancer Genome Atlas (TCGA), low grade glioma (LGG) – glioblastoma (GBM) dataset (2016)

Gene expression levels of the whole panel of human chemokines were inspected: the CC chemokine family (CCL1–5, CCL7, CCL8, CCL11, CCL13–28), the CXC chemokine

family (CXCL1–14, CXCL16 and CXCL17), the two XCL chemokines (XCL1 and XCL2) and CX3CL1 by bulk RNA sequencing (HiSeq). Data were extracted from patient-derived tumour samples from adult-type diffuse gliomas by investigating the LGG-GBM TCGA[30] dataset with the use of the GlioVis platform (http://gliovis.bioinfo.cnio.es/). Patients from this dataset were grouped according to their IDH and 1p/19q codeletion status and clinical grade: (A) IDHmut, 1p/19q co-deleted tumours (WHO grade 2/3, n=169), (B) IDHmut, 1p/19q intact astrocytomas of variable grade (WHO grade 2/3/4, n=256), (C) IDHwt tumours (n=229), that include GBMs (WHO grade 4) but also a subset of tumours that are assessed as WHO grade 2 or 3 in the initial dataset (n=94), mostly based on histological aspects of astrocytomas/oligoastrocytomas. 67% of these samples (57/85) show chr7/chr10 copy number alterations and/or TERT promoter mutations, which rather speaks for GBM IDHwt, WHO grade 4 according to the 2021 WHO CNS classification. However, substantial differences in chemokine expression could be noticed with respect to the initially described tumour grade, which we therefore displayed in two subgroups.

Analysis of the Genotype-Tissue expression (GTEx) normal tissue dataset (and comparison with the TCGA GBM dataset)

In order to further explore chemokine expression in gliomas, with particular focus on GBM, we used Gene Expression Profiling Analysis (GEPIA2)[31] was used to compare bulk RNAseq data from GBM TCGA dataset (163 GBM tissue samples) to normal human brain from GTEx (207 tissue samples). The RNAseq dataset GEPIA2 is based on the UCSC Xena project (http://xena.ucsc.edu), which is computed by a standard pipeline. Bar graphs were generated by querying the whole panel of human chemokines (Supplementary Table 1) as multiple gene comparison. Tumour data and matched normal data were downloaded from http://gepia2.cancer-pku.cn/#analysis.

Analysis of the Ivy Glioblastoma Atlas Project (Ivy GAP) dataset (2018)

Chemokine expression was assessed in different GBM tumour subregions with the use of Ivy Glioblastoma Atlas Project (Ivy GAP)[32]. This dataset associates anatomic structural features and transcriptomes from bulk RNAseq GBM samples (122 samples from 10 GBM patients). In this study, five structures identified by hematoxylin-eosin staining and isolated by laser microdissection were screened. These include the three major anatomic regions of a tumour: (1) the leading edge (LE), namely the outermost margin of the tumour, (2) the infiltrating tumour area (IT), and (3) the cellular tumour core (CT). In addition, structural features can be observed within the tumour core like (4) microvascular proliferation (MVP),

marked by the presence of at least two neighboring blood vessels and (5) pseudopalisading cells around necrosis (PAN), densely aligned tumour cells surrounding necrotic areas. We explored the expression levels of the different chemokines (CCL1–5, CCL7-8, CCL11, CCL17, CCL19–28, CXCL1-14, CXCL16-17, XCL1-2, CX3CL1) focusing on the five tumour subregions that were identified by reference histology as filtering criteria (LE, IT, CT, MVP and PAN). Data were downloaded from https://glioblastoma.alleninstitute.org.

Analysis of the GBmap resource

To decipher and determine chemokine expression in different cell subtypes associated with GBM, we explored the GBmap[33], a curated resource integrating multiple scRNAseq datasets from different published studies. The entire GBmap dataset was downloaded from the data link provided in the corresponding publication on BioRxiv. Here, we used the 'core GBmap' reference dataset (referred to as GBmap onwards) that contains over 330'000 cells from 110 patients[33]. As the GBmap data had already been integrated and pre-processed by the authors, it was not subjected to any additional preprocessing before we generated corresponding dot plots and intercellular ligand-receptor interaction plots. Cellular distribution of the human chemokines (and their receptors) in GBM was investigated using our gene list (Supplementary Table 1) as the values for the "features" parameter of the DotPlot function in the Seurat package for R to visualize gene expression changes in the form of a dot plot. Of note, the chemokines CCL1, CCL3, CCL11, CCL14, CCL15 and CCL16 and CCL21 were not detected in the GBmap dataset and were therefore excluded from the analysis. The chemokines CCL24, CCL25, CCL27, CXCL4 and CXCL17 were represented in the dataset at barely detectable levels, not differentially expressed across the different annotations in the GBmap.

GBmap was used as an input for *CellChat*[34,35] package in R to infer intercellular communication network and signalling pathways of selected chemokines (CCL2, CCL4, CCL5, CCL20, CXCL10, CXCL12, CXCL16, CXC3CL1) and related receptors (CCR1, CCR5, CCR6, CXCR3, CXCR4, CXCR6, ACKR3, CX3CR1). CellChat integrates gene expression data with literature-supported and manually-curated databases of ligand–receptor interactions in human. The exploration, analysis and visualization of inferred networks were performed using default parameters of relevant CellChat functions. Different visualization packages of R were used to improve the quality of plots and plot annotations (Seurat, ggplot2, cowplot and patchwork)[36,37].

Table 1: General information about the datasets used in this study.

	1	2	3	4
Publication, project	[30] ***The Cancer Genome Atlas (TCGA Project)***	[32] ***Ivy Glioblastoma Atlas Project (Ivy GAP)***	[33] **Harmonized single-cell landscape, intercellular crosstalk and tumour architecture of Glioblastoma**	***Genotype-Tissue Expression (GTEx Project)***
Selected information	Gene expression in glioma subgroups (correlated with severity)	Gene expression in five anatomical locations within GBM tumours	Gene expression in different GBM-related cell subtypes	Gene expression in GBM and human brain
Online tool	https://www.cbioportal.org, http://gliovis.bioinfo.cnio.es	https://glioblastoma.alleninstitute.org	NA → R software	http://gepia2.cancer-pku.cn/#index
Method	Bulk RNAseq (HiSeq)	Bulk RNAseq (HiSeq) after laser microdissection	scRNAseq	Bulk RNAseq
Datasets and number of samples and patients	Brain lower grade glioma, LGG (513 patients) Glioblastoma, GBM 154 patients)	Glioblastoma (122 samples/10 patients)	Glioblastoma (338’564 cells/110 patients)	Human brain (207 tissue samples)
Data expressed as	Log2 RSEM	Log2 RSEM	Normalized expression matrix (log-scale)	Log2(TPM+1)

Legend: GBM: glioblastoma; GTEx: Genotype-Tissue Expression; LGG: low-grade glioma; RNAseq: RNA sequencing; scRNAseq: single-cell RNA sequencing; RSEM: RNAseq by expectation maximization; TPM: transcripts per million

The whole panel of human chemokines was investigated across the four datasets, however some genes were not included in Ivy GAP and/or GBmap. For each dataset, gene expression was displayed for the genes for which data were available (Supplementary Table 1). For chemokines showing multiple isoforms, the expression of the major isoform was reported. Original publications, online platforms, RNA sequencing methods, number of samples and patients included in the datasets are listed in the Table 1. Original data were not subjected to any modification or recalculation, existing data were filtered to generate original heatmaps or dot plots.

3. Results

Global profiling of chemokine expression in gliomas

We have previously evaluated chemokine receptor expression in various types of gliomas, in an attempt to decipher the complex chemokine-chemokine receptor network. To deepen this characterization, we here focused on the ligands of these receptors, the chemokines. We therefore investigated the relevance of the 43 human chemokines in glioma tissue by applying an approach similar to our previous analysis[29]. With the use of online platforms, we explored four publicly available datasets providing gene expression data for the target chemokines in glioma patient-derived material[12,30,32,38]. Bulk gene expression data from patient-derived tumour samples from the TCGA LGG-GBM dataset were extracted and their analysis allowed us to monitor the chemokines most abundantly expressed in different adult-type diffuse gliomas.

In GBM tumours, we observed an important expression of several chemokines, namely CCL2, CCL3, CCL4, CCL5, CCL8, CCL20, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL14, CXCL16 and CX3CL1 (threshold arbitrarily placed at average RSEM $\geq$ 3.5). For most of the chemokines, gene expression increases with disease severity (CCL2, CCL5, CCL8, CCL20, CXCL1, CXCL3, CXCL8, CXCL9, CXCL10, CXCL11 and at lower level CXCL14), suggesting their involvement in malignant processes. Note that histology-based grade within the IDHwt tumour group appeared associated with the level of chemokine expression. Other chemokines showed elevated expression regardless of glioma grade (CCL3, CCL4, CXCL2, CXCL5, CXCL12, CXCL16 and CX3CL1). Importantly, the expression level in the normal brain should also be considered to truly spot the transcripts upregulated in the pathological condition. For example, CXCL14 and CX3CL1 showed an increased expression also in the normal brain (Supplementary Figure 1A). Conversely, the chemokines CXCL12 and CXCL16, which were among the most prevalent in all glioma subgroups, showed moderate expression in normal human brain samples (Figure 1 and Supplementary Figure 1A).

CXCL12 was highlighted in our analysis. Its relevance in glioma malignancy together with the related classical and atypical receptors CXCR4 and ACKR3, is largely supported by numerous preclinical and clinical studies[12,26,39-48]. NOX-A12 (olaptesed pegol) an RNA-aptamer neutralizer of CXCL12, has recently been tested in a phase I/II clinical trial (GLORIA trial, NCT04121455) in combination with radiotherapy and immunotherapy in GBM patients.

Our analysis also revealed the IFN-γ-inducible chemokine CXCL16 as highly expressed in different gliomas, including GBM patients. Consistently, CXCL16 was previously reported to be expressed in gliomas, where it was suggested to play a critical role in microglia polarization towards a tumour-supportive phenotype, as well as contributing to glioma cell proliferation, migration and invasion[26,49].

CX3CL1, also known as fractalkine, showed elevated expression across all glioma types (Figure 1). This chemokine is proposed to be involved in CNS homeostasis by reducing brain inflammation. Although elevated in non-tumour brain tissue, its expression in human gliomas increases with tumour progression and correlates with disease severity[49].

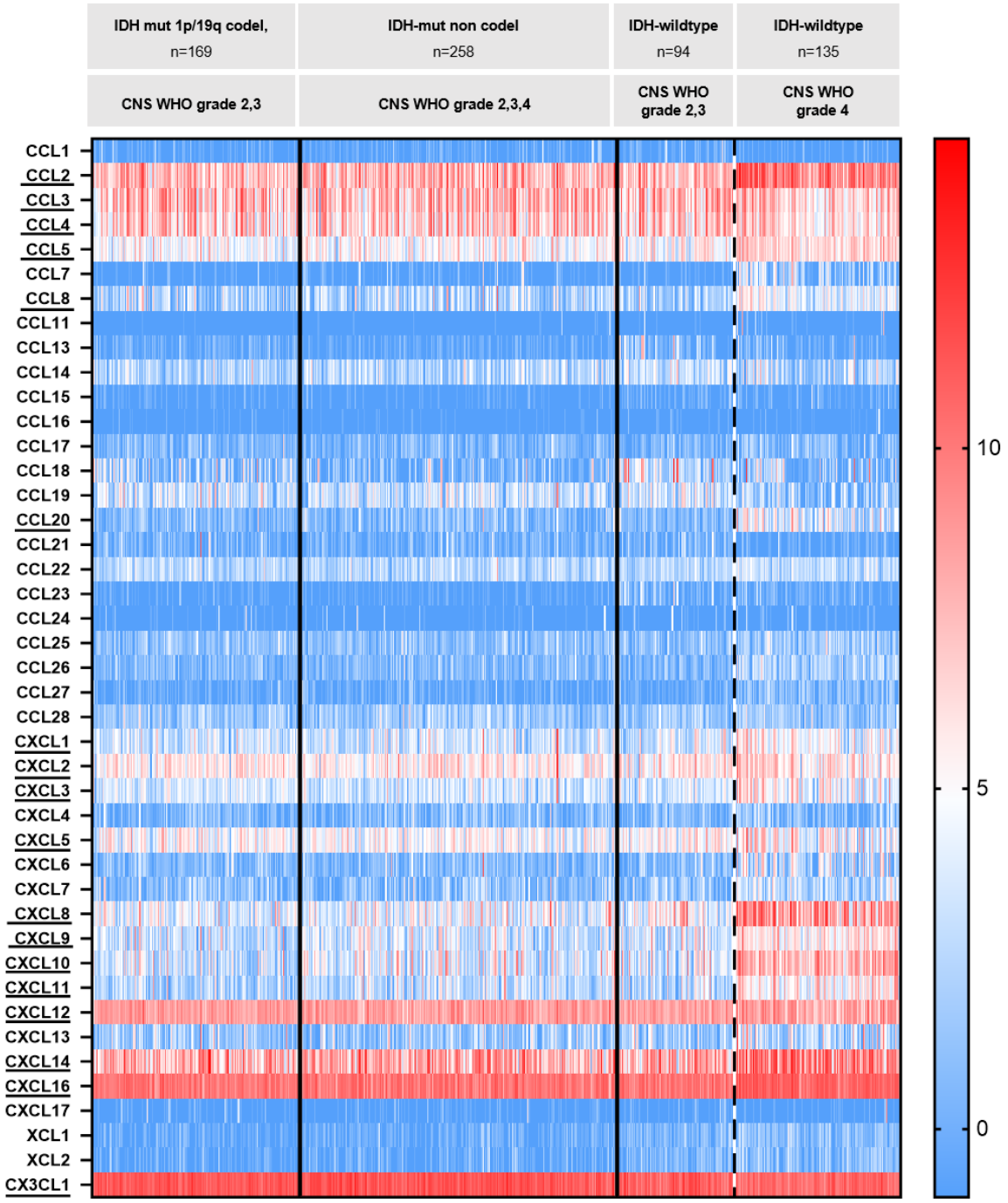

Figure 1. Chemokine expression in glioma patients (TCGA LGG-GBM datasets)[30]. The heatmap displays log2 normalized counts (RSEM) for the 43 human chemokines. Each cell represents one patient. The chemokines selected for further description in this study are underlined.

CCL2 was also spotted among the most abundantly expressed chemokines in the different tumour entities in particular in GBM. These findings are in agreement with previous results showing CCL2 protein expression in tumour samples from different glioblastoma and astrocytoma patient[50]. Serum concentrations of CCL2 were higher in GBM patients when compared to healthy individuals, which was also the case for CCL5[51]. Interestingly, these

aforementioned chemokines CXCL12, CXCL16, CX3CL1 and partially CCL2 were already suggested as important for tumour maintenance[52].

CXCL8, also known as IL-8, was one of the first chemokine to be detected in human brain tumours[53]. Its increased expression was confirmed in our analysis, particularly in GBM (Figure 1). Several studies highlighted its presence in patient-derived glioma tissues and glioblastoma stem cells (GSCs)[54,55].

Despite their accumulation in GBM, CXCL10 and CXCL14 (Figure 1) have not been intensively investigated in patient-derived material. A recent study describes CXCL14 production by tumour cells in different types of astrocytoma[56].

The chemokines CCL3, CCL4, CCL5, CCL8, CCL20, CXCL1, CXCL2, CXCL3, CXCL9 and CXCL11 showed only moderate expression in GBM (Figure 1). Those able to activate the chemokine receptors CCR1 and CCR5, (CCL3, CCL4, CCL5 and CCL8) have been the body of investigation of different studies, with CCL8 being highly produced by glioma-associated macrophages and stimulating invasive abilities of tumour cells[57-59]. Among the CXC-chemokines able to activate CXCR2, a receptor responsible for neutrophils recruitment to inflammatory sites, CXCL1, CXCL2 and CXCL8 were shown elevated in GBM and their upregulation correlates with poor prognosis[60,61]. Also CCL20, together with its cognate receptor CCR6 was detected in brain tumour samples, in contrast to non-neoplastic brain samples[62]. CXCR3 ligands CXCL9, CXCL10 and CXCL11 have not been extensively characterized in gliomas. Only a few divisive studies propose them as antitumour molecules, while others suggested a pro-tumoural role[63-66].

Finally, CCL7, CCL26, and CXCL5, CXCL7, CXCL13 show increased expression in GBM compared to lower-grade tumours, but their expression level is just above threshold, therefore their significance remains to be discussed (Figure 1).

Unravelling chemokine expression in GBM subregions

In light of the notable increase of different chemokines in GBM, we further investigated their expression in this glioma subtype. The intra- and intertumoural heterogeneity of GBM constitutes one of the major challenges in neuro-oncology[13]. Various regions and niches have been described within these tumours, such as invasive, hypoxic, necrotic and vascularized areas. The Ivy Glioblastoma Atlas Project (IvyGAP) correlates the anatomo-histological features of GBM with genomic and gene expression patterns from a panel of GBM patients[32].

This freely accessible atlas allowed us to investigate the chemokine expression in the five different tumour areas, suggestive of their activity in these regions (Figure 2).

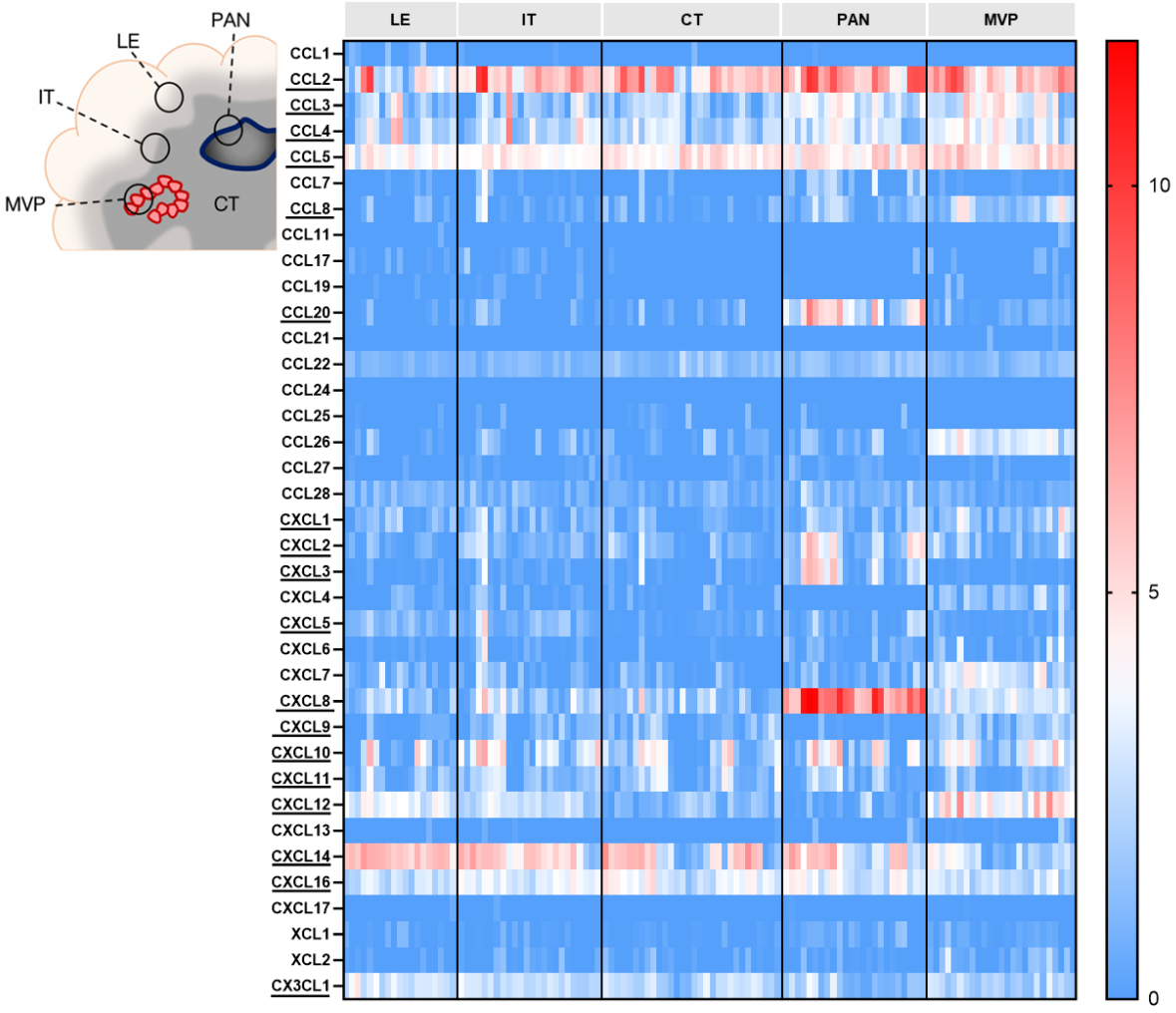

Figure 2. Chemokine expression in various areas of GBM tumours (IvyGAP project). The heatmap displays log2 RSEM normalized counts for each chemokine in the different tumour subregions. Each cell represents one sample. Legend: LE (leading edge); IT (infiltrative tumour); CT (cellular tumour); PAN (pseudopalisading cells around necrosis); MVP (microvascular proliferation). The chemokines found enriched in TCGA analysis (Figure 1) are underlined.

The assessment of RNAseq profiles revealed that certain previously highlighted chemokines displayed similar expression profiles across different tumour areas. The expression of CCL2 and CCL5 was well detectable in almost all areas. CXCL14 also showed noticeable expression in all regions, which is likely due to its basal expression in the brain tissue. On the other hand, we noted region-specific differences in the expression of several chemokines. The chemokines CXCL10 and CXCL16 were also detected in GBM but showed a rather heterogenous distribution within the tumour mass. Chemokine enrichment in certain tumour regions may reveal their role in specific cell types or in regulating tumour-associated

processes, e.g. angiogenesis or cell invasion. It could also indicate how a given chemokine is involved in GBM cell adaptation to its local environment. In the next paragraphs, we develop a few aspects of the existing knowledge about chemokine function with regard to their expression in the areas of interest, mainly MVP and PAN.

- ***Chemokines mostly associated with microvascular proliferation (MVP) regions***

The analysis showed the expression of CCL2 and CCL5 in the MVP regions, and to a lower extent also of CCL3 and CCL4 if compared to other areas (Figure 2). Consistently, *in vitro* results have shown that CCL2, CCL3 and CCL5 were produced by brain endothelial cells upon inflammatory conditions[67], as well as CXCL8 and CXCL10[68]. These chemokines have been also detected in endothelial cells from the brain of patients with multiple sclerosis[68]. Interestingly, CCL3 and CCL4 have been found in glioblastoma patient tissue as predominantly expressed in a specific subset of endothelial cells, associated with an inflammatory phenotype, as identified by single cell sequencing[69]. The inflammatory status of a tumour therefore appears as an important driver of chemokine expression in tumour-associated endothelial cells. CCL3 has also been described to promote the expression of the vascular endothelial growth factor-A (VEGF-A), an important inducer of angiogenesis involved in the progression of different cancer[70].

More evidently, CXCL12 was particularly abundant in the MVP regions (Figure 2), in line with the immunohistochemical CXCL12 detection in proliferating endothelial cells[71] and more generally, tumour-associated blood vessels in patient samples[72,73]. This is in concordance with the well-described CXCL12 production by endothelial cells, underlying their crosstalk with tumour cells through CXCR4 or ACKR3 activation in different models of gliomas[74,75]. CXCL12/CXCR4-mediated angiogenesis inhibition has been the rationale for the clinical studies in GBM patients using plerixafor (CXCR4 antagonist) in combination with bevacizumab[76].

Cancer cell migration and invasion are closely linked to angiogenesis in gliomas[77,78]. Indeed, the infiltrative behaviour of GBM cells is one of the hallmarks of this aggressive tumour[77,79,80]. In physiological conditions, the role of CXCL12 in directing cell migration during neural development has already been described[81,82]. Furthermore, CXCL12 is a well-known driver of cell migration and homing in the hematopoietic system[83]. In addition, CXCL12 is also involved in gliomagenesis, as it was detected in 31 GBM cases with an increased expression in neurons, blood vessels, subpial regions and white matter, consistent with its proangiogenic role[71].

- ***Chemokines mostly associated with pseudopalisading cells around necrotic (PAN) regions***

Gliomas are highly heterogeneous tumours with widely distributed hypoxic areas[84,85], where several chemokines are also implicated. In our analysis, the expression of CCL20 and CXCL8 was elevated in the PAN regions (Figure 2), suggesting a role in modulating cell phenotypes in a hypoxic, necrotic environment, and in regulating the PAN-neighbouring environment. Accordingly, immunochemical analyses have highlighted CXCL8, also named IL-8, in perinecrotic areas in GBM patient tissue[85]. Mechanistically, *in vitro* evidence suggested indeed that necrotic cells favour CXCL8 secretion in GBM cells via NF-kB and AP1 signaling[86]. CXCL8 has also been endowed with proangiogenic activities[87], together with CXCL2, especially on brain endothelial cells[88]. Of note, CXCL2 and CXCL3 seemed to be enriched in the PAN region in comparison to other tumour regions (Figure 2). Additionally, CXCL8 was found to be secreted by mesenchymal (MES) glioma stem-like cells (GSCs) which stimulated endothelial proliferation and tube formation, suggesting a proangiogenic role especially in the MES tumours that are associated with an increased vascularization compared to other tumour subtypes[89].

Similarly, CCL20 has been shown to be produced by GBM cells upon contact with necrotic cells, which was associated with improved microglial infiltration[90]. Another *in vitro* study revealed that CCL20 was secreted by astrocytes in hypoxic conditions and acted on glioma cells via CCR6 and NF-kB to induce the expression of the hypoxia-inducible factor 1α (HIF-1α), thus sustaining tumour growth[91].

Of note, CXCL8 and CCL20 both appeared upregulated in the WHO grade 4 IDHwt tumours, compared to WHO grade 2/3 (Figure 1). This observation supports the idea that particular GBM-associated histological features (e.g. necrotic areas) may be related to chemokine expression in the tumour sample.

Chemokine in diverse cell entities within the GBM ecosystem

It is considered that one of the main causes of treatment resistance and relapse in GBM lies in the huge complexity of this TME[11,13]. The TME is made up of different cell types, such as vascular cells and immunosuppressive cells which favour tumour growth, proliferation and maintenance. This close interaction between GBM cells and surrounding stroma influences disease progression and patient outcome[92]. Myeloid cell types within the GBM TME include microglia, infiltrating monocytes and macrophages, neutrophils, etc[93-97]. In contrast, GBM is characterized by a low number of tumour-infiltrating lymphocytes

(TILs)[98]. TME composition and molecular features vary across tumours[99] but also over time, along tumour development[100]. Chemokines and their receptors tightly regulate immune cell recruitment and activity, and therefore may have important implications in the GBM TME.

To determine chemokine expression in different cell subtypes associated with GBM, we explored the GBmap, a curated resource integrating multiple scRNAseq datasets from published studies[33]. GBmap constitutes the largest integrated scRNAseq database of GBM samples to date[33].

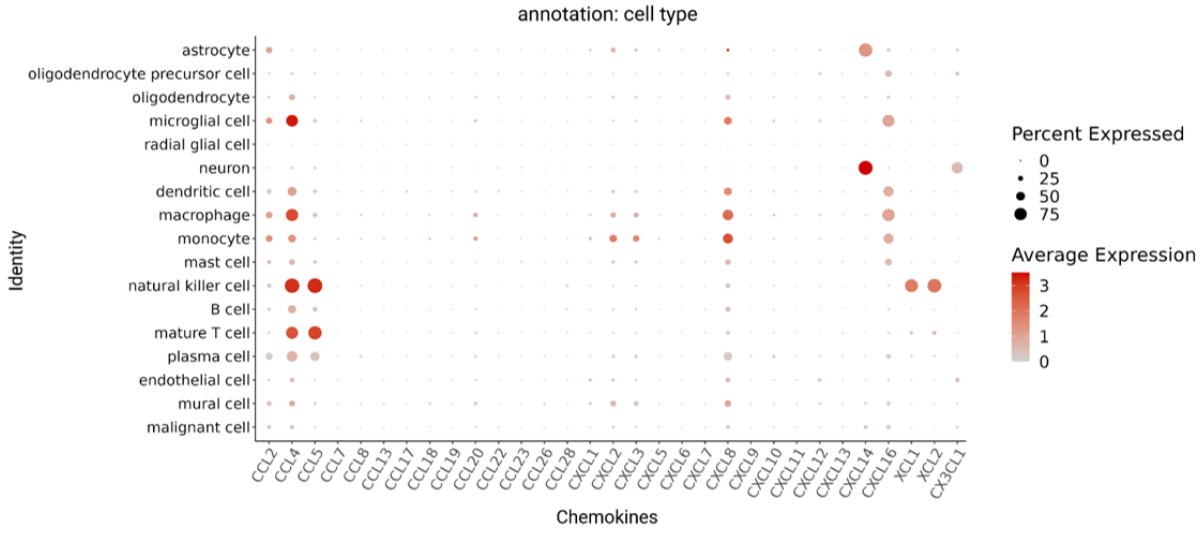

Figure 3. Chemokine expression in different cell types detected within GBM tumours from GBmap[33]. The size of the dots corresponds to the percentage of the cells within a cell type expressing the gene, while the intensity of the colour represents the average expression level.

We examined the expression of all human chemokines in different cell-type related annotations reported in the dataset (Figure 3 and Supplementary Figure 2A). Chemokine expression patterns varied across the cell types, with differences in both expression intensity and proportion of positive cells. Whereas several chemokines were broadly distributed across multiple cell types, the expression of others appeared more restricted to distinct cell entities. Among the most represented chemokines, CCL2 was predominantly expressed in monocytes, macrophages, microglial cells and astrocytes, with lower expression observed in other cell types. CCL2 has especially been associated with CD163-positive, M2-like macrophages in human GBM tissue[101]. CCL4 exhibited a strong expression in NK cells, microglial cells, macrophages, and T cells (Figure 3). This analysis also revealed an elevated expression of CCL5 by NK cells and mature T cells. CCL4 and CCL5 were also detected in various cell types such as plasma cells, B cells and mast cells albeit with considerably lower expression. CXCL8 showed a predominant expression by monocytes and macrophages, but

is also present in numerous other cell types, including natural killer cells and plasma cells. CXCL16 also demonstrated a relatively uniform expression across cell types but was predominantly found in myeloid cells, including microglial cells, monocytes, macrophages and dendritic cells. Finally, CXCL14 and CX3CL1 were mainly found in neurons and/or astrocytes, further supporting their association with the normal brain tissue rather than the tumour. It is also noteworthy that despite their low expression levels detected in glioma tissues by bulk RNAseq (Figure 1), the scRNAseq database analysis revealed a highly specific association of XCL1 and XCL2 with NK cells (Figure 3), as previously described in other cancer models[102]. CCL20 showed mild expression in monocytes and macrophages. CXCL2 and CXCL3, showed similar expression profiles, with a slightly higher expression in monocytes, but they were also weakly detected in various other cell types like macrophages and mural cells.

Overall, T lymphocytes, NK cells as well as microglia, monocytes and macrophages appeared as the major chemokine source in GBM tissue. Other more restricted cell populations (e.g. neurons) also specifically expressed several chemokines. In contrast, the malignant cells which are the principal tumour components did not show consistently elevated expression of any chemokine.

Relevant chemokines – chemokine receptors axes in GBM

Chemokines play a crucial role in the recruitment and activation of leukocytes in a spatiotemporal manner, shaping the TME and strongly influencing tumour cell proliferation and dissemination[103-105]. They exert these functions mainly through a paracrine action on various cell types differentially expressing their respective receptors. In our previous analysis[29], we elaborated on the role that these chemokine receptors could play in the different GBM-associated cell types, based on supporting literature data. Here, we also further examined the relation between the receptors that emerged in our first analysis[29] and the high expression level of chemokines in GBM. We observed a good concordance of the ligand–receptor axes. For the majority of chemokines revealed by the present study, the related receptors also showed higher expression as exemplified by CXCL1, CXCL2, CXCL3, CXCL5, CXCL8 and their receptor CXCR2; CXCL12 and CXCR4/ACKR3; CXC3CL1 and CX3CR1; CCL3, CCL4, CCL5 and CCR5, CCR1; CCL8 and CCR1; CCL20 and CCR6; CXCL1, CXCL3, CXCL5, CXCL8, CCL2, CCL5 and ACKR1; and finally CCL2, CCL3, CCL4, CCL5, CCL8, CXCL10 and ACKR2 (Figure 4).

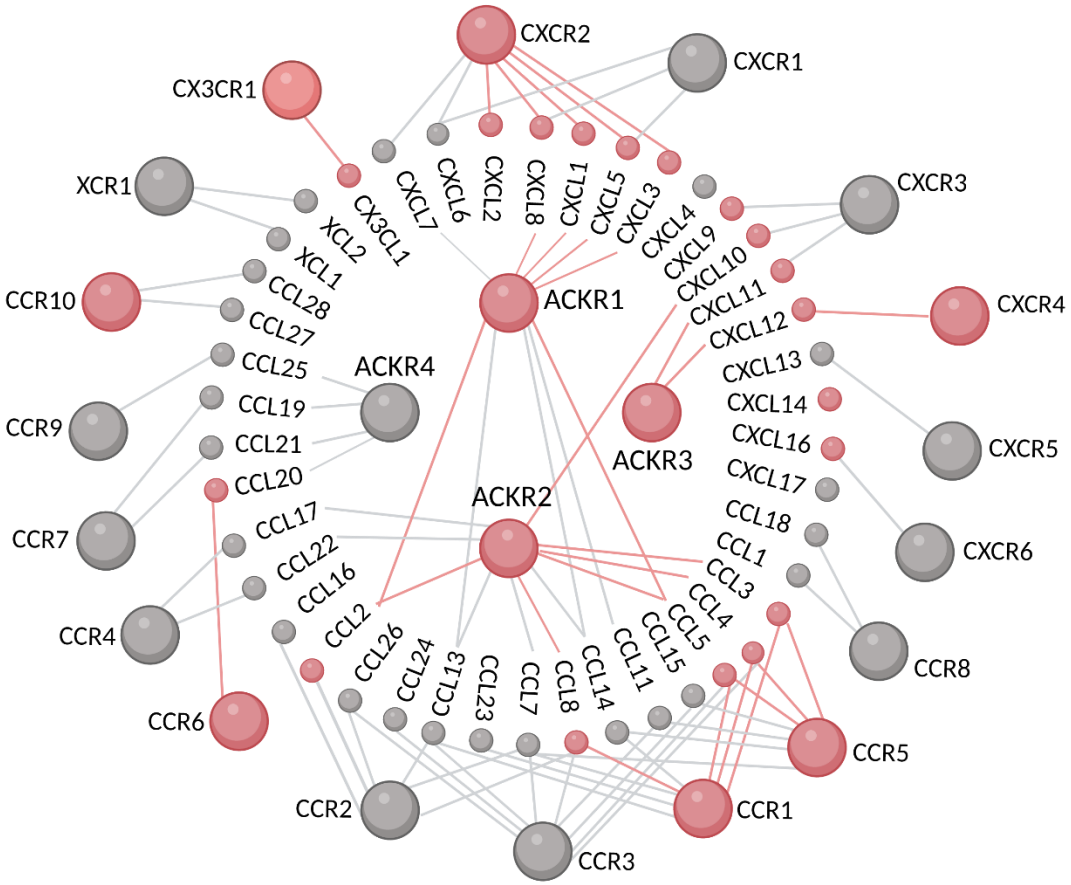

Figure 4. Representative image of the chemokine-chemokine receptor interaction network in gliomas. The chemokines receptors most abundantly expressed in gliomas from our previous analysis[29] are shown in red. Chemokines which emerged as highly expressed in GBM in the present study are visualized in red. For both the chemokine receptors and the chemokines, the selection is based on bulk RNAseq analysis from TCGA and arbitrary thresholds were set. When both the ligand and related receptor are found expressed above the related thresholds the connecting line is colored in red.

In contrast, while the chemokines CCL2, CXCL9, CXCL10, CXCL11, and CXCL16 were found in GBM (Figure 1-2), the expression level of their respective receptors CCR2, CXCR3 and CXCR6, although higher than in normal human brain (Supplementary Figure 1B), was below the threshold in our previous analysis[29]. However, these receptors may be present in minor cell subpopulations, which would result in an undetectable tumour expression at the bulk level. We therefore further explored the presence of these receptors at the single-cell level using the newly available, exhaustive GBmap dataset[33] (Supplementary Figure 2B) which revealed the expression of CXCR3 and CXCR6 in the mature T cell compartment. CCR2 was also detected, but at low level, in monocytes and dendritic cells (Supplementary Figure 2B). Of note, the receptor for CXCL14 is still unknown, which hinders the elucidation of the mechanism of action and role this chemokine plays in tumours.

With the aim to better understand the chemokine–chemokine receptor axes, we further exploited the GBmap[33] to analyse relevant molecular crosstalks between definite cell types. Specifically, normalized GBmap gene expression matrix was used as input for CellChat analysis to infer putative intercellular communication networks and signalling pathways focusing on the major chemokines identified in the present study, and on the receptors that we have shown as relevant[29] (Figure 5 and Figure 6). This analysis allowed us to visualize the putative interactions between different cellular actors within GBM tissue, based on chemokine and receptor expression levels.

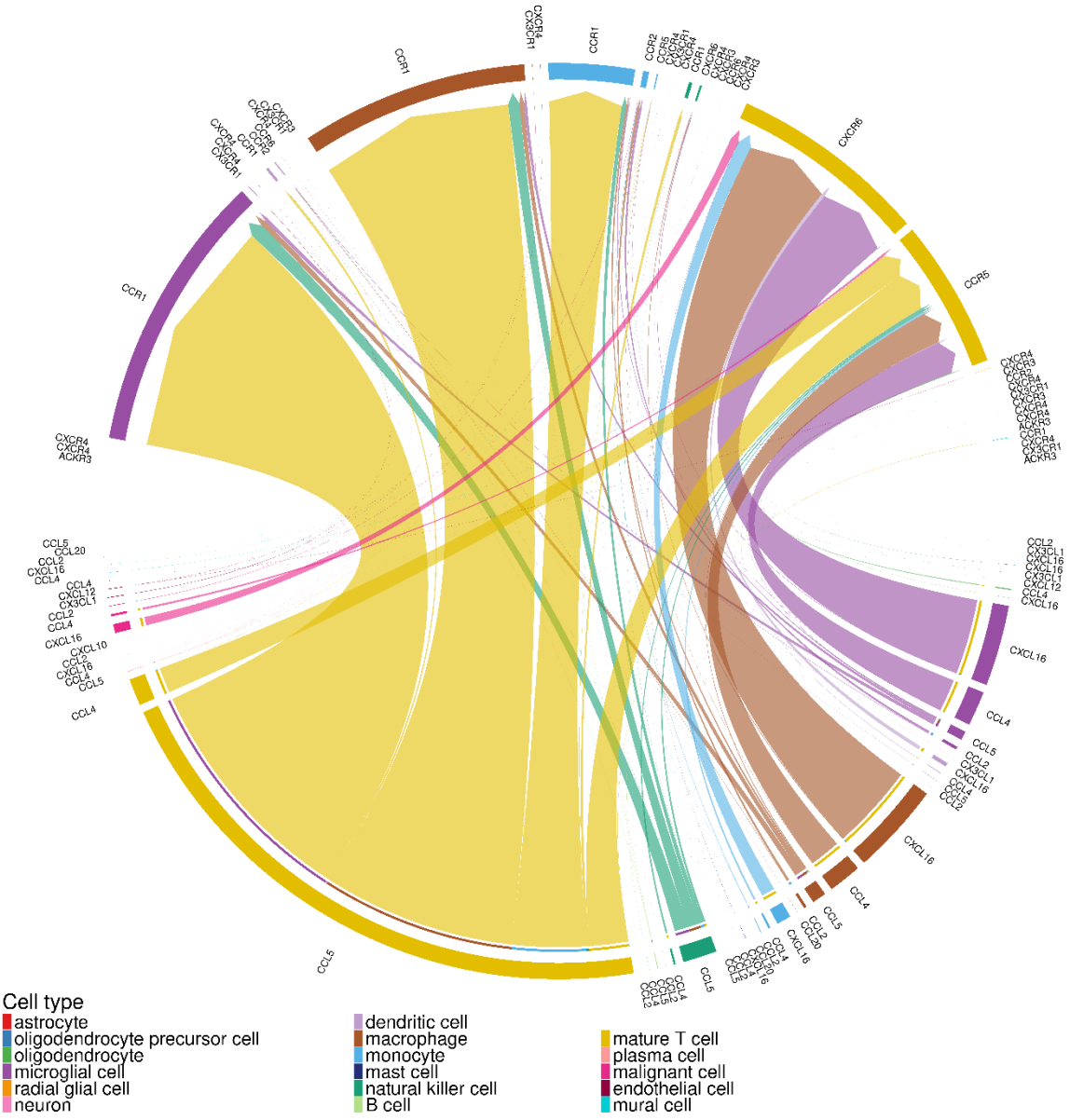

Figure 5. The chord diagram of the inferred cell-cell interactions. All the significant interactions (p-value < 0.05) shown were inferred through the CellChat calculations using the GBmap data as input. The direction of communication is represented by arrows and the strength of communication is indicated by the width of the connecting lines.

It showed a higher probability for interactions to occur between T cells that secrete CCL5 and microglia, macrophages and monocytes that express CCR1 (Figure 5, yellow arrows). These myeloid subsets were in turn proposed to interact with T cells based on their production of CCL4 and CXCL16 that respectively act on CCR5 and CXCR6 (Figure 5, brown and purple arrows). In line with these observations, another study using scRNAseq on multi-sector biopsies demonstrated that *CXCL16* was highly expressed in tumour-associated macrophages (TAMs)[106]. On the other hand, malignant cells express CXCL16, as previously observed[106], with a possible influence on T cells via CXCR6 (pink arrows). A recent study has shown that the glioma cells produce CXCL16 that acts via CXCR6 on microglial cells, driving them towards an anti-inflammatory states[50], however a crosstalk with T cells has not yet been described. Although these interactions appeared less strong, annotated NK cells also express CCL5, also potentially modulating microglia, macrophages and monocytes via CCR1 (green arrows).

Other ligand–receptor pairs were highlighted, albeit with a "reduced" likelihood, as potentially involved in a wide diversity of cell–cell interactions. For instance, a putative action of CXCL12 produced by endothelial cells and oligodendrocyte precursor cells (OPCs) on various CXCR4-expressing cell types within the TME could be revealed (Supplementary figure 2B). Moreover, the involvement of CX3CL1/CX3CR1 signalling in the communication between astrocytes and neurons, with diverse other tumour-associated cell types was suggested (Supplementary figure 2B).

In a similar fashion, a recent study emphasized the significance of the chemokine–receptor network in GBM. The receptor expression profile of GBM-infiltrating T cells, and the chemokine expression profile of non-lymphocyte GBM-associated cells was characterised using scRNAseq. It revealed that tumour infiltrating T cells were enriched in certain chemokine receptors (e.g. CCR2, CCR5, CXCR3, CXCR4, and CXCR6) suggesting their role in directing T cell migration into GBM. As for the non-lymphocyte GBM-associated cells, various chemokines such as CCL2, -4, -5, -20, CXCL1, -2, -3, -10, -11, -12, -16, and CX3CL1 were detected, each with different enrichment scores. Notably, CCL4 and CXCL16 were predominantly expressed by GBM-associated macrophages and microglia[107].

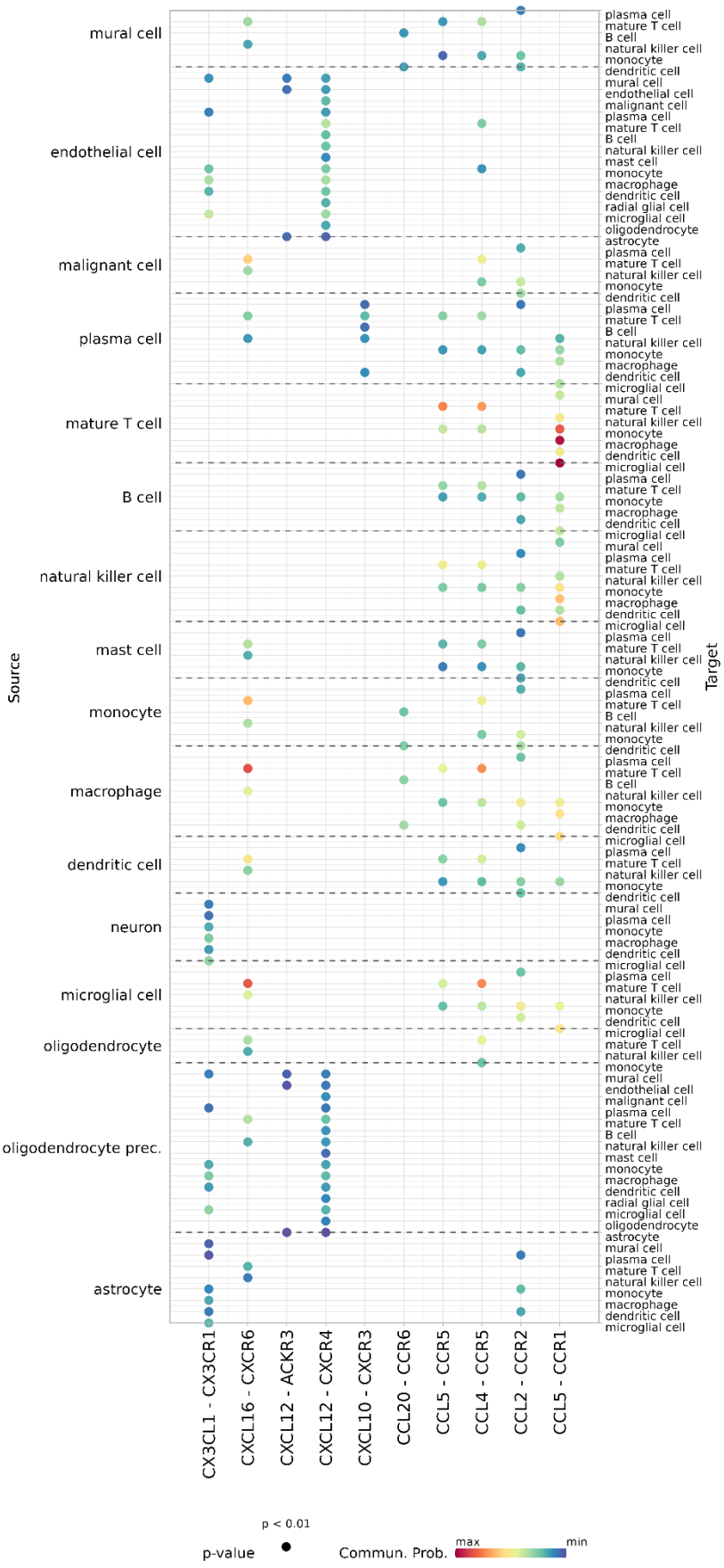

Figure 6. The dot plot of inferred CellChat interactions from the GBmap. The dot color and size represent the calculated communication probability and p-values.

4. Conclusions

Glioblastoma (GBM) tumour microenvironment (TME) is extremely important in driving tumour progression and response to therapy. Unlike in other tumour types, most of the immunotherapeutic approaches failed in demonstrating global efficacy in GBM patients, owing to the immune-privileged brain tissue but also the acquired tumour cell resistance to therapy[108]. A better understanding of the mechanisms that dictate GBM TME organization and evolution is therefore needed for establishing effective treatments. In this study, we aimed to investigate the expression of chemokines and their receptors in an exhaustive, patient sample-based approach with unique single-cell resolution and representation.

The first part of the study was based on bulk RNAseq analyses of snap-frozen glioma tissue from different patient cohorts. It revealed that many chemokines were expressed in these tissues, some of them correlated with glioma severity. The Ivy GAP dataset offered a greater resolution by exploiting microdissected tissue regions to highlight the chemokine expression in discrete functional subregions within the tumours (e.g. pseudopalisading cells around necrosis, microvascular proliferation, etc). Among them, the presence of CCL20 and CXCL8 appeared localised to perinecrotic areas. This illustrates how histological specificities may influence chemokine expression, which was also reflected in chemokine expression in tumours from different histological grades (CCL2, CCL5, CCL8, CCL20, CXCL8 and CXCL10).

Therefore, the more recent gene expression data based on dissociated, single cells and excluding peripheral blood cell contamination, provided an unprecedented knowledge on cellular functions within the tumours. We explored the GBmap resource, which, to date, constitutes the largest available scRNAseq dataset harmonized from previous studies. The most abundant cell annotations in this dataset are the malignant cells, macrophages, microglial cells and T-cells (Supplementary figure, 2A), whereas other neural or immune cell annotations are less represented. This scRNAseq data analysis essentially showed that chemokine expression is mostly associated with cells from the TME, including T cells, macrophages, or microglia. Consistently, the CellChat chemokine–receptor crosstalk analysis also linked these immune cell types, highlighting putative interactions with NK cells and monocytes. Our results show CCL5/CCR1 and CXCL16/CXCR6 axes as key duets in these immune cell crosstalks, which warrant further investigation. In parallel, the widely described CXCL12/CXCR4/ACKR3 axis appeared to dictate cell interactions within the tumour in a less prominent, but a rather universal manner. CXCL12 indeed appeared to be

expressed by endothelial cells and OPCs, and to act on a plethora of cell types expressing variable levels of CXCR4. This data also showed that malignant GBM cells had reduced chemokine expression, and also lower expression of chemokine receptors compared to non-malignant, TME cells.

It is crucial to note that the respective depiction of each annotation in the GBmap dataset does not reflect the genuine proportion of corresponding cell types within GBM tumours *in situ*. Malignant cells remain the major components, while macrophages are the most abundant immune cells in the TME, representing up to 30% of the tumour content. In contrast, infiltrative lymphoid cells are much scarcer[109,110]. Although this study provides key information about the cell types to which chemokine/receptor expression appear the most relevant (i.e. immune cells from the TME), it remains to be addressed to what extent it is also reflected clinically by immunomodulatory functions. Considering that gene expression data are captured as a tumour snapshot, further investigation using functional and dynamic GBM models is also warranted to determine whether chemokine-receptor signalling and related immune cell interactions actually drive tumour progression, emerge as a consequence of host tissue antitumour responses, or both.

Altogether, this analysis provides a comprehensive, in-depth assessment of chemokine and chemokine receptor expression in GBM cell types, using patient-based data, with important elaborations on how relevant cell types may work together in directing tumour outcome. The CCL5/CCR1, CXCL16/CXCR6, and CXCL12/CXCR4/ACKR3 pathways emerge from this analysis as relevant drivers of cell interactions. The study may serve as a handbook for those interested in the relevance of chemokines in cancer, and will help to pave the way for novel immunomodulatory and antitumor approaches.

Conflict of Interest

The authors declare no conflict of interest.

Authors contribution

Design of the study: GD'U, DI, AC, VN and MS. Investigation: GD'U, DI, BN, VN. Writing—original draft preparation: GD'U, DI, BN, MW, VN. Writing—review and editing: GD'U, DI, AK, MW, AG, BR, AC, VN, MS. Supervision: PN, AG, BR, AC, VN, MS. Funding acquisition: AG, BR, AC, VN, MS. All authors have read and agreed to the published version of the manuscript.

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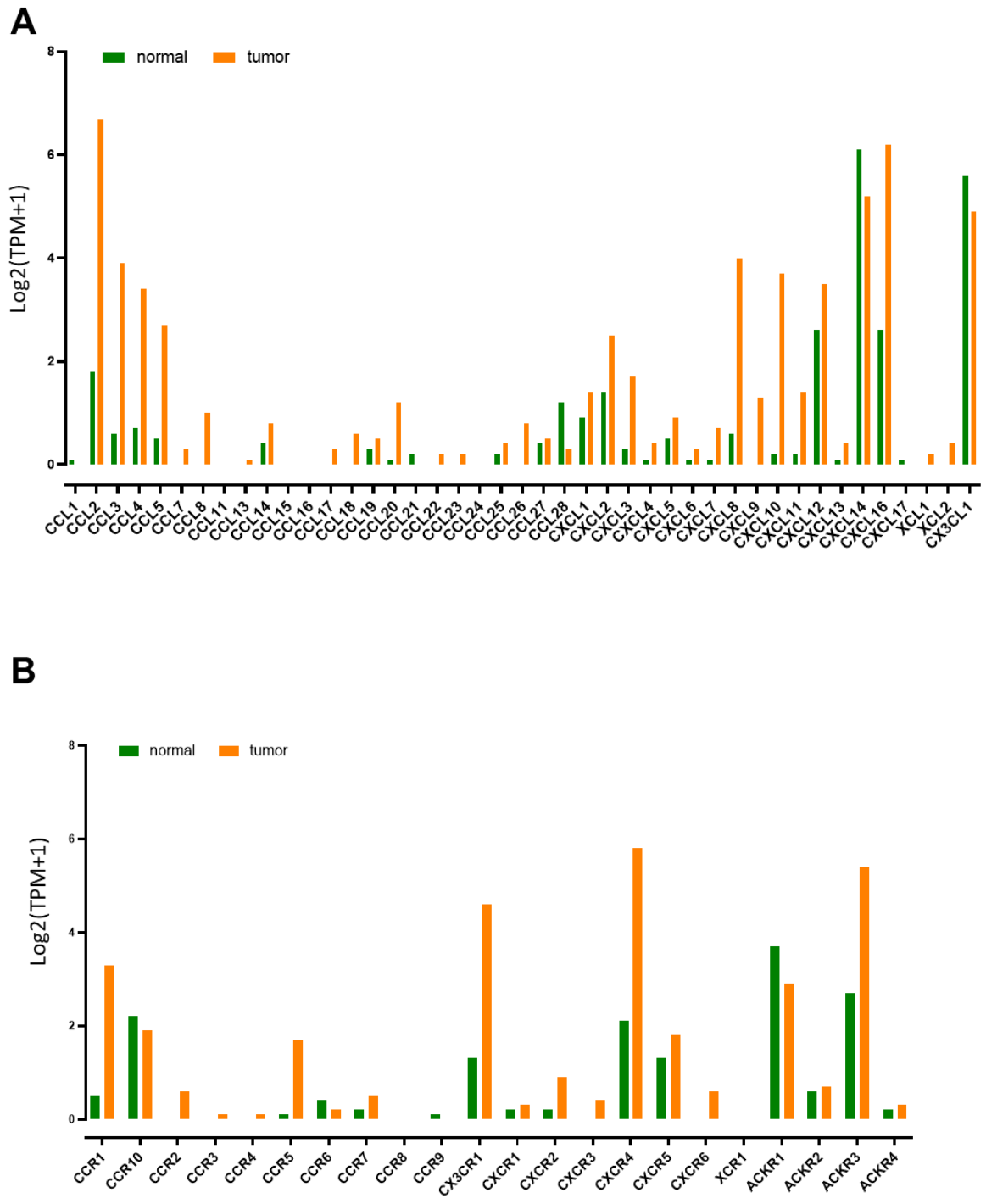
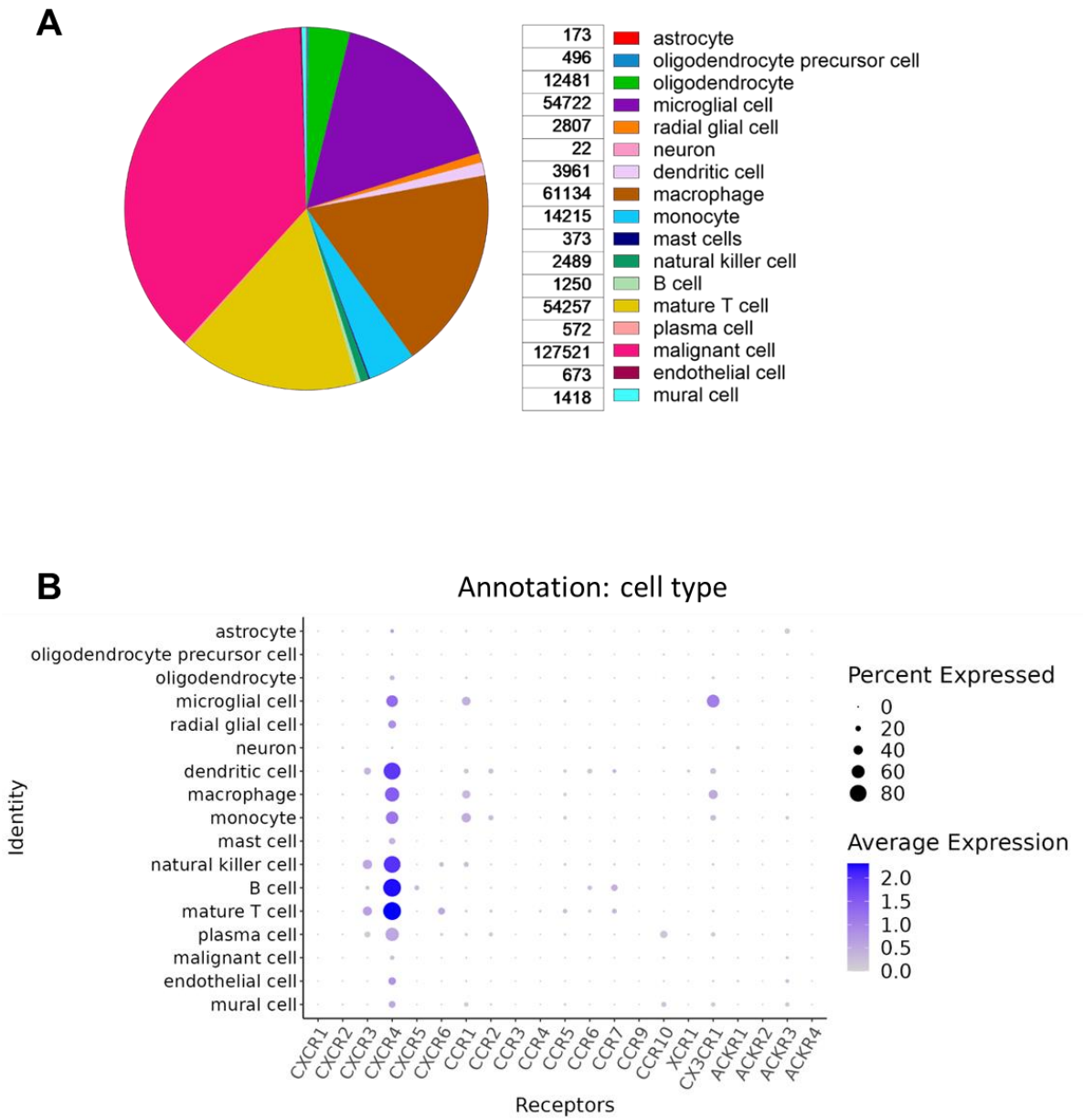
Supplementary material



Supplementary Figure 1. Comparison of gene expression in GBM (163 tissue samples from TCGA GBM dataset) to normal brain (207 tissue samples from GTEx Brain) for (A) the chemokines and (B) their receptors. Bar graphs display data expressed as log2(TPM + 1).



Supplementary Figure 2. (A) Cells distribution in each category of the cell type annotation. The precise number of cells per cell type gathered in the harmonized GB dataset is reported. The individual 16 studies made use of different purification cell purification strategies, therefore the proportions are not retained. (B) Expression of chemokine receptors in different cell types from GBmap. The panel of human chemokines is used as values for the "features" parameter of the DotPlot function in the Seurat package for R to visualize gene expression changes in the form of a dot plot. The size and color of each dot in the dot plots represent the percentage of cells within a class and the average expression of a gene within a class, respectively.

Supplementary Table 1 – chemokine list

	TCGA	Ivy Gap	Gbmap	GTex
CCL1	x	x		x
CCL2	x	x	x	x
CCL3	x	x		x
CCL4	x	x	x	x
CCL5	x	x	x	x
CCL7	x	x	x	x
CCL8	x	x	x	x
CCL11	x	x		x
CCL13	x		x	x
CCL14	x			x
CCL15	x			x
CCL16	x			x
CCL17	x	x	x	x
CCL18	x		x	x
CCL19	x	x	x	x
CCL20	x	x	x	x
CCL21	x	x		x
CCL22	x	x	x	x
CCL23	x		x	x
CCL24	x	x	x	x
CCL25	x	x	x	x
CCL26	x	x	x	x
CCL27	x	x	x	x
CCL28	x	x	x	x
CXCL1	x	x	x	x
CXCL2	x	x	x	x
CXCL3	x	x	x	x
CXCL4	x	x	x	x
CXCL5	x	x	x	x
CXCL6	x	x	x	x
CXCL7	x	x	x	x
CXCL8	x	x	x	x
CXCL9	x	x	x	x
CXCL10	x	x	x	x
CXCL11	x	x	x	x
CXCL12	x	x	x	x
CXCL13	x	x	x	x
CXCL14	x	x	x	x
CXCL16	x	x	x	x
CXCL17	x	x	x	x
XCL1	x	x	x	x
XCL2	x	x	x	x
CX3CL1	x	x	x	x

Supplementary Table 2 – chemokine receptor list

	Gbmap	GTex
CCR1	x	x
CCR2	x	x
CCR3	x	x
CCR4	x	x
CCR5	x	x
CCR6	x	x
CCR7	x	x
CCR8		x
CCR9	x	x
CCR10	x	x
CXCR1	x	x
CXCR2	x	x
CXCR3	x	x
CXCR4	x	x
CXCR5	x	x
CXCR6	x	x
XCR1	x	x
CX3CR1	x	x
ACKR1	x	x
ACKR2	x	x
ACKR3	x	x
ACKR4	x	x

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Conclusions and Perspectives

In this cumulative thesis, different aspects of the chemokine network have been investigated, with a particular focus on CXCR3, atypical chemokine receptors and glioblastoma. The different chapters describe new technologies, original findings or contextualize discoveries that, taken together, shed light on the intricate cross-talk governing leukocyte migration and positioning in health and diseases. I had the possibility to learn, develop, apply and optimize several Nanoluciferase-based technologies that contributed to expand the current understanding of chemokine receptor function, regulation, signalling, trafficking and pharmacology (*Chapters I, II, III and appendix*). Additionally, thanks to collaborative studies with the research group of Prof. Bernard Rogister and Dr. Virginie Neirinckx from the University of Liège (Belgium), I had the opportunity to deeply assess chemokine and chemokine receptor expression from a more translational perspective and contextualize their involvement in hard-to-treat brain malignancies *(Chapters V.i and V.ii).*

Starting my PhD thesis in 2020 during the pandemic was rather unusual and it affected the whole first year my doctoral studies. Despite this adverse event, I was able to integrate into the group and work in close collaboration with several PhD students of our laboratory but also external partners.

I started my PhD journey by contributing to an already-initiated analysis (*Chapter III*). The use of the methodologies detailed in *Chapter I* was critical for the discovery of CXCL10 as the first CXC chemokine ligand for the atypical chemokine receptor ACKR2 (*Chapter III*). Before this finding, CXCL10 had only been shown to bind CXCR3, the receptor on which my PhD research would be centred. Because of my expertise in confocal microscopy, acquired during my previous studies, I was involved in the confirmation of CXCL10–ACKR2 interaction using microscopy as imaging approach. The findings of this study demonstrate that ACKR2 is a scavenger of both inflammatory CC and CXC chemokines, representing an additional regulatory mechanism of chemokine receptor activity. In our analysis, the ligand–receptor pairing was described for both human and murine versions. As future perspective, it would be interesting to investigate the effects and relevance of this interaction in mouse models, especially in inflammatory conditions where CXCL10 is known to be massively upregulated.

At the beginning of the PhD, I took over an analysis (*Chapter II)* initiated by a former PhD student in our group, which over time became a very important part of my research. Our group has a long-standing interest in atypical chemokine receptors, related knowledge and tools proved critical for this project. In particular, this analysis can be considered as a thorough molecular comparison of a classical and an atypical chemokine receptor deriving from the same gene. To increase the reliability of our findings, I was involved in the design and validation of new tools, mostly assessing the G protein activation. Of note, the related experiments were crucial in discriminating the opposing signalling features of CXCR3-A and CXCR3-B. The identification of atypical features for the latter, namely the dramatic impairment in G protein coupling and signalling, the predominantly intracellular localization, as well as the ability to scavenge chemokines, were crucial to highlight its similarity to atypical chemokine receptors. We were able to show that the N-terminal extension specific to CXCR3-B is at the origin of those atypical features. With our findings, not only did we confirm the impaired signalling for CXCR3-B suggested by *Berchiche et al. 2016* and *Smith et al. 2017,* but also comprehensively characterized the cellular events triggered by this enigmatic isoform. Moreover, we profiled and compared the ligand selectivity of CXCR3-A and CXCR3-B, thereby confirming CXCL11, CXCL10, CXCL9 as shared ligands but not CXCL4. Despite GPCR isoforms being shown to have specific tissue distribution (*Marti-Solano et al. 2020*), the precise differential expression of CXCR3-A and CXCR3-B remains an unanswered question. This partly arises from the absence of reliable isoform-specific antibodies or alternative tools. The strong similarity of the two isoforms, only differing by 51 amino acids at the N terminus of CXCR3-B, renders the generation of such probes particularly challenging. The development of novel tools would be critical to elucidate which organs and cell types express the classical or atypical isoforms and therefore further explain how the latter could regulate the former.

As for the two book methodological chapters, *Chapter I.i and Chapter I.ii,* they constitute comprehensive descriptions of techniques largely used in the GPCR field to study receptor activation. Together with two other PhD students from our group, we worked to develop and optimize the assays, perform and analyse the experiments, and prepare detailed guidelines for those methods. In particular, alternative approaches to investigate receptors lacking the ability to signal via G proteins were detailed in *Chapter I.i* using ACKR2 and ACKR3 as example receptors. By following the reported protocols, it is possible to monitor β-arrestin recruitment using technologies based on BRET or Nanonoluciferase complementation. In addition, ACKR activity can be studied by assessing the receptor fate, specifically its

internalization, i.e. its disappearance from the plasma membrane or subsequent endosomal delivery. In *Chapter I.ii,* rigorous instructions are provided to study the regulatory protein family of G protein-coupled receptor kinases (GRK), which have been recently gaining a lot of interest but remain to be fully understood. The tools we described can be used to profile GPCR–GRK interactions with the help of Nanoluciferase complementation technology and investigate the possibility of specific GRK activity patterns for receptor classes. So far, the focus of most studies has been the assessment of the relevance of single or multiple GRK isoforms with the use of cell lines devoid of those genes in terms of arrestin recruitment or cellular trafficking of the receptor. The use of our methods represents a complementary or as cross-confirmation approach to profile the specific GRK isoforms directly interacting with the receptor of interest, with the hope of broadening our understanding of these important GPCR regulators.

In *Chapter IV,* a systematic overview of the recent pairings among atypical chemokine receptors was presented. In the past five years, several reports involving ACKRs suggested new ligands for the known four members (ACKR1, ACKR2, ACKR3 and ACKR4). With this mini-review article, we summarize and emphasize the relevance of such discoveries. Of note, the identification of novel interactions, like the CXCL12 dimer for ACKR1, CXCL2, CXCL10, CCL26 for ACKR2, vCCL2/vMIP-II and several opioid peptides for ACKR3, and the chemokines CCL20 and CCL22 for ACKR4, were obtained by independent research groups, including our team, highlighting the shared interest in broadening the current understanding of the chemokine system. Identification of new ligand–receptor pairs, especially for non-signalling receptors, acknowledges the presence of additional levels of regulation. In the future, a better comprehension of these regulatory receptors may be exploited in terms of therapeutic opportunities seeking to target chemokine receptor signalling, by acting in an indirect manner on their atypical counterpart.

The CXCR3─arrestin manuscript (in the *annexes*), despite not being included in the main body of this thesis, represents an important part of my thesis especially during these last two years. It started rather recently as a side project and it rapidly evolved into a particularly interesting object of analysis that was developed in collaboration with Prof. Carsten Hoffmann from the University of Jena (Germany). Arrestins were initially described as crucial for their abilities to stop G protein signalling leading to receptor internalization. However, more and more studies have shown that, for some receptors, the internalization process is not entirely dependent on arrestins (*Holliday et al. 2007, Von Moo et al. 2021*). In

general terms, arrestins are defined as scaffolds serving all GPCRs, regardless of which downstream signalling pathway is activated, if any. With the aim to better understand the elements driving basal and ligand-induced GPCR─arrestin interaction, we undertook a thorough analysis and used the chemokine receptor CXCR3 as a model. For this receptor, β-arrestin-1 and -2 show different levels of basal interaction. In the presence of the related ligands, both isoforms are recruited to CXCR3. However, with the use of GRK knockout cells and receptor mutants lacking phosphorylation sites in ICLs or C-terminal tail, we observed some differences in how the two arrestin isoforms interact with CXCR3. To confirm and expand these findings, I had the opportunity to join the partner laboratory of Prof. Hoffmann for six weeks and work there with Noureledine Youssef, a PhD student, to analyse CXCR3-mediated conformational changes in β-arrestin isoforms with innovative BRET-based technologies. The resulting data highlighted some differences in the binding interfaces of the two β-arrestins with CXCR3. The data allowed to identify molecular determinants that are required for β-arrestin-1, which while also involved, are dispensable for the interaction of β-arrestin-2 with the receptor.

It would be interesting to verify whether this difference can be considered a feature of this particular receptor or a shared characteristic among GPCRs. To this aim, other receptors, belonging to distinct GPCR families should be investigated and screened with the hope of finding patterns able to shed more light on arrestin functions. In *Drube et al. 2022*, the authors have shown that β-arrestin-2 is able to form GRK-independent complexes with GPCRs more readily than β-arrestin-1, suggesting that the difference we observed for CXCR3 might be conserved for several other receptors.

With the intent to study the chemokine receptor system from a more translational perspective, we initiated a collaborative project with the group of Nervous System Disorders and Therapy from the University of Liège, known for their expertise in primary brain tumours. Thanks to the combined efforts of a consortium with complementary competencies in chemokine receptors, from our group, and gliomas, from the Belgian group, I could experience a particularly stimulating working environment. In *Chapter V*, the most abundantly expressed chemokines and chemokine receptors in glioma and glioblastoma patients have been the object of study. This part of my PhD thesis was initiated with a dual purpose. One aim was to further investigate the possibility of targeting the chemokines as a therapeutic approach for these incurable brain tumours. The second aim of this analysis was CXCR3-centred, specifically to expand our characterization of this chemokine receptor from

a molecular perspective (*Chapter II and annexes*) to its potential function in this disease. To address our questions, we focused on evaluating the most relevant players of the chemokine system, both from the receptor side (*Chapter V.*i) and from the chemokine side (*Chapter V.ii*). We inspected publicly available patient-derived transcriptomic data by subgroups reflecting different glioma types, different regions of the GBM tumour mass to explore the possibility of tissue-specific roles, and the different cell types of the GBM tumour microenvironment. With this approach, we could confirm the relevance of the well-established trio CXCR4–CXCL12–ACKR3 in GBM, which was abundantly expressed in GBM patients and also showed enrichment in the microvascular proliferation regions, suggesting a potential role in support of tumour angiogenesis. The therapeutic potential of targeting this pathway is emphasized by multiple efforts assessing the effects of distinct agents blocking CXCL12–CXCR4 axis as a combined therapy in clinics (*Thomas et al. 2019; Lee et al 2018*). In our analysis, additional chemokine-receptor axes were found as relevant in GBM, specifically the CCL5–CCR1 and CXCL16–CXCR6 driving the interactions of immune cells from tumour microenvironment. These two duets are not largely characterized in GBM and in our CellChat analysis emerged as the most prominent chemokine–chemokine receptor communication in the tumour mass. Of interest, cancer cells do not represent the source of ligands nor receptors for these axes. Further studies are needed to confirm and illuminate the relevance of these new potential targets. It is also important to clarify the tumour-supportive or tumour-suppressive nature of these pathways. With this study, we built a patient-oriented guide detailing expression profiles of the chemokines and their receptors in glioma and glioblastoma patients.

Altogether, this work contributes to better elucidate the interaction network within the chemokine system under several aspects: 1) in terms of chemokine–receptor crosstalk, 2) regulation exerted by the atypical chemokine receptors and finally 3) identification of the most promising targets in in the context of malignancies. With these investigations, not only had I the opportunity to learn several techniques analysing scientific questions from different perspectives, including molecular, cellular and large database-based approaches, but I could also perform and contribute to enhance our comprehension of this complex system and have potential to improve the targeting of its players in clinical practice.

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Annexes

CXCR3–β-arrestin interactions

This Chapter represents a manuscript under preparation

Different requirements and interaction modes of β-arrestin isoforms for basal and ligand-induced interactions with CXCR3

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Short title: Differential binding mode of arrestin isoforms to CXCR3

Abstract

β-arrestins are fundamental scaffold proteins for the regulation of G protein-coupled receptor (GPCR) signalling and trafficking. β-arrestin–GPCR interaction is largely considered to occur upon receptor activation by cognate ligand and subsequent phosphorylation of GPCR intracellular regions by G protein-coupled receptor kinases (GRKs). However, the precise underlying mechanisms are not entirely understood and recent results suggest that β-arrestin isoforms could differently act on the same receptor. Here, we characterize the molecular determinants driving the interaction between the two β-arrestin isoforms with the chemokine receptor CXCR3, following ligand stimulation but also under basal conditions. Interestingly, we demonstrate that only β-arrestin-2 is able to interact with CXCR3 in the absence of ligands whereas, upon chemokine stimulation, both isoforms are recruited to the active receptor and undergo similar conformational changes. By using GRK knock-out cells and CXCR3 mutants lacking phosphorylation sites in the intracellular loops (ICLs) or C terminus, we show that β-arrestin-1 and β-arrestin-2 have different requirements for binding and rely on different receptor determinants. Our results indicate that, despite having similar interaction interfaces, β-arrestin-1 is more sensitive to perturbations, while β-arrestin-2 reveals a higher degree of plasticity for receptor binding. Overall, our study demonstrates that CXCR3–β-arrestin-1 coupling is coherent with the general mechanism of GPCR–β-arrestin complex formation being largely dependent on ligand-induced GRK-mediated receptor phosphorylation. Conversely, CXCR3–β-arrestin-2 interaction is more complex and while GRKs and receptor phosphosites are involved, they appear dispensable.

1. Introduction

G protein-coupled receptors (GPCRs) are seven transmembrane receptors which transduce signals across the plasma membrane following extracellular ligand binding. β-arrestins control GPCR function by serving as a scaffolding platform that regulates receptor signaling in time and space[1-3]. There are two isoforms of β-arrestins (β-arrestin-1, or arrestin-2 and β-arrestin-2, or arrestin-3) that were described to play a major role in the termination of G protein signaling, receptor internalization and recruitment of other scaffold or effector proteins.

β-arrestins are generally considered to be recruited to the receptor following its activation and subsequent phosphorylation of its intracellular loops and C terminus by G protein-coupled receptor kinases, also known as GRKs. Different receptors have been shown to display heterogeneous phosphosite content in terms of number and positioning[4-9]. Remarkably, despite this variety, a combination of only four ubiquitously expressed GRKs (GRK2, -3, -5 and -6) and two β-arrestins orchestrate the regulation of all non-visual GPCRs, accounting for over 800 receptors[1,10,11]. A great effort has therefore been invested in recent years to better understand the exact molecular determinants driving GPCR─β-arrestin interactions[3,12-15]. Precise mapping identified important clusters of phosphorylated serine and threonine located in the C terminus and/or intracellular loops, which were proposed to differentially contribute to arrestin recruitment, receptor trafficking, and effector protein activation[13,16,17].

Structural analysis revealed that GPCR phosphosites interact with the positively charged groove of the N-domain of β-arrestins following the disengagement of β-arrestins C-tail from the N-domain groove[18]. Among the diversity of interactions reported, some receptors were shown to interact through their transmembrane domains and intracellular loops, altogether defined as "*GPCR core*", with the central crest loops of β-arrestins, including the finger loop region[19-21]. Other receptors were described to interact with β-arrestin solely using their phosphorylated C terminus defining the "*tail only*" engagement. Alternatively, some receptors were reported to interact with β-arrestin using both their phosphorylated C terminus and their core, leading to the so-called "*tight*" engagement. Additionally, biophysical studies showed that different β-arrestins regions like the N-domain, the lariat loop and the C-tail undergo conformational rearrangements upon receptor interaction[18,22,23]. The specific β-arrestins conformational changes differ depending on the GPCRs[24], underlying the flexibility of β-arrestins that can act as scaffold proteins for the interaction

with clathrin, clathrin adaptor protein AP2, Src kinases, phosphatases, E3 ubiquitin ligases, PIPs and many others[2,25,26]. It was recently demonstrated that the two β-arrestin isoforms can undergo different conformational rearrangements upon interaction with the same activated receptor[27]. Moreover, some receptors show affinity towards β-arrestins under basal conditions, resulting in constitutive GPCR─β-arrestin interactions[28,29]. However, it is unclear whether this phenomenon relies on the basal state of receptor phosphorylation.

The chemokine receptor CXCR3, which is highly important for immune effector functions, is activated by three endogenous ligands CXCL11, CXCL10 and CXCL9, which display different affinity[30-34]. Previous studies reported the importance of CXCR3 phosphosites in the C terminus and intracellular loops for the induction of cell migration and receptor internalization[35,36]. It was recently suggested that CXCR3 ligands induce different phosphorylation patterns of CXCR3 C terminus and that all the ligands shifted the equilibrium from single to multiple phosphorylated sites[37]. Altogether those observations and the novel notions around GPCR─β-arrestin interactions[16,38] motivated us to systematically explore the molecular determinants for CXCR3–β-arrestin interactions.

In this study, we systematically assessed CXCR3─β-arrestin interactions in the absence and presence of chemokine ligand. In particular, in our analysis, we combined the use of CRISPR-Cas9-edited cell lines lacking GRK2,-3,-5,-6 with CXCR3 mutants lacking phosphorylation sites in ICLs or C-terminal tail, and a unique set of BRET-based β-arrestin conformational biosensors. Here, we show that, while CXCR3–β-arrestin-1 interaction follows the generally accepted interaction model being largely dependent on ligand-induced, GRK-mediated receptor phosphorylation. In contrast, our results indicate that CXCR3–β-arrestin-2 interaction is more complex, can already occur under basal conditions, and that upon ligand stimulation, phosphorylation by GRKs is important but not an essential requirement.

2. Material and Methods

2.1. Reagents

Chemokines were purchased from Protein Foundry, LLC.

2.2. Plasmids and constructs

For the receptor mutants the following phosphosites were mutated to alanine.

CXCR3 C ter ST-A	S349, S350, S351, S355, S356, S358 T360, S361, S364, S366
CXCR3 ICL ST-A	S80, T83, S86, S87, T88, T157, S245
CXCR3 Full ST-A	S80, T83, S86, S87, T88, T157, S245, S349, S350, S351, S355, S356, S358 T360, S361, S364, S366

2.3. Cell lines

HEK293T cells (Abcam), and HEK293, knock-out for GRK2, -3, -5, -6 (ΔQ-GRK) and parental cell lines[39] were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma) and penicillin/streptomycin (100 units/mL and 100 µg/ mL).

2.4. Intermolecular NanoBRET

Basal and ligand-induced GPCR─β-arrestin interaction was monitored in HEK293 or ΔQ-GRK cells by saturation curves. The cells were seeded in clear bottom 96-well plates ($4x10^4$ cells per well), co-transfected with fixed amount β-arrestin N-terminally fused to Nanoluciferase (BRET donor) and increasing amount of receptor C-terminally fused to the fluorescent protein mScarletI (BRET acceptor). 24 hours after transfection, the medium was removed and the cells were incubated with coelenterazine H in Opti-MEM. Upon chemokine stimulation (CXCL11 30 nM) or vehicle treatment, BRET signal was measured with Mithras LB940 luminometer (Berthold Technologies) equipped with 460/70 filter for donor luminescence emission and 600 LP filter for acceptor fluorescence emission.

2.5. Intramolecular NanoBRET

HEK293 and ΔQ-GRK cells were seeded into 6-well plates and transfected with 600 ng untagged receptor, 60 ng of the respective β-arrestin FlAsH-tagged biosensor[27] C-terminally coupled to NanoLuc and an empty vector to adjust the total amount of DNA to 1 µg. 24 h after transfection, we seeded $4x10^4$ cells per well into poly-D-lysine coated 96-well plates

and incubated overnight at 37 °C. Cells were washed twice with PBS, then incubated with 250 nM FlAsH in labelling buffer (150 mM NaCl, 10mM HEPES, 25 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10mM glucose; pH7.3), complemented with 12.5 µM 1,2-ethane dithiol (EDT) for 60 min at 37 °C. After aspiration of the FlAsH labelling or mock labelling solutions, the cells were incubated for 10 min at 37 °C with 100 µl of 250 µM EDT in labelling buffer per well. The cells were washed twice with measuring buffer (140 mM NaCl, 10 mM HEPES, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.3) and NanoLuc substrate furimazine (Promega) was added 1:35000 in measuring buffer. The ligand-dependent BRET change was measured using a Synergy Neo2 plate reader (Biotek), containing a BRET2 filter (emission 400 nm, 510 nm, dichroic mirror 470 nm. Initial BRET changes were calculated by the division of the stimulated values by baseline values. The initial BRET change was then corrected for labelling efficiency via subtraction of values generated by mock labelling. To achieve the final Δ net BRET change, the corrected BRET change was divided by the vehicle control.

2.6. Intracellular calcium mobilization

Ligand-induced intracellular calcium release was monitored by nanoluciferase complementation assay (NanoBiT), relying on the Ca^{2+}-dependent interaction between calmodulin and the calmodulin binding protein MYLK2S[32]. HEK293T were seeded into 10 cm^2 dishes and transfected with 200 ng of untagged receptor, 200 ng of calmodulin C-terminally fused to SmBiT and 200 ng of MYLK2S N-terminally fused to LgBiT and empty vector to adjust the total amount of DNA to 4 µg. 24h after transfection cells were incubated in PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 10 minutes at 37°C. Cells were incubated with coelenterazine H and distributed in a 96-well plate ($1x10^5$ cells per well). After 10 minutes at 37°C, the baseline luminescence was recorded for 2 minutes. Calcium fluxes were monitored and quantified by assessing the changes in luminescence upon stimulation with chemokines or the calcium ionophore A23187 (1 µM). Luminescence was measured on a GloMax Discover plate reader (Promega).

2.7. Flow cytometry

Presence of the receptor at the cell surface was evaluated by flow cytometry in HEK293 or ΔQ-GRK cells. The cells were seeded into 6-well plates and 24 hours later transfected with 100 ng of receptor C-terminally fused to the fluorescent protein mScarletI. 48 hours after transfection 1.5 $x10^5$ cells/well were seeded in a 96-well plate and stained with anti-CXCR3

(1C6) mAb or isotype control. After 1 hour incubation at 4°C, cells were washed twice with FACS buffer (PBS, 0.1% sodium azide, 1% BSA) and the fluorescence was measured on a Quanteon Flow Cytometer (NovoCyte).

2.8. Confocal microscopy

To visualize and localize receptor expression, confocal microscopy images were obtained. 5 $x10^4$ cells were transfected in suspension with 15 ng of receptor C-terminally fused to ScarletI and seeded on poly-L-lysine coated 8-well chamber slides (Ibidi). After 48 hours, cells were washed with PBS, fixed with fixed with 3.5 % (w/v) paraformaldehyde for 20 minutes at room temperature. Cells were again washed with PBS for two times and subsequently subjected to nuclear staining with Hoechst 33342 dye (1 µg/mL) by incubating it for 20 minutes at room temperature, finally 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).

2.9. Data and statistical analysis

Saturation curves were fitted to one site-specific binding equation and for each data set, this model was compared to the line through origin equation to determine the preferred model, P-value, $BRET_{50}$ and $BRET_{max}$. Concentration–response curves were fitted to the three-parameter Hill equation using an iterative, least-squares method. All curves were fitted to the data points generated from the mean of at least three independent experiments. All statistical tests i.e. comparison of fits, one-way and two-way ANOVA were performed with GraphPad Prism 9.3.1.

3. Results

The two β-arrestin isoforms show different levels of interaction with CXCR3 under basal and ligand-induced conditions

It was recently proposed that β-arrestins are able to spontaneously translocate from the cytosol to the plasma membrane forming pre-association complexes with the lipid bilayers before interacting with activated receptors[38]. Based on these observations, we wondered whether CXCR3 could constitutively interact with β-arrestins under basal conditions. To investigate this question, we tested, by BRET-based saturation curves, the ability of CXCR3 to interact with the two β-arrestin isoforms in the absence of chemokine stimulation. Interestingly, under basal conditions, we observed different profiles for the two β-arrestin

isoforms, with β-arrestin-2 significantly binding to CXCR3 (one site-specific binding model, P < 0.0001) but not β-arrestin-1 (line through origin model, P = 0.540) (Figure 1A). Furthermore, the calculation of the R-squared confirmed the difference in the profiles observed, with β-arrestin-2 showing a strong preference towards the one-site specific binding model whereas β-arrestin-1 retained very similar R^2 for the two regression models (Figure 1B). We next repeated the experiment in the presence of saturating concentration of CXCL11 (30 nM), the strongest agonist chemokine for CXCR3, and observed an important ligand-induced interaction for both β-arrestin isoforms (β-arrestin-1: $BRET_{50}$ ~10 fold, $BRET_{max}$ ~3.5 fold and β-arrestin-2: $BRET_{50}$ ~1.7 fold, $BRET_{max}$ ~8.2 fold) (Figure and Supplementary table).

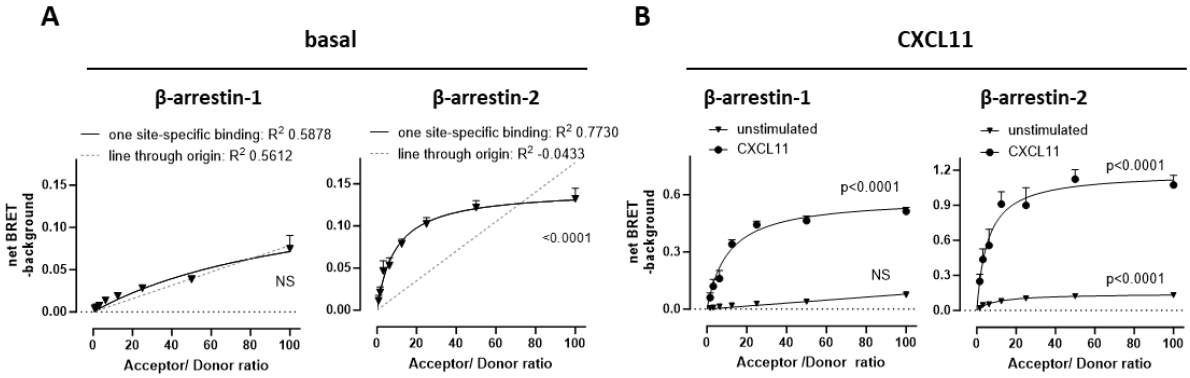


Figure 1. β-arrestin-2 but not β-arrestin-1 can constitutively interact with CXCR3. BRET-based saturation curves describing CXCR3 interaction with β-arrestin-1 or β-arrestin-2 were obtained upon transfection of HEK293 cells. Saturation curves relies on acceptor/donor titration where β-arrestin is N-terminally fused to Nanoluciferase, constituting the BRET donor, whereas CXCR3 is C-terminally tagged to the fluorescent protein mScarletI, representing the BRET acceptor. Profiles describing (**A**) basal and (**B**) CXCL11 (30 nM)-induced CXCR3─arrestin interactions are shown for β-arrestin-1 (left panels) and for β-arrestin-2 (right panels), by BRET-based saturation analyses. Data are expressed as net BRET and represent mean ± SEM of at least four independent experiments. Statistical significance was calculated by comparison of fits. R squared and significant P values are indicated.

Different contributions of GRKs and receptor determinants for β-arrestin isoforms interaction with CXCR3 under basal and ligand-induced conditions

To investigate the importance of GRK-mediated phosphorylation to the interactions between CXCR3 and the two β-arrestin isoforms, under basal and ligand-induced conditions, we repeated these experiments by employing the GRK2,-3,-5,-6 knock-out cell line[39] (ΔQ-GRK). Additionally, to evaluate the contribution of the different phosphorylation sites, we combined this analysis with the use of a panel of mutated receptors in which the serine and threonine residues located within the intracellular loops (ICL ST-A), or the C-terminus (C ter ST-A), or both (full ST-A) were replaced by alanine residues (Figure 2). Before analyzing

the impact on β-arrestin binding, we confirmed the presence of these mutated receptors within the cells by assessing their surface and total expression by confocal microscopy and flow cytometry (Supplementary Figures 1A-C). Mutants' functionality was verified by comparing their ability to activate G protein signaling upon stimulation with different CXCR3 ligands (Supplementary Figures 1D and 1E).

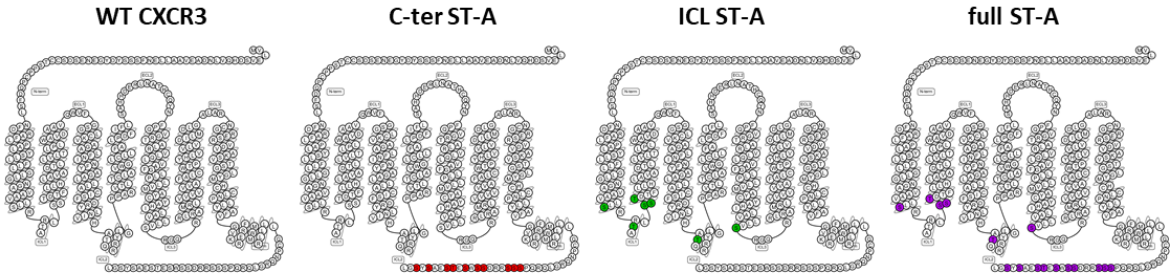


Figure 2. CXCR3 mutants. Schematic representation of CXCR3 mutants in which phosphosites at the C terminus or the intracellular loops or both are mutated to alanine. Mutated amino acids are color-coded: red for C-term ST-A, green for ICL ST-A, purple for full ST-A. The snake diagrams were generated using GPCRdb[40].

GRKs and GPCR phosphosites are dispensable for the interaction between β-arrestin 2 and CXCR3 under basal conditions

Under basal conditions, we observed that the binding of β-arrestin-2 was conserved, although reduced, in the absence of GRK2,-3,-5,-6, suggesting that GRK-mediated phosphorylation favors but is not required for basal interaction of β-arrestin-2 with the receptor. This observation was confirmed with the mutants as, none of the mutations, abolished basal receptor interaction with β-arrestin-2 (Figure 3). ICL ST-A mutant appeared to behave similarly to the WT receptor retaining an identical $BRET_{max}$ but a reduced $BRET_{50}$ value, suggesting an even higher affinity for the receptor (Figure 3 and Supplementary table). For β-arrestin-1, we also observed that the mutant devoid of ICL phosphosites showed slightly better affinity for the arrestin compared to WT whereas the WT, in the presence or absence of GRKs, and other mutants did not interact with β-arrestin-1 under basal conditions.

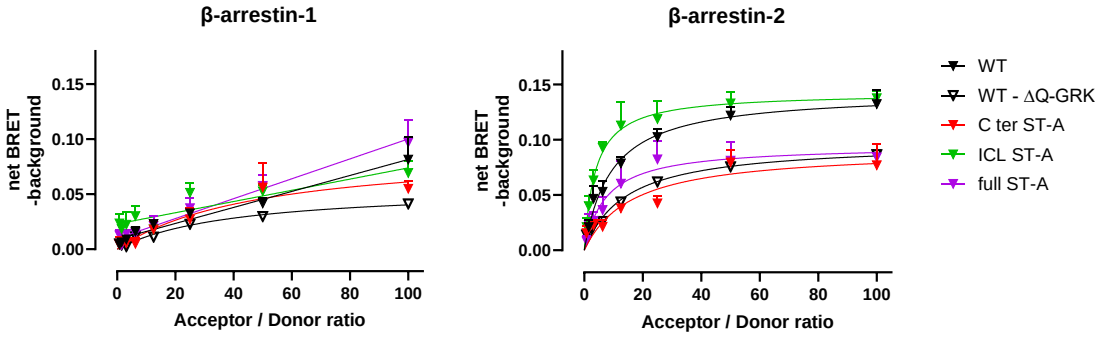


Figure 3. GRKs and receptor phosphosites are dispensable for constitutive CXCR3—β-arrestin-2 interaction. BRET analysis describing interaction between CXCR3, tagged with mScarletI, and β-arrestins, tagged with NLF, in the absence of ligands, in HEK293 for CXCR3 WT or mutants and in ΔQ GRK cells for the WT receptor. The saturation curves are shown for β-arrestin-1 (left panel) and for β-arrestin-2 (right panel). Data are expressed as net BRET and represent mean ± SEM of at least four independent experiments.

GRKs and C-terminal phosphosites are crucial for CXCL11-induced β-arrestin-1 recruitment to CXCR3

We next studied the interactions between the two β-arrestins and CXCR3 under chemokine-induced conditions. First, we profiled the binding of β-arrestin-1 and evaluated the impact of GRKs and mutations of phosphosites on CXCL11-induced recruitment. The saturation profiles, revealed the vital role of serine and threonine residues from the C terminus for efficient recruitment of β-arrestin-1 compared to ICL phosphosites (Figure 4A). In the absence of GRKs, CXCR3 WT showed a marked impairment in β-arrestin-1 recruitment, which was completely lost in the mutants (Figure 4B). These results suggest a critical role for the C-terminal residues, whose absence is sufficient to abolish arrestin-receptor binding and an important, but not complete, dependency on GRKs.

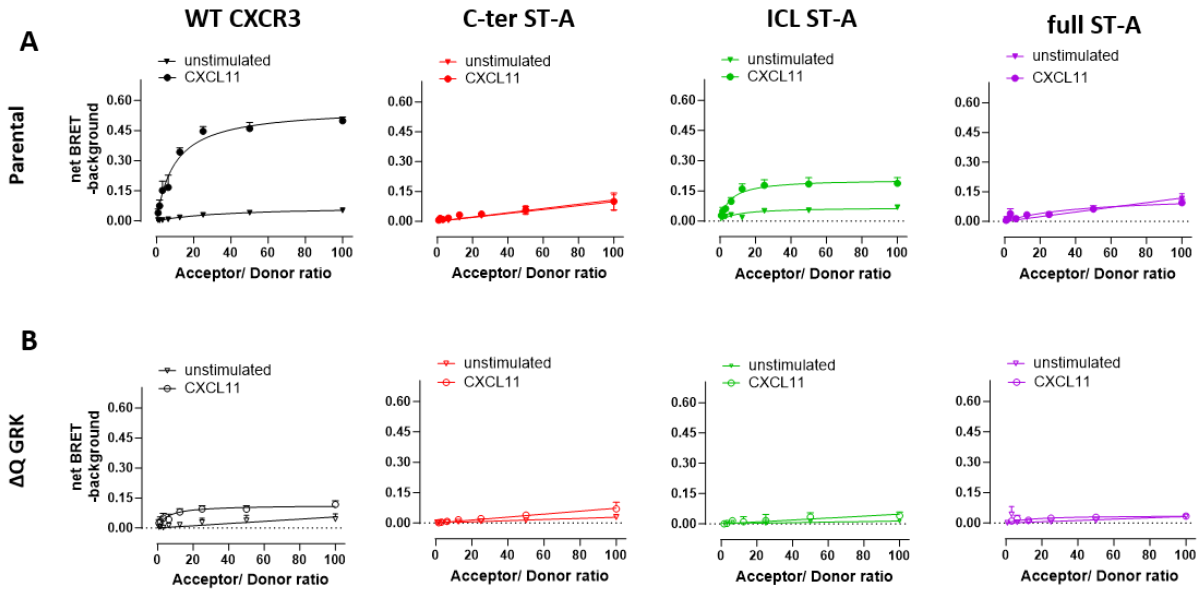


Figure 4. β-arrestin-1 strongly relies on the C-terminal phosphosites and GRKs for CXCR3 interaction. BRET analysis describing the interaction between CXCR3 mutants and β-arrestin-1. Saturation curves describing CXCL11-induced (30 nM) recruitment of β-arrestin-1 (NLF-tagged) to CXCR3 mutants (mScarletI-tagged) in HEK293 (parental, **A**) GRK2, -3, -5, -6 knock-out (ΔQ GRK, **B**) cell. Results are expressed as net BRET. Data represent mean ± SEM of at least four independent experiments.

GRKs and receptor phosphosites are dispensable for CXCL11-induced β-arrestin-2 recruitment to CXCR3

We then undertook an identical profiling for β-arrestin-2 in the presence of CXCL11. The BRET-based saturation analysis, revealed some differences in terms of phosphosite and GRK dependencies. Compared to β-arrestin-1, β-arrestin-2 retained partial binding capacity to the receptor despite the removal of the phosphosites from the different domains (Figure 5A). The effects of the mutations seemed to indicate an additive behaviour, with the following recruitment levels: CXCR3 WT ($BRET_{max}$ ~8.2 fold) > ICL ST-A ($BRET_{max}$ ~3.6 fold) > C-ter ST-A ($BRET_{max}$ ~3 fold) > full ST-A ($BRET_{max}$ ~1.7 fold) (Supplementary table).

GRK contribution was also evaluated, and while their presence enhanced the binding of β-arrestin-2 to the activated receptor, they remained largely dispensable (Figure 5B). When absent, the recruitment hierarchy of the receptor mutants was the following: CXCR3 WT ($BRET_{max}$ ~7.6 fold) > C-ter ST-A ($BRET_{max}$ ~4.6 fold) > ICL ST-A ($BRET_{max}$ ~4.2 fold) > full ST-A ($BRET_{max}$ ~1.8 fold) (Supplementary table).

Overall, this analysis indicates that despite GRKs and receptor phosphosites being involved in mediating both β-arrestin-1 and β-arrestin-2 interactions with CXCR3, the underlying hierarchies governing the related binding dynamics and the contribution of phosphorylation sites differ for the two arrestin isoforms. In particular, the C-terminal phosphosites appear to be essential for β-arrestin-1, with a strong influence from GRKs, whereas β-arrestin-2 appears less stringent, being able to bind to the receptor in the absence of GRKs or phosphosites.

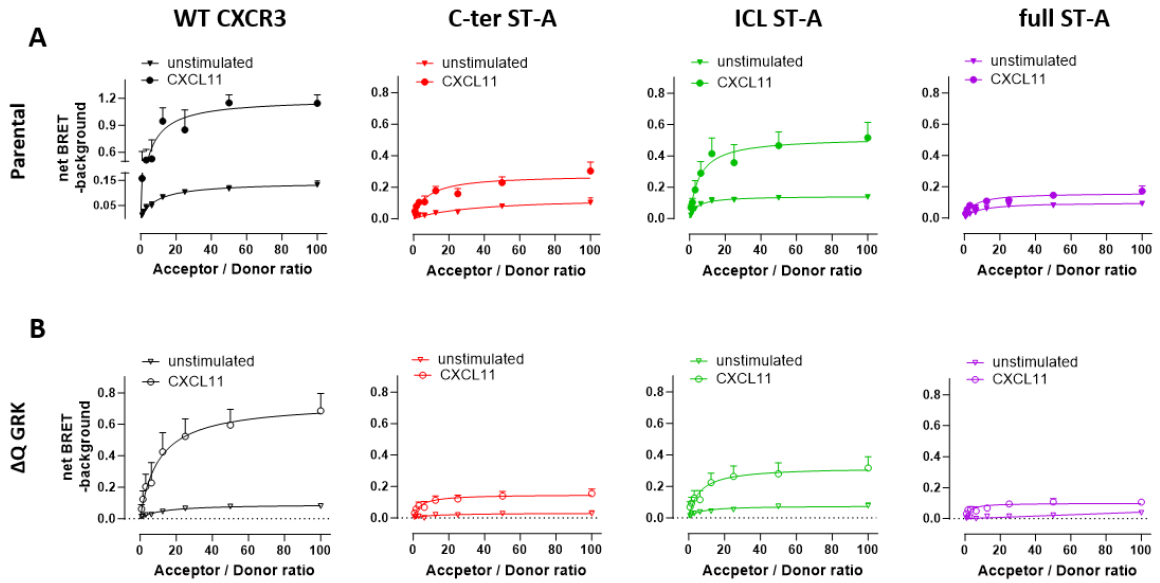


Figure 5. β-arrestin-2─CXCR3 interaction is enhanced by receptor phosphosites and GRKs, however they remain dispensable. BRET analysis describing the interaction between CXCR3 mutants and β-arrestin-2. Saturation curves describing CXCL11 (30 nM)-induced recruitment of β-arrestin-2 (NLF-tagged) to CXCR3 mutants (mScarletI-tagged) in HEK293 (parental, **A**) or GRK2, -3, -5, -6 knock-out (ΔQ GRK) cells, **B**. Results are expressed as net BRET. Data represent mean ± SEM of at least four independent experiments.

The two β-arrestin isoforms undergo different conformational changes following binding to activated CXCR3

Arrestins are subjected to conformational changes upon interaction with active GPCRs, in which the receptor C terminus plays a central role where distinct phosphorylation clusters show synergistic effects[12]. Furthermore, a recent study revealed that β-arrestin-1 and β-arrestin-2 could undergo different rearrangements upon ligand-induced receptor activation[27].

In light of our results, proposing different hierarchies and requirements for the two β-arrestin isoforms in terms of receptor phosphosites and GRK involvement, we investigated whether the difference observed for the binding the two β-arrestins is also associated with their different conformational changes. With this aim, we monitored β-arrestin-1 and β-arrestin-2 conformational changes upon CXCL11-induced CXCR3 activation using a unique set of intramolecular FlAsH-based BRET[27]. These tools rely on the presence of both the donor and acceptor within the same β-arrestin molecule, i.e. an N-terminal fusion to full Nanoluciferase and a six-amino-acid FlAsH labelling motif (CCPGCC) inserted in different positions, respectively[27] (Figure 6A). With this set of biosensors, we compared the conformational fingerprints of the two β-arrestins upon binding to the activated CXCR3.

This systematic profiling revealed important conformational changes in the β-arrestin N-domain for both isoforms (Figure 6B). For β-arrestin-1, the mean BRET changes appeared

homogenous throughout the N-domain. On the contrary, β-arrestin-2 showed larger re-arrangements for F3 and F5 over F2 and F4 located within the N-domain (Figure 6B). Interestingly, the C-domain showed rather variable conformational changes. For β-arrestin-1, we observed re-arrangements only in F1 and F10 biosensors, which are located in the C-edge loop 2 and the outward loop, respectively. For the C-domain of β-arrestin-2, we detected changes for F1 and F10 biosensors as for β-arrestin-1, but also for F9, which is positioned in the outward loop (Figure 6B).

In the absence of GRKs, the C-domain appeared significantly impaired for both β-arrestin isoforms, whereas the N-domain largely varied for the two regulatory proteins. For β-arrestin-2, signal was detected for all the sensors but with reduced BRET changes. While β-arrestin-1 retained conformational change abilities only for F3. These data suggest that, in agreement with the observations made for recruitment to receptor, β-arrestin-2 is less affected by the absence of phosphorylation than β-arrestin-1.

To further investigate the contribution of the different receptor phosphorylation sites on β-arrestin conformational changes, we repeated the analysis on the three ST-A CXCR3 mutants (Figure 6C). For this comparison, we focused on the biosensors for which a signal on the WT receptor was detected for both arrestins, namely F2, F3, F4, F5 from the N-domain and F1, F10 from the C-domain. In agreement with the recruitment data, we observed a general reduction in the intensity of the conformational changes for all mutants, regardless of the distribution of the modified phosphosites and the β-arrestin isoform.

For β-arrestin-1, the absence of receptor phosphosites was detrimental, with only the ICL ST-A showing conformational change abilities in one sensor, F3, for which no significant difference could be observed when compared to WT CXCR3 (Figure 6C and Supplementary Figure 2). Interestingly, the F3 biosensor from β-arrestin-1 was also unaffected by the absence of GRKs (Figure 6B). For β-arrestin-2, the impact of receptor phosphosites was rather heterogeneous, where the interaction with both C-ter and ICL mutants was still leading to conformational changes in the N-domain. Remarkably, the full mutant failed to induce conformational changes in β-arrestin-1 but not β-arrestin-2 further pointing towards a less important role of CXCR3 phosphorylation for β-arrestin-2 interaction and activation.

Finally, we took advantage of the similar changes observed for the biosensor F5 probing the re-arrangements in the N-domain to evaluate the impact of the presence or absence of the endogenous GRKs in the induction of the conformational changes in the N-domain (Figure

6D). Even though these data provide information only on one particular region of the N-domain and are therefore not sufficient to exhaustively exemplify the changes and requirements for the other regions, it seems to support the β-arrestin binding data (Figures 4 and 5). Notably, these findings confirmed the differential importance of C terminus phosphosites for β-arrestin-1 and β-arrestin-2, while supporting the observation that the presence of GRKs is more critical for β-arrestin-1 than β-arrestin-2.

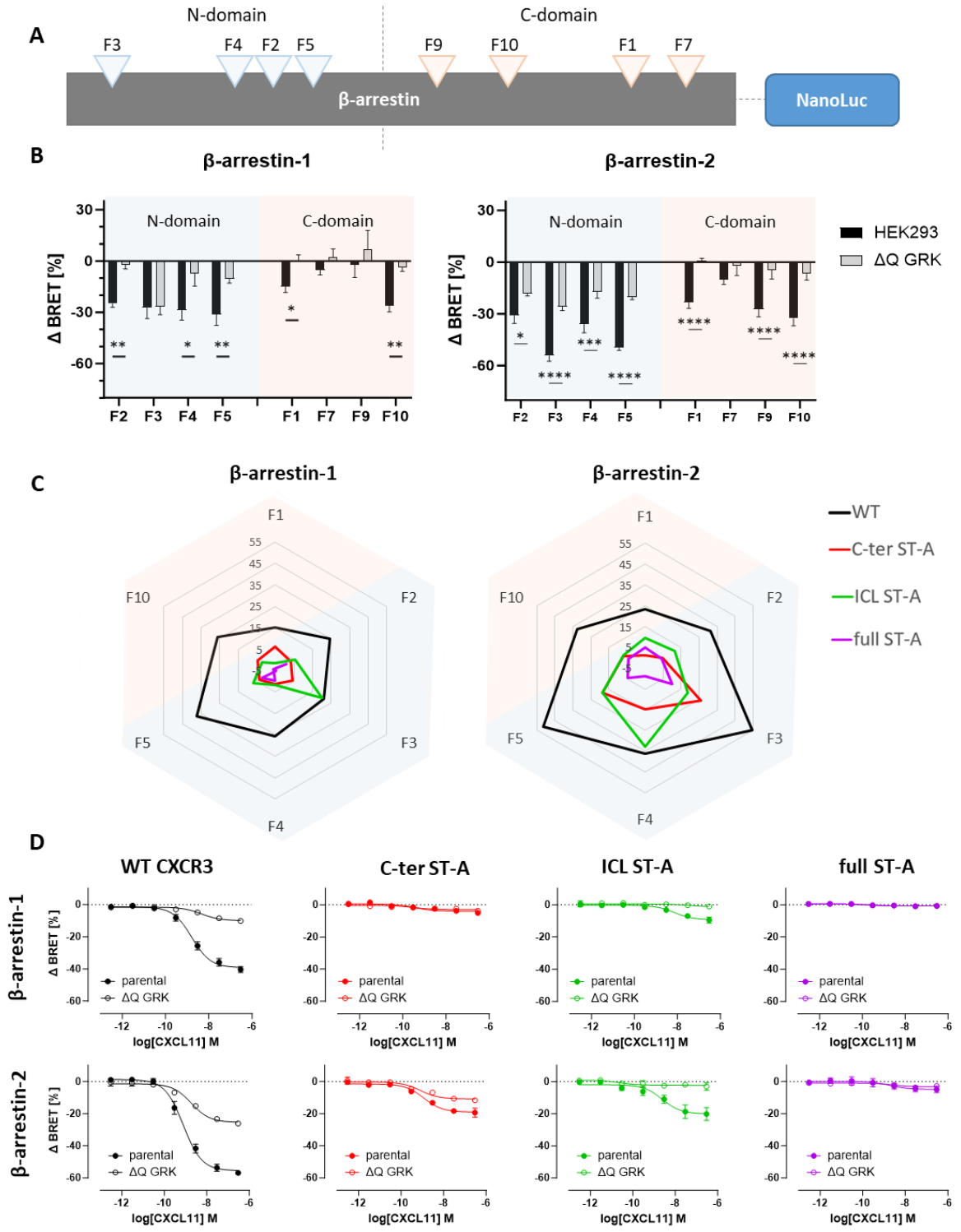


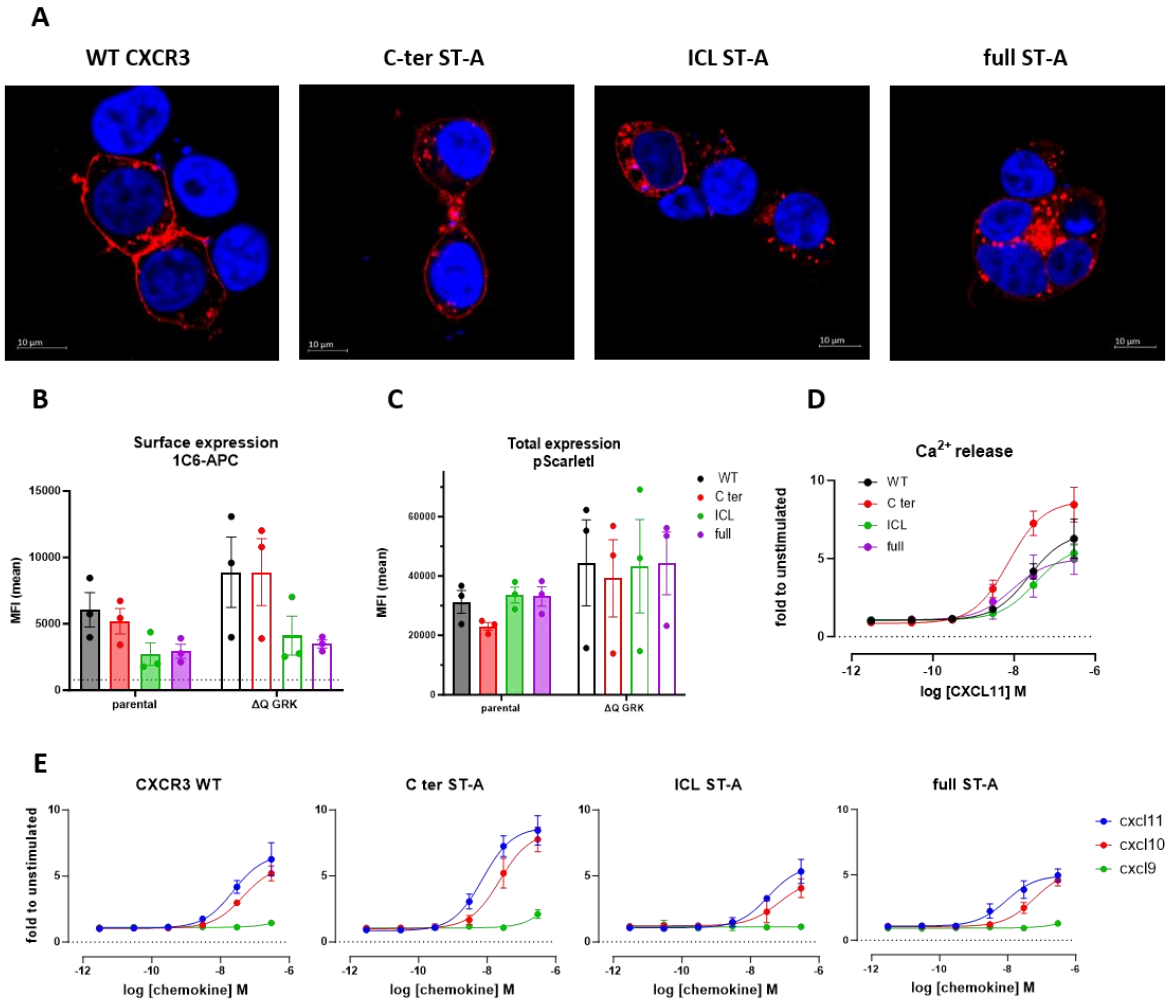
Figure 6. β-arrestin-1 and β-arrestin-2 undergo different conformational changes upon CXCR3 activation relying on different combinations of receptor phosphosites. (**A**) Schematic representation of the β-arrestin conformational biosensors[27]. The biosensors rely on intramolecular BRET and are composed of the NanoLuc donor fused to the β-arrestin C terminus and fluorescent acceptor (F) bound to the FlAsH-binding motifs inserted at different positions in the N-domain (blue triangles) or C-domain (orange triangles). (**B**) Conformational fingerprint of β-arrestin-1 and of β-arrestin-2 upon CXCL11 (30 nM)-induced CXCR3 activation in HEK293 (parental) or GRK2,-3,-5,-6 knock-out (ΔQ GRK) cells obtained using FlAsH-based BRET biosensors inserted at the N-domain (blue background) and C-domain (orange background). (**C**) Radar plots of CXCR3 mutants by β-arrestin conformational change in HEK293 cells. Only the biosensors that

induced detectable conformational changes in both β-arrestins, in the presence of the wild-type receptor, were tested with CXCR3 mutants: F1, F2, F3, F4, F5 and F10. (**D**) β-arrestin-1 (upper panel) and for β-arrestin-2 (lower panel) CXCL11 concentration–response analysis for CXCR3 wild-type and mutants in HEK293 (parental) or GRK2,-3,-5,-6 knock-out (ΔQ GRK) cells using FlAsH5 biosensor. Results are expressed as Δ net BRET percentage and represent mean ± SEM of at least three independent experiments. Statistical significance was calculated by two-way ANOVA with Tukey's post hoc analysis (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

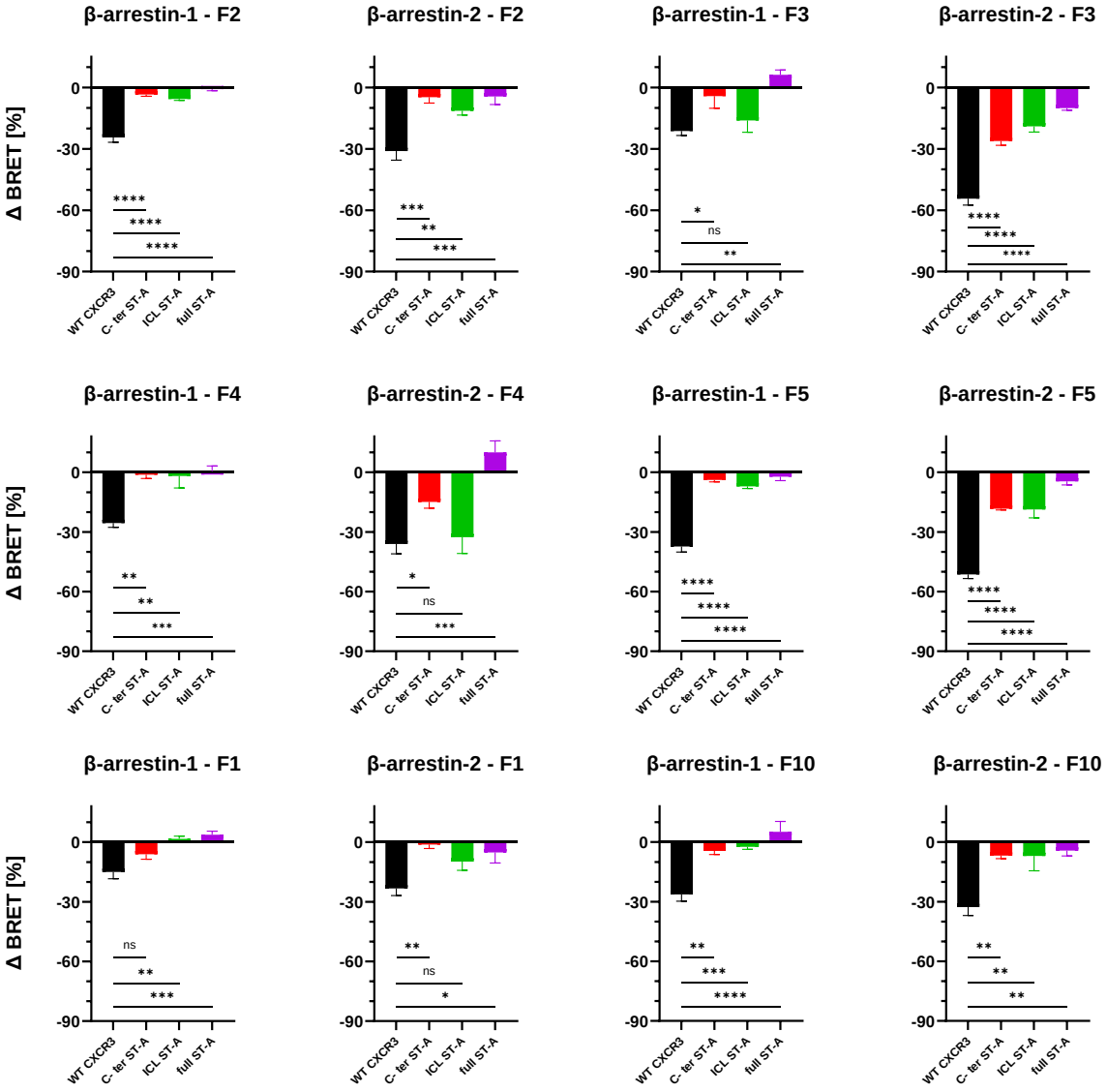
4. Conclusions

- β-arrrestin-2 but not β-arrestin-1 can interact with CXCR3 in the absence of ligands;
- CXCL11 efficiently induces β-arrrestin-1 and β-arrestin-2 recruitment to CXCR3;
- In the absence of GRKs, ligand-induced CXCR3─β-arrestin-1 interaction is strongly impaired;
- Under basal conditions and upon ligand stimulation, GRKs, enhance CXCR3─β-arrestin-2 interaction, although they are not critical;
- β-arrestin-1 and β-arrestin-2 undergo different conformational changes upon CXCL11-induced CXCR3 activation;
- CXCR3 C-terminus phosphosites are crucial for ligand-induced interaction with β-arrestin-1 but not with β-arrestin-2;
- CXCR3 C-terminus phosphosites are crucial for ligand-induced conformational changes for β-arrestin-1 but not for β-arrestin-2;

5. Supplementary material



Supplementary Figure 1. (**A**) CXCR3 WT or mutants cellular distribution by confocal microscopy. HEK293T cells were transiently transfect with mScarletI receptor (shown in red), Hoechst 33342 was used for nuclear staining (shown in blue). Pictures are representative of acquired images from three independent experiments. Scale bar: 10 µm. (**B**, **C**) Receptor expression, CXCR3 WT and mutants, was analysed by flow cytometry upon transient transfection of HEK293 (parental) or GRK2/3/5/6 KO (ΔQ GRK). (**B**) Surface expression revealed by CXCR3-specific mAb (clone 1C6), (**C**) total expression was evaluated as fluorescent intensity from mScarletI tag (**D**). Concentration–response profiles of CXCR3 WT or mutant-mediated intracellular calcium mobilization upon stimulation with CXCL11, monitored by Nanoluciferase complementation assay. Comparison of CXCL11-, CXCL10- and CXCL9-mediated receptor activation by intracellular calcium release by Nanoluciferase complementation assay. In (**D**, **E**), data are expressed as fold increase over background and were generated in HEK293T cells and calcium ionophore A23187 (1 µM) was used as receptor-independent positive control (not shown). Data represent mean ± SEM of at least three independent experiments.



Supplementary Figure 2. Conformational change of β-arrestin-1 and β-arrestin-2 biosensors (F2 and F3 up, F4 and F5 in the middle, F1 and F10 down) in presence of CXCR3 WT, C-ter ST-A, ICL-ST-A or full ST-A upon stimulation with CXCL11 (30nM). Data are expressed as Δ net BRET percentage and represent mean ± SEM of at least four independent experiments. The statistical significance was calculated by one-way ANOVA, followed by Dunnett's multiple comparison test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Supplementary table – CXCR3─β-arrestin binding affinities

	BRET max	$BRET_{50}$ (Kd)	preferred model	P value
β-arrestin-1				
WT CXCR3, HEK293	0.161 (0.085 - 10^9)	114 (34.39 - ∞)	A	0.054
WT CXCR3, HEK293 + CXCL11	0.563 (0.494 - 0.643)	9.563 (6.147 - 17.73)	B	<0.0001
C-ter ST-A, HEK293	0.348 (ND)	269.1 (ND)	A	0.5466
C-ter ST-A, HEK293 + CXCL11	0.178 (ND)	85.56 (12.14 - ∞)	A	0.1939
ICL ST-A, HEK293	0.067 (0.046 - 0.107)	9.658 (1.791 - 39.51)	B	<0.0001
ICL ST-A, HEK293 + CXCL11	0.208 (0.175 - 0.246)	5.727 (3.022 to 10.52)	B	<0.0001
full ST-A, HEK293	0.474 (ND)	316.6 (ND)	A	0.3795
full ST-A, HEK293 + CXCL11	0.129 (0.069 - 0.963)	44.46 (7.903 - 816.1)	B	0.0298
WT CXCR3, ΔQ GRK	0.056 (0.086 - 12.67)	39.8 (35.41 - 10^4)	A	0.0634
WT CXCR3, ΔQ GRK, CXCL11	0.114 (0.085 - 0.155)	5.435 (1.536 - 17.00)	B	<0.0001
C-ter ST-A, ΔQ GRK	0.106 (0.013 - ∞)	284.7 (15.01 - ∞)	A	0.6662
C-ter ST-A, ΔQ GRK + CXCL11	0.218 (ND)	214.1 (ND)	A	0.4958
ICL ST-A, ΔQ GRK	0.105 (ND)	645.5 (ND)	A	0.9474
ICL ST-A, ΔQ GRK + CXCL11	0.059 (ND)	45.48 (ND)	A	0.3076
full ST-A, ΔQ GRK	0.017 (0.003 - 10^9)	1.543 (0.567 - ∞)	A	0.4622
full ST-A, ΔQ GRK + CXCL11	0.038 (0.023 - 0.077)	13.62 (3.085 - 81.80)	B	0.0027
β-arrestin-2				
WT CXCR3, HEK293	0.143 (0.126 - 0.164)	9.280 (5.875 - 14.52)	B	<0.0001
WT CXCR3, HEK293 + CXCL11	1.177 (1.016 - 1.364)	5.587(3.115 -9.667)	B	<0.0001
C-ter ST-A, HEK293	0.091 (0.067 - 0.132)	16.25 (6.148 - 41.00)	B	<0.0001
C-ter ST-A, HEK293 + CXCL11	0.278 (0.215 - 0.375)	7.671 (2.698 - 21.65)	B	<0.0001
ICL ST-A, HEK293	0.143 (0.128 - 0.159)	3.808 (2.421 - 5.891)	B	<0.0001
ICL ST-A, HEK293 + CXCL11	0.518 (0.409 - 0.657)	5.322 (2.122 - 12.90)	B	<0.0001
full ST-A, HEK293	0.095 (0.074 - 0.123)	3.808 (3.005 - 17.18)	B	<0.0001
full ST-A, HEK293 + CXCL11	0.161 (0.128 - 0.206)	5.226 (1.903 - 13.64)	B	<0.0001
WT CXCR3, ΔQ GRK	0.097 (0.082 - 0.117)	14.16 (8.208 - 24.41)	B	<0.0001
WT CXCR3, ΔQ GRK, CXCL11	0.739 (0.570 - 0.984)	10.23 (4.143 - 24.94)	B	<0.0001
C-ter ST-A, ΔQ GRK	0.032 (0.016 - 0.086)	11.84 (0.653 - 130.0)	B	0.0096
C-ter ST-A, ΔQ GRK + CXCL11	0.149 (0.114 - 0.196)	3.832 (1.114 - 11.69)	B	<0.0001
ICL ST-A, ΔQ GRK	0.077 (0.058 - 0.103)	5.944 (1.865 - 17.80)	B	<0.0001
ICL ST-A, ΔQ GRK + CXCL11	0.324 (0.239 - 0.441)	5.889 (1.669 - 18.17)	B	<0.0001
full ST-A, ΔQ GRK	0.057 (0.029 - 10^9)	59.09 (11.23 - ∞)	A	0.0653
full ST-A, ΔQ GRK + CXCL11	0.101 (0.076 - 0.134)	2.503 (0.548 - 8.925)	B	<0.0001

A: line through origin model

B: one site- specific binding model

ND: Not determinable since saturation was not reached

Author contributions

G.D.U., M.S. and A.C. designed the study. GDU and N.Y. performed the experiments, analyzed and interpreted the data. G.D.U. and A.C wrote the manuscript. G.D.U., R.L., A.A.B., M.S. and A.C, revised the manuscript. M.S., A.C. and C.H. acquired the funding. All authors approve the manuscript.

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