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Molecular insights into recognition, activation and function of the atypical chemokine receptor CXCR7/ACKR3

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Science

by

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2015-2016

I'm shut tight.

Even if you break me to pieces,

we'll all still be closed.

You can grind us to sand,

we still won't let you in.

Wislawa Szymborska,
Conversation with a Stone

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my promoter Prof. Jacques Piette for his continuous support, his assistance and the advice he has provided from the very beginning of the project.

I would also like to thank my co-promoter, Dr. Jean-Claude Schmit as well as the director of the Department of Infection and Immunity, Prof. Markus Ollert for allowing me to carry out my past and future projects at the Luxembourg Institute of Health.

I would particularly like to thank my supervisor, Dr. Andy Chevigné for his guidance, support and enthusiasm, the stimulating discussions and the long evenings before deadlines.

I would also like to thank the other members of my thesis committee, Prof. André Matagne, Prof. Nico van Nuland and Dr. Julien Hanson for their insightful comments and constructive criticisms at different stages of my work.

I wish to thank Prof. Dominique Schols, Dr. Julien Hanson, Nadine Dupuis and Thomas D'Huys for thought-provoking discussions and our fruitful collaboration.

My sincere thanks goes to the team of Molecular Signalling and Virus-Host Interaction and in particular to Manuel Counson, Nadia Beaupain and Julie Mathu for their invaluable help throughout my PhD, as well as to Anouar Derj and Pauline Lambert for the contribution they made to this project during their Master's training.

Last but not with less gratitude, I would like to thank my parents and my brother for their unfailing support and continuous encouragement throughout my years of study.

The Ph.D project was supported by the Luxembourg National Research Fund, FNR through the "Aides à la Formation-Recherche" grant scheme.

Preface

Over the last years, the field of research on chemokines and their receptors has made significant progresses. As the crystallography techniques improve, the number of difficult-to-obtain three-dimensional chemokine receptor structures begins to increase. In parallel many, sometimes groundbreaking, functional data have recently been reported. As a consequence, the convictions within the chemokine-receptor community evolve considerably as the previously widely accepted models fail now to withstand the weight of both structural and functional evidences pointing out their oversimplification. The two-site/two-step binding model for chemokine-receptor interactions is being replaced with a more complex, multistep one, the concept of biased signalling is gaining ground and the growing number of novel unsuspected non-chemokine ligands for chemokine receptors regularly refine our understanding on how these receptors work and how they are regulated, bringing about new paradigms. CXCR7, for which two new ligands have recently been discovered and for which the signalling potential, 10 years after its identification as a chemokine receptor, remains a debatable subject, integrates really well in the current changing landscape of the chemokine-receptor field.

The present thesis gathers the fruit of my four years of work as a PhD student at the LIH in the group of Molecular Signalling and Virus-Host Interactions. The aim of my project was to provide new insights into the molecular and structural determinants that dictate CXCR7 ligand recognition and activation, with the final goal to better understand the functions and biology of this atypical and fascinating receptor, how it may affect CXCR4 and CXCR3, with which it shares its ligands and, more generally, the role it may play in the chemokine receptor network.

This thesis will be divided as follows: After a general introduction, the work done for my project will be presented in eight chapters. A short introductory section will precede each chapter, explaining the motivations or goals and the context of the related work. Each chapter will be also followed by a brief concluding section. Chapters 1, 2, 4 and 7 correspond to work already published and a copy of each article is appended at the end of this thesis. Some of the paragraphs have been adapted, taking into account the most recent data. Chapters 5 and 8 correspond to submitted articles, whereas chapter 6 is based on a manuscript currently in preparation. Chapter 3 briefly summarises the preliminary results of an ongoing study that need to be further explored. The final

section will be devoted to a short general conclusion allowing to situate the data accumulated during the project in a larger context and suggesting possibilities for follow-up studies.

Although a growing body of evidence indicate that the two-step/two-site binding model is not sufficient to faithfully reflect how chemokines interact with their receptors, it still provides a valuable conceptual framework to understand these interactions and therefore reference to site 1 and site 2 will be regularly made in this thesis. Furthermore, according to the IUPHAR, CXCR7 has recently been officially classified among the atypical chemokine receptors and renamed ACKR3, throughout this work, however, its previous name will be used.

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Abbreviations

ACKR: atypical chemokine receptor

ADCC: antibody-dependent cell-mediated cytotoxicity

AIDS: acquired immune deficiency disorder

cAMP: cyclic adenosine monophosphate

CCL: chemokine CC motif ligand

CRS: chemokine-recognition site

CXCL: chemokine CXC motif ligand

CX3CL: chemokine CX3C motif ligand

DARC: Duffy blood group antigen

EBV: Epstein–Barr virus

ECL: extracellular loop

ERK: extracellular signal-regulated kinase

GAG: glycosaminoglycan

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

HCMV: human cytomegalovirus

HEK: human embryonic kidney

HHV: human herpesvirus

HIV: human immunodeficiency virus

HTLV-1: human T-lymphotropic virus-1

ICL: intracellular loop

KS: Kaposi's sarcoma

KSHV: Kaposi's sarcoma-associated herpesvirus

mAb: monoclonal antibody

MAP: mitogen-activated protein

MCD: multicentric Castelman disease

NK: natural killer

NMR: nuclear magnetic resonance

ORF: open reading frame

PEL: primary effusion lymphoma

PET: positron emission tomography

PI3K: phosphoinositide-3 kinase

PTM: post-translational modification

PTX: pertussis toxin

TM: transmembrane segment

Th1/Th2: Type 1 or 2 T helper cells

vCCL2/vMIP-II: viral CC motif chemokine 2/viral macrophage inflammatory protein-II

XCL: chemokine C motif ligand

Introduction

I. Chemokines and their receptors

1. Structure and classification of chemokines and their receptors

Chemokines are small (8-14 kDa) secreted proteins that play a central role in guiding directional migration (chemotaxis) of leukocytes in immunosurveillance and immune responses. Despite their low level of sequence identity, all chemokines display a common structure consisting of a flexible N terminus, a cysteine motif followed by an N-loop, three antiparallel β-strands and a C-terminal α-helix (Fig. 1). This structure is stabilised by one or two disulphide bonds linking the cysteine motif with the β1-β2 turn (30s loop) and the β3-strand. Based on the disposition of the cysteines within the conserved motif, chemokines are divided into four families designated XC, CC, CXC and CX3C. The CXC family can be further subdivided on the basis of the presence of an N-terminal motif glutamate-leucine-arginine (ELR) adjacent to the cysteine motif, into ELR-positive and ELR-negative chemokines.

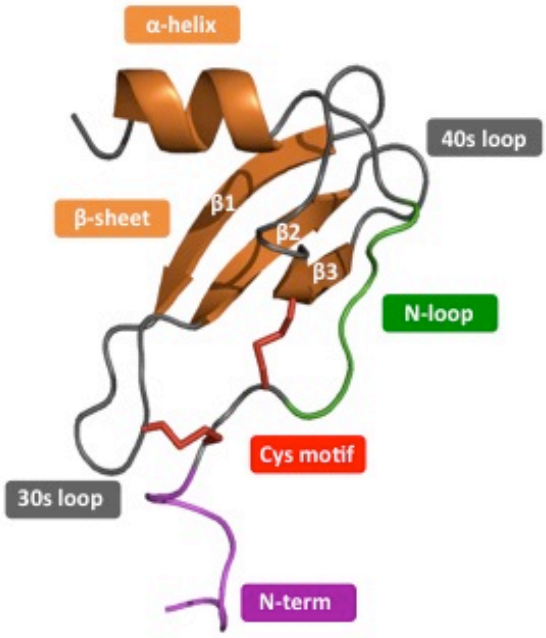

Fig. 1. Chemokine three-dimensional structure. Three-dimensional structure of CXCL12 resolved by X-ray crystallography (PDB ID: 1SDF) showing the structural arrangement of chemokine features, including the highly disordered N terminus (purple), the N-loop (green), the conserved cysteine motif (red) and the core of the chemokine with the three antiparallel β-strands and the C-terminal α-helix (orange). The secondary structure elements are connected by turns known as 30s, 40s and 50s loops, reflecting the numbering of residues in the protein.

Chemokine exert their functions by interacting with chemokine receptors, which belong to the superfamily of class A (or rhodopsin-like) heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs). Chemokine receptors show the typical GPCR structure consisting of seven hydrophobic transmembrane (TM) α--helices separated by alternating hydrophilic extracellular (ECL) and intracellular (ICL) loops,

with the N terminus situated on the outside of the cell and the C terminal tail on the intracellular side (Fig. 2). Chemokine receptors are stabilised in a barrel-like structure by two disulphide bridges linking ECL1 (or the top of TM3) to ECL2 and the N terminus of the receptor to ECL3 (or the top TM7). Based on the chemokines that they bind, chemokine receptors are classified into four families: XCR, CCR, CXCR and CX3CR.

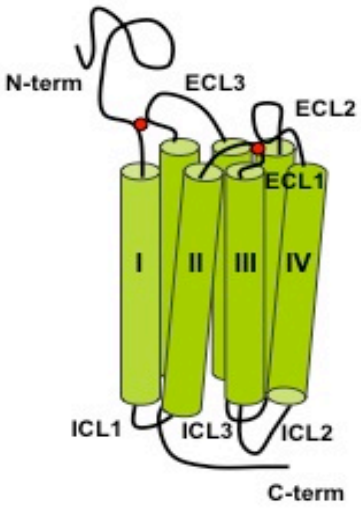

Fig.2. Chemokine receptor architecture. The three extracellular loops (ECL1-3) and the N terminus are situated in the extracellular region and the three intracellular loops (ICL1-3) and the C terminus, in the intracellular region. The transmembrane segments arranged in counter-clockwise manner are numbered with Roman numerals. The two disulphide bridges are represented as red dots.

To date, 47 chemokines and 19 receptors have been identified in humans. They form a highly intricate and precisely regulated network, where a chemokine may bind to and activate several receptors, while a chemokine receptor usually has multiple ligands. In addition, other receptors referred to as atypical chemokine receptors (ACKR1-4) can recognise chemokines and act as scavengers or signal through alternative pathways, further contributing to the complexity of the chemokine network (Fig. 3).

Chemokine-receptor interactions may be further regulated on different levels including by tissue-specific expression patterns of both partners, receptor and chemokine proteolysis, oligomerisation and interactions with glycosaminoglycans (GAGs) that are part of extracellular proteoglycans [1].

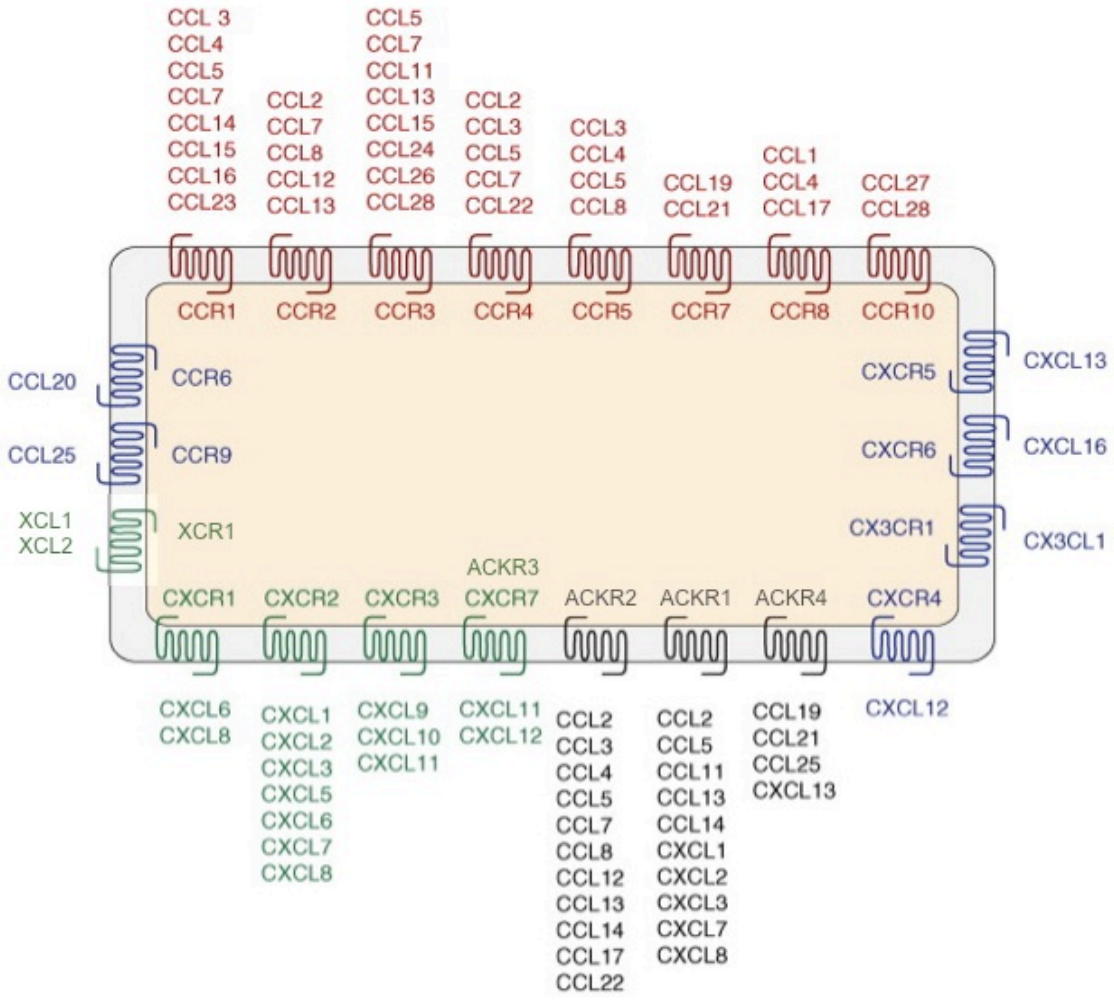

Fig. 3. Chemokine and chemokine receptor families. Most chemokines can interact with multiple receptors, and a single receptor can interact with multiple chemokines. This is the case for most CC (red) and CXC (green) chemokines. Decoy receptors (black) can also bind multiple chemokines. By contrast, a minority of receptors (blue) have only one ligand. Adapted from Lazennec and Richmond 2010 [2].

2. G protein signalling

In their inactive state, chemokine receptors can associate with heterotrimeric GDP-bound G proteins. Upon ligand binding the G protein trimers dissociate into an active GTP-bound Gα and a Gβγ dimer, each independently triggering downstream signalling pathways that result in a variety of cellular responses. Chemokine receptors typically signal through the pertussis toxin-sensitive $G\alpha_{i/o}$ subtype, resulting in adenylate cyclase inhibition, decrease of intracellular cAMP levels and activation of PKA. Other events have been shown to occur in response to chemokine binding, e.g., phosphorylation of ERK1/2 (part of the MAP kinase cascade), activation of phosphoinositide-3 kinase (PI3K)/Akt pathways [3] as well as increase in Ca^{2+} flux, most likely through the Gβγ

subunit, which activates PLC-β [3]. These various signalling responses lead to modulation of transcription factors that mediate changes in the cytoskeletal apparatus, regulate cell growth or production of other cytokines.

3. Arrestin recruitment

It is essential for cellular homeostasis to restore the receptor basal state following its activation and signal transduction. This process is initiated by PKC or G protein-coupled receptor kinases (GRK), which leads to the phosphorylation of multiple sites, mainly threonine or serine residues, in the C terminus of the receptor. This phosphorylation allows for to the recruitment of an adaptor molecule, β-arrestin, which sterically inhibits further binding of G proteins to the receptor. β-arrestin is then ubiquitinilated, which leads to receptor endocytosis through clathrin-coated pits and receptor degradation or recycling back to the membrane. Furthermore, over the last decade, a new paradigm has emerged for chemokine receptors, and more generally for GPCRs, according to which arrestin, besides its role in receptor desensitisation, is itself an essential signal transducer molecule, leading to activation of cellular pathways and a variety of physiological outcomes.

4. Function of chemokines and their receptors

Chemokines and their receptors regulate vital cellular mechanisms including migration, adhesion as well as growth and survival [4, 5]. Functionally, chemokines are often classified as either homeostatic or inflammatory. Homeostatic chemokines regulate processes such as haematopoiesis, development of lymphoid organs, immunosurveillence as well as embryonic development and angiogenesis. Inflammatory chemokines are induced under stress and play crucial roles in adaptive and innate immune response, wound healing and organ repair by attracting effector cells to the site of infection or injury [6-8]. The inflammatory responses driven by chemokines, when deregulated, are also source of pathological processes including inflammatory and autoimmune diseases as well as cancer [2, 5, 9-12].

5. Pathogens targeting chemokine receptor system

Various pathogens have evolved ways to subvert and exploit the immune processes regulated by chemokines and their receptors to promote their survival and propagation.

Large DNA viruses (poxviruses, herpesviruses) have hijacked host chemokine or chemokine receptor genes or have evolved their own chemokine-binding proteins to interfere with the host immune response thereby increasing their chances of survival and efficiency of dissemination [13, 14].

The human herpesvirus 8 encodes three chemokines vCCL1/vMIP-I, vCCL2/vMIP-II and vCCL3/vMIP-III. vCCL2 is a case of viral molecular mimicry par excellence and will be discussed more in detail in Chapter 4. HHV-8 and other hepersviruses have also pirated genes encoding viral G protein-coupled receptors (vGPCRs) such as ORF74, US28 or BILF, which are expressed on infected host cells and have acquired unique properties such as constitutive signalling and the ability to bind a broad range of human chemokines [13].

Similar immune evasion strategy is used by viruses like herpesviruses or poxviruses but also the parasitic worm *Schistosoma mansoni* and ticks that encode diverse soluble chemokine-binding proteins (CKBP) able to bind chemokines with high affinity, despite no sequence similarity to cellular chemokine receptors [15-17]. The vast majority of CKBPs bind directly to the receptor-binding region of the chemokine, often to the N terminus, but others can also indirectly inhibit chemokine activity by interfering with their GAG binding domain thereby preventing the formation of chemokine gradients.

Chemokine receptors can also be hijacked by pathogens to allow their entry into specific cell types. One of the best-known examples is the human immunodeficiency virus (HIV-1), the causative agent of AIDS, which uses CCR5 and CXCR4 as entry co-receptors through interactions with its envelope protein, gp120 [18, 19].

Another striking example of chemokine receptor piracy are the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* that use the atypical chemokine receptor ACKR1/DARC (Duffy blood group antigen) to invade human erythrocytes [20-22]. The molecular details of chemokine receptor interactions with gp120 of HIV and the Duffy binding protein of malaria parasites are elaborated on in Chapter 1.

6. Chemokines and their receptors as drug targets

To date, only a small number of drugs developed to target the chemokine-receptor network have made it through the clinical trials. It was at first all the more surprising and disappointing for chemokine receptors, as they belong to the GPCR family, which is currently the target of about 30 % of marketed drugs [16]. The still-limited understanding of the biology and selectivity of the highly promiscuous chemokine-receptor network is certainly one of the causes of the poor success rate in developing efficacious treatment strategies. Another issue often raised in this context is that the majority of therapeutic targets are validated in rodent disease models, which may be misleading considering the substantial differences between rodents and humans in the role played by particular chemokine-receptor pairs [16].

Several strategies have been used to target pathology-implicated chemokine-receptor interactions, either by blocking the receptor, the chemokine or indirectly by inhibiting their binding to GAGs [23].

To date, only two small molecules targeting chemokine receptors have reached the market and are approved in Europe and the United States. The first is the antiretroviral drug maraviroc, targeting CCR5 and used as HIV entry inhibitor blocking the interaction between the virus envelope protein gp120 and the co-receptor. The second molecule is the CXCR4 antagonist AMD3100 (plerixafor), a bicyclam derivative used for mobilisation of hematopoietic stem cells from the bone marrow to the bloodstream and post-chemotherapy autologous transplantation in patients with lymphoma and multiple myeloma.

In addition, many attempts have been made at developing therapeutic antibodies either directly blocking the target, be it the chemokine receptor, its cognate ligand or a receptor-binding pathogen protein, or having an indirect effect by triggering host-dependent responses like antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Alternatively, antibodies can be used as carriers to deliver cytotoxic agents or radioisotopes [24].

The first and, at the moment, only marketed antibody targeting a chemokine receptor is the humanised mouse monoclonal anti-CCR4 antibody. It recognises the N terminus of CCR4 and does not inhibit the interaction of CCR4 with its ligands but rather induces ADCC (see Chapter 1, section 4 for further discussion on the therapeutic potential of

chemokine receptor N terminus). KW-0761 has been marketed in Japan in 2012 for the treatment of relapsed and refractory adult T cell leukemia and its safety and efficacy is currently being further evaluated, also in Europe and the United States, against various types of leukemia and lymphoma [25].

Various other antibodies or small molecule inhibitors are also being investigated but a better understanding of the molecular basis of the promiscuity of chemokine-receptor interactions and their physiological and pathological implications could offer possibilities to improve therapeutic strategies [26].

II. Chemokine receptor interactions

1. Two-site/two-step model and beyond

The two-step/two-site model was proposed almost 20 years ago through the work of three concurrent functional and structural studies using chimeric chemokines and receptors as well as peptides derived from the receptor N terminus [27-29]. This model describes the chemokine-receptor interactions *functionally* (two-step) and *spatially* (two-site). Spatially, site 1 refers to interactions between the receptor N terminus (chemokine recognition site 1, CRS1) and the chemokine globular core, and site 2 refers to contacts between residues in the receptor transmembrane (TM) domain (CRS2) and the unstructured chemokine N terminus [30] (Fig. 4). Functionally, site 1 provides affinity and specificity, whereas site 2 interactions lead to receptor activation.

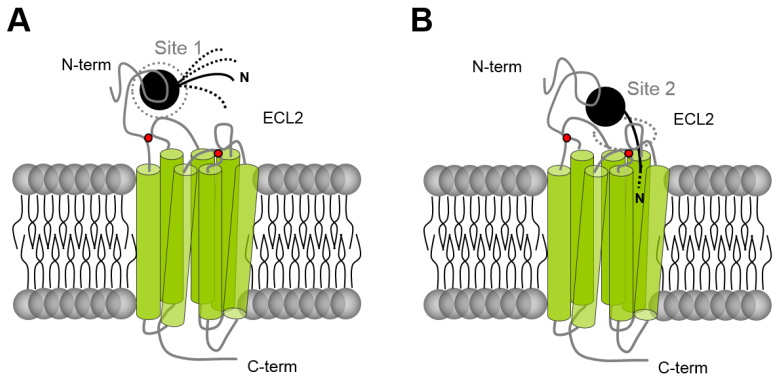

Fig. 4. Putative two-step/two-site mechanism for the interaction between chemokines and chemokine receptors. (A) First step: interactions between the N-loop of the chemokine and the N-terminal domain of the receptor. **(B)** Step two: interactions between the flexible N terminus of the chemokine and the extracellular loops as well as the transmembrane segments of the receptor. The disulphide bridges between N-term/ECL3 and ECL1/ECL2 are depicted as red dots.

Although the two-step/two-side model has for years provided a useful conceptual framework in which to understand the interactions between chemokines and their receptor, it often overlooks other receptor regions that also play crucial structural and functional roles. Notably, interactions between chemokines and the receptor extracellular loops (ECLs) are variously ascribed to site 1, site 2, or not included in these models at all [31-35] (see Chapters 2, 6 and 8).

Furthermore, the first two chemokine-receptor crystal structures showed that in contrast to recognising spatially distinct receptor domains, the chemokines can form interactions spanning from the receptor N terminus (site 1) to the receptor TM domain (site 2) [34]. This interaction region between the chemokine's cysteine motif and the receptor N-terminal base does not corresponds to either site 1 or site 2 of the two-step/two-site model and was named *chemokine recognition site 1.5* (CRS1.5) [34-36]. The existence of multiple intermediate interfaces challenges thus the assumed *spatial* and *functional* separation between sites 1 and 2.

Various reports on oligomerisation of both chemokines and receptor also calls into question the current binding model, as they suggest that the stoichiometry of their interactions may be different, at least in some instances, than the 1:1 relation (see chapter 8).

2. Chemokine and receptor oligomerisation

2.1 Chemokine dimerisation

Initially thought to be a crystallisation artefact, chemokine dimerisation has been re-examined over the past years in numerous structural and biochemical studies. It appears now that the vast majority of chemokines are able to form dimeric structures with the monomer-dimer equilibrium being regulated by factors such as pH, anions and interactions with glycosaminoglycans [37, 38]. Additionally, some chemokines can form tetramers or higher-order oligomers [39-42]. Heterodimers of two different CC or CXC chemokines as well as cross-family CC/CXC heterodimers have also been reported [37, 38].

The biological relevance of chemokine dimerisation is still a matter of debate and its exact impact on receptor binding, stoichiometry and signalling remains to be unravelled [38, 43-45]. It has however been demonstrated that monomeric and dimeric CXCL12

induce different intracellular signalling and opposite effects on cell migration suggesting yet another level of regulation of chemokine-receptor interaction [46].

2.2 Receptor dimerisation

It has been for long assumed that chemokine receptors exist as monomers, which behave as fully competent signalling units. The first structural evidence of chemokine receptor dimerisation was provided by the first inactive-state crystal structures of CXCR4 in which the receptor was present as a dimer with the interface between the subunits located at the top of TM5 and TM6 and stabilised by hydrogen bonds [47]. Chemokine receptors from all four subfamilies (C, CC, CXC, CX3C) have now been described to form homo- or heterodimers *in vitro* [48-51]. Receptor dimerisation has been shown to modify their ligand binding properties [52, 53] and signalling [54-57] as well as intracellular trafficking [58]. However, so far there is no *in vivo* data reporting the existence of chemokine receptor dimers and therefore their biological relevance remains a controversial question [59, 60].

3. Functional selectivity – biased signalling

Compared to the rather exclusive ligand-receptor paring characterising other GPCR families, the interaction network of chemokines and their receptors is highly complex. Usually, a chemokine is able to bind and activate several receptors, while a receptor can have multiple chemokine agonists. This promiscuity of interactions has long been regarded as simple redundancy but it has now become apparent that it allows to achieve a great variety of crucial functions through distinct effects of chemokine-receptor pairs depending on their spatio-temporal expression [61]. In addition, until recently chemokine receptors were commonly accepted to signal exclusively through the canonical G protein pathways and to be coupled to the $G_{\alpha i/o}$ subtype. However, a growing body of evidences show that, depending on the ligand and the cellular context, some chemokine receptors can also signal through other G protein subtypes (G_s, $G_{q/11}$ or $G_{12/13}$) or activate signalling independent of G proteins such as arrestin-mediated signalling pathways [3, 62, 63]. This emerging paradigm is known as biased signalling or functional selectivity and appears to be ubiquitous among chemokines and chemokine receptors, complexifying their crosstalk and diversifying the possible

signalling outcomes. Biased signalling can originate from three main phenomena: ligand bias, receptor bias and tissue or cell bias [64-67] but in fact it may occur at all the steps of chemokine-receptor interactions.

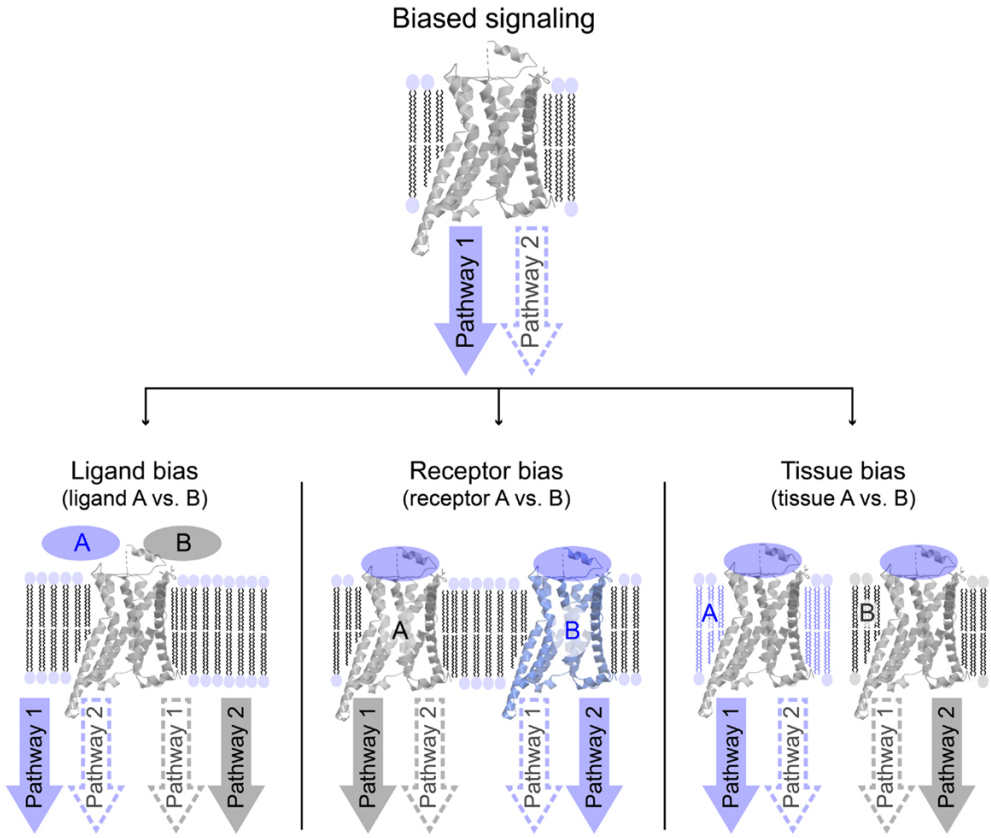

Fig. 5. Simplified overview of biased signalling at chemokine receptors. Biased signalling describes a situation in which one signalling pathway is activated over another. **(Left)** Ligand bias: different chemokines binding to the same receptor activate distinct cellular responses **(Centre)** Receptor bias: the same chemokine activates different pathways depending on the receptor it binds. **(Right)** Tissue bias: the same chemokine-receptor pair triggers distinct signalling pathways depending on the cellular context. From Steen *et al.* 2014 [65].

Ligand bias can be best illustrated by the situation when different chemokines binding to the same receptor activate distinct cellular responses. In addition, ligand bias may depend on chemokine post-translational modifications, such as truncation, citrullination or dimerisation [46, 68]. The poor sequence identity between chemokines, especially in their N terminus, which bears important receptor binding and activation determinants, in part explains the existence of ligand bias. Indeed, chemokines may engage the same receptor through slightly different binding modes and thereby stabilise distinct active forms of the receptor, defining to which cellular signalling effectors it will preferentially couple. The recently uncovered molecular basis of ligand bias in other receptor families

also suggests an important role of the interactions with specific structural determinants, the so-called micro-switches, and of distinct helical movements of TM5 and TM6 or TM7, favouring G protein coupling or arrestin signalling, respectively [65, 69, 70].

Biased signalling may also rely on the functionality of the *receptor* and the presence of motifs that ensure on one hand chemokine binding and on the other hand the coupling of the receptor to various effectors. So far, receptor bias has mainly been observed between conventional and atypical chemokine receptors or for viral chemokine receptors [71]. For instance, CXCL12 binding to CXCR4 induces canonical G protein signalling but also arrestin signalling [3, 72], whereas its binding to CXCR7 is proposed to trigger arrestin-mediated, G protein-independent signalling [73].

Bias can also occur at the *cellular* level as the same chemokine-receptor pair may trigger distinct signalling pathways or cellular responses, depending on the cellular context [74, 75]. Such cellular bias is unsurprising considering the large variety of cells expressing chemokine receptors and having different expression patterns of signalling partners (G proteins subtypes and arrestin isoforms), receptor modifying enzymes (GRKs, TPSTs) or effector molecules as well as of other chemokine receptors or receptors modulating partners.

Altogether, these different aspects of bias signalling complexify tremendously the responses of chemokine receptors to their cognate ligands and reveal how finely regulated their interactions are. The various levels of bias are tightly linked and should not be regarded as independent mechanisms when chemokine-receptor interactions are considered. Although the binding of a particular chemokine to the receptor may dictate the functional outcome by stabilising a particular active state of the receptor, it equally depends on the intrinsic functionality of the receptor as well as the expression patterns of second messenger and signalling effector molecules.

4. Non-chemokine ligands

Some chemokine receptors were also shown to bind to endogenous or virus-encoded ligands other than chemokines. These unconventional ligands vary extremely in terms of size, ranging from large proteins to peptides, and often have no sequence or structural similarities with chemokines [76-79]. They trigger signalling pathways similar to or different from those induced by the endogenous chemokines [76-82]. For some of

these non-chemokine ligands, the binding and signalling rely on the chemokine receptor alone [79], while for others, the chemokine receptor operates in tandem with another membrane protein that usually serves as primary receptor [18, 80].

Intracellular signalling induced by the HIV envelope protein gp120 (120 kDa) following interactions with CCR5 and CXCR4 is well documented. Another HIV protein, the matrix protein p17, was reported to bind to CXCR1 and CXCR2 inducing chemokine-like activity on monocytes [83, 84].

More recently, the pseudo-chemokine MIF (macrophage migration inhibitory factor), a pleiotropic and proinflammatory chemotactic cytokine of 12.3 kDa highly expressed by tumour cells has been shown to induce signalling and chemotaxis through CXCR2 [77], CXCR4 [78] and CXCR7 [80].

CXCR4 has also been shown to bind extracellular ubiquitin (eUb, 8.6 kDa), leading to G protein signalling similar to that induced by CXCL12 [79]. Other endogenous non-chemokine ligands such as human β3-defensin (HDB-3) (5.1 kDa) [85] and EPI-X4 (1.8 kDa) a 16-amino acid peptide derived from human albumin [86] were also demonstrated to interact with CXCR4 but failed to induce intracellular signalling.

The identification of non-cognate ligands for chemokine receptors, some exclusive of one receptor, others interacting with several receptors not necessarily belonging to the same family, further emphasises the complexity of the chemokine receptor network, which seems now even more promiscuous and predisposed to bias than initially thought. These new ligands will certainly help to uncover other important physiological and pathological functions for this family of receptors, explain past observations and provide new therapeutic opportunities to modulate chemokine/receptor activity.

We are only now starting to really appreciate the complexity of chemokine-receptor interactions through the numerous recent paradigm-changing discoveries based on chemokine-receptor three-dimensional structures, the existence of biased signalling or the identification of several new non-chemokine ligands for chemokine receptors. The last section of the introduction will present the atypical chemokine receptor, CXCR7, which was the focus of this thesis and whose biology and functions have also recently been reassessed.

III. CXCR7

1. Historical overview and ligand identification

CXCR7 was originally isolated from a dog thyroid cDNA library and named RDC1 (receptor dog cDNA) [87]. Mouse and a human orthologues were subsequently found and later the observation that RDC-1 is found on the same chromosome and shares high degree of similarity with CXCR2 and CXCR4 lead to presume that RDC-1 was an orphan CXC chemokine receptor [88].

Ever since it was initially isolated, several ligands had been proposed for RDC-1, including the vasoactive intestinal peptide (VIP) or calcitonin gene-related peptide (CGRP) but these interactions could never be confirmed [89, 90]. In addition the peptide hormone adrenomedullin was also suggested to recognise and activate RDC1 [91].
It was only a decade ago, when CXCL12 and CXCL11 were identified as its high-affinity ligands that RDC-1 was deorpahinsed and classified among the chemokine receptors as CXCR7 [92]. Since then, an ever-increasing number of studies have been pointing to the involvement of CXCR7 in many physiological and pathological processes and its possible crosstalk with CXCR4 and CXCR3, which also bind to CXCL12 and CXCL11, respectively.

Furthermore, more recently it also emerged that CXCR7 can interact with two other non-chemokine proteins. The first one is the pseudo-chemokine MIF (macrophage migration inhibitory factor), a pro-inflammatory chemotactic cytokine highly expressed by tumour cells and proposed to bind and activate CXCR7, although the presence of another membrane protein, CD74 is required [80]. The second recently identified ligand is the peptide hormone belonging to the calcitonin gene-related peptide family, adrenomedullin, which in fact has already been proposed to interact with CXCR7 in an early, unnoticed study [91]. Similarly to MIF, adrenomedullin may need another membrane protein to efficiently bind CXCR7.

The exact molecular bases of CXCR7 interactions with both its chemokine and non-chemokine ligands remain unexplored. Moreover, due to its unusual biology (see further), CXCR7 has now been classified as an atypical chemokine receptor and renamed ACKR3 [73, 93].

2. Biological role

CXCR7, is expressed in various cells such as B and T lymphocytes, neurons and endothelial cells and plays a crucial role in many processes including cardiovascular and neuronal development as well as in migration and homing of hematopoietic stem/progenitor cells [92, 94-99]. *Cxcr7* knockout mice die perinatally due to cardiac defects. It has recently been proposed that the lethality of CXCR7 depletion may be accounted for by the receptor's involvement in the control of adrenomedullin levels, required for normal cardiovascular development [91, 98]. CXCR7 is also proposed to play a role in immune responses and in tissue repair although the exact mechanisms are not well understood.

An increasing number of studies point to the involvement of CXCR7 in many cancers as it is expressed in various cancer cell types as well as on tumour-associated vasculature and accumulating evidence demonstrates its involvement in metastasis development [100-103].

CXCR7 was also shown to be upregulated upon infection by several cancer-inducing viruses including HHV-8, EBV, HTLV-1 and to play an important role in cell transformation and proliferation [104, 105].

3. Signalling vs. scavenging

Divergent data exist in the literature regarding the signalling properties of CXCR7 [92, 106, 107]. The predominant view in the field is that CXCR7 is unable to trigger G protein signalling, as shown by the lack of the classical responses induced by chemokine (intracellular calcium release, cAMP modulation and chemotaxis [92, 107]. Instead, it is proposed that CXCR7 can trigger arrestin-dependent signalling [3, 72, 108].

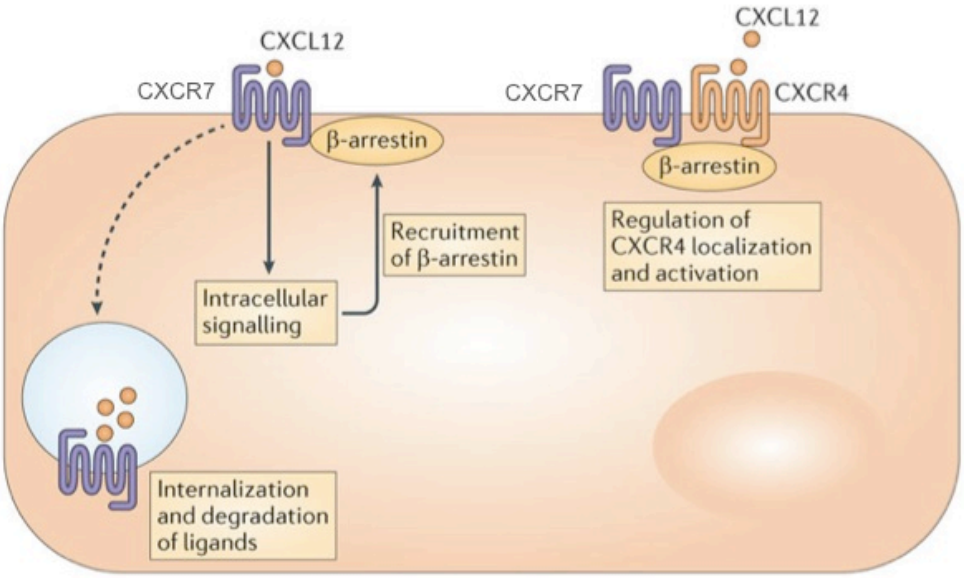

Fig. 6. Cellular functions of CXCR7. CXCL12 binding induces arrestin recruitment to the receptor leading to its internalisation and subsequent ligand degradation. On some cellular contexts, this interaction may also trigger intracellular signalling. CXCR7 can also regulate the trafficking and signal transduction of CXCR4, thereby modulating the biological responses that are mediated by this conventional chemokine receptor. Adapted from Nibbs and Graham, 2013 [109].

CXCR7 is also commonly proposed as a non-signalling receptor acting exclusively as a chemokine scavenger. Through its continuous cycling between the plasma membrane and endosomal compartments and its capacity to efficiently internalise and degrade chemokines CXCR7 can regulate the availability of CXCL12 and CXCL11 for CXCR4 and CXCR3 [110-114]. Endorsing the hypothesis of CXCR7 role as a scavenger receptor is its 10-fold higher affinity for CXCL12 compared with CXCR4 and the observation that after internalisation following chemokine binding, CXCR7 rapidly recycles back to the cell surface and is therefore continually available to clear chemokines [92, 115, 116]. In addition, CXCR7 was shown to regulate other aspects of CXCR4 functions through heterodimerisation [113, 117] (Fig.6).

CXCR7 has recently been renamed ACKR3 and classified among the atypical chemokine receptor family, which is currently composed of three other members, ACKR1 (formerly DARC), ACKR2 (D6) and ACKR4 (CCRL1 or CCXCKR), all characterised by their inability to signal through the classical G protein pathways [93, 118]. They are also commonly referred to as the decoy receptors owing to their ability to internalise and degrade chemokines. However, while the other ACKRs are highly promiscuous and

bind a large number of mostly CC chemokines CXCR7 has a more limited ligand repertoire (Fig. 3).

Currently, there is no clear explanation for the contradicting reports regarding CXCR7 activity and signalling properties however, cell type or cell context differences may be a contributing factor.

Various sections presented in this introduction will be further elaborated in the following chapters. First, an overview on the chemokine receptor N termini will be given, emphasising the presence of the second cysteine bridge between the N terminus and the top of TM7 and the formation of a fourth extracellular loop as well as molecular signatures, which we identified in this project. The existence and the potential role of several particularities of the N terminus of CXCR7 will be then discussed and supported by preliminary experimental data. The properties and functions of a viral chemokine, vCCL2, will be then outlined, followed by its characterisation as a new ligand for CXCR7. A comparative study on the importance of various features of the N terminus of chemokines, including vCCL2, for the interactions with CXCR7 but also CXCR3 and CXCR4 will also be presented. Finally, from receptor's viewpoint, our study on the structural ligand-binding determinants of CXCR4 will be described. The closing chapter will illustrate how the present models for chemokine-receptor interactions are being challenged, leading to changes in paradigms.

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Chapter 1

Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors

Adapted from Szpakowska M, Fievez V, Arumugan K, van Nuland N, Schmit J-C and Chevigné A. Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors. Biochemical Pharmacology 2012 Nov 15;84(10):1366-80.

This first chapter originates in a thorough comparative analysis of the N terminus of CXCR7 and other chemokine receptors, performed at the very beginning of my project. The aim was to understand the characteristics of this region of chemokine receptors in general but also to determine distinctive features of CXCR7, which could help to explain its atypical properties. Interestingly, we were also able to identify new molecular signatures within the N-terminal domains of chemokine receptors.

Chemokine-receptor interactions are particularly intricate and require precise orchestration. The seemingly redundant network of many chemokines binding to multiple receptors and vice versa reflects in fact a high degree of regulation, resulting in myriad of functional outcomes. Although a growing body of evidence suggests that the early two-step/two-site model for chemokine-receptor interactions is highly oversimplified, the binding of the receptor N terminus to the chemokine globular core remains an important component of chemokine-receptor interactions and has regularly been demonstrated to hold a crucial role in the initial recognition and selective binding of the receptor ligands. The length and the amino acid sequences of the N termini vary considerably among different receptors but they all show a high content of negatively charged residues and are subject to post-translational modifications such as O-sulfation and N- or O-glycosylation. In addition, a conserved cysteine that is most likely engaged in a receptor-stabilising disulphide bond delimits two functionally distinct parts in the N terminus, characterised by specific molecular signatures. Structural analyses have also shown that for many chemokine-receptor pairs the N terminus of chemokine receptors recognises a groove on the chemokine surface and that this interaction is stabilised by high-affinity binding to a conserved sulfotyrosine-binding pocket.

The diversity of human chemokine receptor N-terminal domains will be discussed in this chapter and illustrated in a comprehensive annotated inventory of their sequences, laying special emphasis on the presence of post-translational modifications and functional features. Various attempts to develop therapeutic strategies targeting the receptor N terminus interactions will also be described.

1. Introduction

Chemokine receptors are rhodopsin-like G protein-coupled receptors (GPCRs) displaying a structure typical of this family that consists of seven hydrophobic membrane-spanning α-helices separated by alternating hydrophilic extracellular (ECL) and intracellular (ICL) loops. The N terminus of the receptor is situated on the outside of the cell and participates in ligand binding whereas the C-terminal tail is located on the intracellular side. Upon ligand binding, chemokine receptors activate intracellular heterotrimeric G proteins triggering downstream signalling pathways that result in a variety of cellular responses. Additionally, non-signalling receptors such as decoy receptors were shown to control the cellular response to chemokines by sequestration and modulation of their local concentration [1, 2].

Chemokines and their receptors regulate vital cellular mechanisms including migration, adhesion as well as growth and survival [3, 4]. Chemokines control processes such as embryonic development, angiogenesis and haematopoiesis but can be also released under stress. These inducible chemokines play crucial roles in adaptive and innate immune response, wound healing and organ repair by attracting effector cells to the site of infection or injury [5-7]. Many chemokines are also involved in pathological processes including inflammatory and autoimmune diseases as well as cancer [4, 8-10]. In addition, some pathogens interfere with the host chemokine/chemokine receptor network to promote their own survival by either encoding chemokines, chemokine receptors or other chemokine-binding proteins or by co-opting chemokine receptors for host cell entry.

Chemokines interactions with their cognate receptors are often described with a simple two-step/two-site model [11, 12]. According to this model, the initial step corresponds to the anchoring of the chemokine to the N terminus of the receptor and is followed by the binding of the flexible chemokine N terminus to the extracellular loops and the transmembrane segments of the receptor. Numerous studies illustrate the great importance of the extracellular parts, and in particular the receptor N terminus, in discriminating between the various chemokine ligands.

Despite their low level of sequence identity, all chemokines display a common monomeric structure consisting of a flexible N terminus followed by an N-loop, three anti-parallel β-strands and a C-terminal α-helix [13, 14]. The N-terminal domain contains one or two cysteines implicated in structure-stabilising disulphide bonds. Based on the positioning of these cysteines, chemokines are divided into four subfamilies: XC, CC, CXC and CX_3C [15]. Accordingly, chemokine receptors are named XCR, CCR, CXCR or CX_3CR. The chemokine-receptor network is very complex and a given chemokine may bind to several receptors, while a chemokine receptor usually has multiple ligands. To date, over fifty chemokines and twenty receptors have been identified in humans.

The N-terminal domains vary considerably in length between different chemokine receptors, also within subfamilies. They do however display a number of common features, including high content of negatively charged residues, tyrosine sulfation motifs and N-glycosylation sites. In addition, a highly conserved disulphide bond links the N terminus and the third extracellular loop. Some of these characteristics of the chemokine receptor N-terminal domain have been shown to strongly influence ligand binding as well as the cellular responses.

Given the implication of the chemokine network in many pathologies, a better understanding of the mechanisms driving ligand binding to chemokine receptors is essential for the development of highly specific therapeutic molecules targeting either the receptors or chemokines. To accurately comprehend these interactions, three-dimensional structures of chemokine receptors are needed. Yet, their resolution has proven particularly arduous mainly due to the difficulties in purifying and crystallising these proteins. During the last five years, several three-dimensional structures of chemokine receptors have been resolved. For the earlier co-complexes with small molecule ligands or short peptidic derivatives, the spatial arrangement of the receptor N terminus could not be determined due to the high flexibility of this region [16, 17]. The very recent first two crystal structures of chemokine receptors bound to chemokine ligands (CXCR4 in complex with the viral chemokine vCCL2 and the viral chemokine receptor US28 in complex with CX3CL1) provided valuable molecular details on these interactions, yet the exact way the receptor N

terminus engages the chemokine, especially its most proximal flexible part, remains difficult to determine (see also chapter 8) [18, 19].

Multiple alternative approaches have also been used to investigate the interactions of the N terminus of chemokine receptors with their ligands. Chimeric, mutated or truncated receptors have long been widely exploited [20-26]. In parallel, soluble synthetic peptides derived from the N termini of chemokine receptors have been used as models for the binding of ligands to full-length receptors [27, 28]. In particular, the NMR studies of interactions between the receptor N terminus-derived peptides and chemokines have provided substantial functional and structural information in this regard [29-36]. Additionally, grafting of the N terminus together with another extracellular loop on the B1 domain of protein G soluble scaffolds allowed examining ligand interactions in contexts reminiscent of native receptors [37, 38]. Other approaches aimed to investigate the N terminus of chemokine receptors in more membrane-like environments such as micelles or phospholipid bilayers as well as in fusion with membrane proteins [27, 39, 40]. Thanks to this constantly growing arsenal of methods and increasingly powerful tools, remarkable progress has been made towards the elucidation of ligand interactions with chemokine receptors.

The present review gives an outline of the information currently available on the diversity and function of human chemokine receptor N-terminal domains. Additionally, it provides a comprehensive annotated inventory of the chemokine receptor N-terminal sequences, laying special emphasis on the presence of post-translational modifications, sequence signatures and functional features.

2. Sequence diversity of chemokine receptor N-terminal domains

2.1 Length and molecular signatures

Chemokine receptors present relatively short N-terminal domains ranging from 26 (CX3CR1) to 65 (ACKR1) amino acids compared to the N-terminal domains of up to 600 amino acids in other GPCRs. Notably, in all chemokine receptors except for CXCR6, the N-terminal domains bear a conserved cysteine residue in their second moiety. This cysteine is

likely to be engaged in a disulphide bridge with the third extracellular loop of the receptor (ECL3 or top of TM7) and delimits two functional parts characterised by different sequence features: the M-C part including residues from the N-terminal methionine (M) to the cysteine (C) and the C-TM part including the residues from the cysteine to the first transmembrane segment (TM). While the M-C parts are in general described as very flexible, the C-TM parts link the TM1 and TM7 through a disulphide bridge forming a pseudo-loop at the surface of the receptor (Fig. 1).

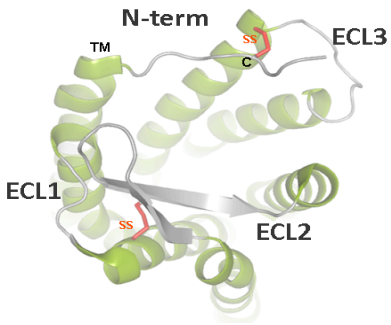

Fig. 1. Top-down view of chemokine receptor surface. X-ray structure of CXCR4 (PDB ID: 3OEO). The seven transmembrane (TM) segments are represented in green. The two disulphide bridges connecting the N terminus to ECL3 (top of TM7) and ECL1 (top of TM3) to ECL2 are coloured in red and indicated by SS. The N-terminal part of the N terminus (M-C part) is flexible and unstructured in the absence of chemokine. Clear electron density was only observed for C-TM part, starting at residue P28.

In all chemokine receptor families, the M-C parts show variable length, low sequence identity, overall negative charges and contain multiple tyrosine and asparagine residues that are post-translationally modified. The size of the M-C parts varies, also within the receptor families, from 21 to 51 amino. Moreover, there seems to be no correlation between their length and the selectivity of the receptor. Low identity observed in the M-C parts supports their implication in ligand selectivity. Except for their overall negative charges, the presence of sulfotyrosines (see section 2.2.2) and of potential N-glycosylation sites (see section 2.2.3), no specific signatures seem to be present and conserved in the M-C parts.

The C-TM parts are shorter (5 to 20 residues), display variable net charges within the CC and decoy receptor families and are neutral or negative in CXC receptors, contain no sulfated tyrosines or glycosylation sites. The only exception is CXCR7, which bears a putative N-glycosylation site two residues before the predicted TM1. Despite the low identity and size variation, we identified new signatures conserved within the C-TM parts of different chemokine receptor families (Tables 1-4). Receptors CCR1, CCR2, CCR3, CCR4, CCR5 and CCR9 present longer C-TM parts characterised by a length of 18 residues and the conservation of a scattered motif K-X_3-K/R-X_7-PPLYS/W separated from the cysteine by

one residue. In contrast, CCR6, CCR7, CCR8, CCR10, all CXC receptors, ACKR2 and ACKR4 display shorter C-TM parts (10 or 11 residues) characterised by the conservation of a negative charge ($E/D_{+3/+4}$) 3 or 4 residues after the cysteine and a positive charge ($K/R_{+9/+10}$) preceding the TM1. In other receptors such as XCR1, CX3CR1 and ACKR1, no particularities or features allowing their classification in one of these two families were found.

2.2 Post-translational modifications

2.2.1 Disulphide bridges

Chemokine receptors typically bear one cysteine residue in each extracellular domain. While the two cysteines present in ECL1 and ECL2 are a characteristic of nearly all rhodopsin-like GPCRs and form a structurally and functionally critical disulphide bridge [41], the other two cysteines situated in the N terminus and ECL3 are a particularity of chemokine receptors and their role is not as well established. Indeed, although the conservation of these residues as a pair in all chemokine receptors except CXCR6 indicates their importance for receptor biology, most likely through disulphide bridge formation, somewhat diverging results have been reported in the literature.

In an early study, it was shown that CXCR1 treatment with diamide, a bifuctional sulfhydryl reagent that oxidises thiol groups and leads to formation of disulphide bonds, resulted in a functionally inactive receptor and reduced CXCL8 (IL-8) binding [42]. Approaches using alkylating agents also pointed to the existence of free thiols in the extracellular domains of CXCR1 [42] and in the N terminus and ECL3 of CCR6 [43].

The results from numerous other studies, however, strongly put forward the role of the N terminus-ECL3 cysteine pair in receptor functions. Indeed, the mutation of one or both cysteines in the N terminus and ECL3 of CCR2, CCR5, CXCR4, CXCR1 and CXCR2 reduced the binding and signalling by CCL2, CCL5, CXCL12 and CXCL8, respectively [40, 44-47] as well as ACKR1 interactions with various chemokines [48]. However, it was shown for CCR2 that the cysteine present in the N terminus is not directly involved in the interactions with CCL2 [40]. Similarly, a study with a constitutively active N119S-CXCR4 demonstrated

that mutants carrying a salt bridge C28R/C274E or an aromatic pair C28F/C274F retained some of the activity of the receptor. It was further proposed that the N terminus-ECL3 cysteine pair may stabilise the active state of CXCR4 [49]. Interestingly, the mutation of this cysteine pair in the two major HIV-1 co-receptors, CXCR4 and CCR5, seems to have little effect on the binding of the gp120 to the receptors [44, 50].

The first direct evidence of the existence of a disulphide bridge between the N terminus-ECL3 cysteines arose from one of the CXCR4 X-ray structures (PDB ID: 3OEO), in which the N terminus cysteine at position 28 is linked to ECL3 cysteine at position 274 (Fig. 1). Interestingly, the helix VII (TM7) of CXCR4 is two turns longer than in other GPCR structures, allowing the optimal positioning of C274 for this interaction [16]. Moreover, the proline residue directly preceding C28, also present in many other chemokine receptors, may play a crucial role in orienting the N terminus regions in the vicinity of this cysteine to facilitate the disulphide bridge formation. The presence of a N terminus-ECL3 bridge was confirmed in all chemokine receptor structures available so far [17-19, 51]. Long-time molecular dynamics simulation suggested that its formation may be favoured by the interactions between other residues from the M-C part and ECL3 (unpublished results). The constraint imposed by the disulphide bond may fashion the chemokine binding pocket and/or be of importance in the correct positioning of the M-C part for chemokine binding or for its further interactions with the receptor (site 2) (see section 3.1). Indeed, in the case of CXCR4, the disulphide bond delocalises the M-C part from TM1 to the top of TM7 and facing the second extracellular loop, which is proposed to participate in the second step of the binding mechanism. Additionally, by linking the TM1 and TM7 the disulphide bridge may stabilise the three-dimensional structure of chemokine receptors by locking the transmembrane segments in a circular arrangement (Fig. 1). Moreover, since ECL3 connects TM6 and TM7, which are proposed to participate in conformational changes that trigger receptor activation, the N terminus-ECL3 disulphide bridge was suggested to have a role in the coupling of ligand binding to receptor activation [52].

2.2.2 Tyrosine sulfation

In addition to their high glutamate and aspartate content, all M-C parts of chemokine receptor N termini display at least one tyrosine residue that may potentially be post-translationally modified by the addition of a negatively charged sulfate to their hydroxyl groups. The reaction of tyrosine O-sulfation is catalysed by the Golgi tyrosylprotein sulfotransferases (TPST-1 and TPST-2) and has been shown to play important roles in the regulation of protein-protein interactions of many secreted and transmembrane proteins [53]. Studies with sulfated chemokine receptors however have proven to be difficult mainly due to the lability of the sulfate group. To date, the presence of sulfated tyrosines has been demonstrated for only six human chemokine receptors: CCR2b, CCR5, CXCR3, CXCR4, CX_3CR1 and ACKR1 (see Table 1) [25, 54-59]. By means of various approaches including site-directed mutagenesis, treatment with sulfation inhibitors or sulfatases, using both whole receptors and N terminus-derived peptides, it could be shown that O-sulfation of the N terminus is critical for high-affinity binding to chemokines as well as for the recognition of the HIV-1 gp120 protein [25, 54-59]. Notably, all these chemokine receptors bear a sulfated tyrosine located approximately nine residues before the conserved cysteine. Sequence analysis indicates that this potential sulfation site ($_{p}$sY) is present in almost all the receptors, arguing for the existence of a common sulfotyrosine-dependent ligand-binding mode. Although the exact importance of sulfotyrosines within the chemokine receptor N termini is not fully understood, the distribution of highly polarisable electrons on both the sulfate and the phenyl group make sulfotyrosines perfectly suitable to be accommodated by the positively charged pocket at the surface of the receptor ligands [35, 36, 60, 61]. Indeed, recent structural modelling and NMR measurements suggest that all chemokines harbour a conserved sulfotyrosine-binding pocket, providing a molecular basis for sulfotyrosine conservation observed among chemokine receptors (Fig. 4). The presence of such sulfotyrosine-binding pocket was experimentally determined for four chemokines representative of the different families (XCL1, CCL5, CXCL12 and CX3CL1) [62]. In particular, for CXCL12, structural data demonstrated that the sulfotyrosine-binding pocket is defined by the residues $V18^{CXCL12}$, $R47^{CXCL12}$ and $V49^{CXCL12}$ located near the hydrophobic groove delimited by the N-loop and the third β-strand (see section 3.1, Fig. 4B).

However, besides the presence of the conserved potential sulfation site, many chemokine receptors bear multiple tyrosine residues whose post-translational modification is not equally important for ligand recognition [57, 63, 64]. These sulfotyrosines however also seem to contribute to the high-affinity chemokine binding as illustrated for the CXCR4/CXCL12 interactions, in which sulfation of the receptor tyrosine 7 and 12 in addition to the conserved sY21 increases the affinity for the chemokine over six fold. (K_DsY_{21}≈1.3 μM versus vs. $K_DsY_{7/12/21}$=0.2μM) [65]. However, while the interacting partner of sY12, the $K27^{CXCL12}$, is well identified on the monomeric form of the chemokine, the interaction site of sY7 is not clearly defined and may involve a pocket formed upon chemokine dimerisation or interaction with other receptor extracellular domains (Fig. 4C and 4D). Interestingly, the involvement of $K27^{CXCL12}$ in heparin binding may also suggest that the N terminus negatively-charged residues and in particular sulfotyrosines play a role in heparin displacement prior to receptor binding [32]. Sulfation of tyrosines may additionally favour an extended conformation of the M-C part of the N terminus. Indeed, we performed long time molecular dynamics for CXCR4, with or without sulfate groups at position 7, 12 and 21 and demonstrated that repulsive interactions caused by the negative charges of the sulfate groups prevent the internal collapse of the N-terminal domain thereby maintaining it in an open conformation accessible for ligand binding (Fig. 2).

The prediction of protein tyrosine sulfation sites remains problematic. Nevertheless, although a specific signature could not be clearly identified among the proteins that are O-sulfated, several consensus features seem to be required for TPSTs activity. (a) Acidic residues are generally found in the vicinity of sulfated tyrosines, whereas basic amino acids abolish the reaction [66, 67]. Another possible determinant for TPST activity is (b) a certain degree of flexibility of the peptide chain, as small or turn-inducing residues are often present close to sulfation sites [66, 67]. Moreover, (c) disulphide bridges and N-glycosylation sites have been proposed to interfere with tyrosine sulfation [67, 68]. Similarly, in silico identification of modified tyrosines remains challenging, as sulfation prediction algorithms are often very restrictive. The sulfation prediction tool Sulfinator [69] for instance fails to identify the sulfation of tyrosines 7 and 12 of CXCR4, which has been determined experimentally. Moreover, in vitro sulfation of N terminus peptides derived

from receptors bearing multiple sulfotyrosines was shown to be sequential but also incomplete, giving rise to products displaying a variety of sulfation patterns that differentially affect the binding to chemokines. These observations point to the existence of a mechanism for regulation of ligand affinity/specificity towards sulfated receptors [59]. Moreover, TPST-1 and TPST-2 show different tissue expression patterns and play distinct but overlapping biological roles [68, 70-72]. The two isoenzymes also display different kinetic properties and show differences in substrate specificities as well as pH optima, which strengthens the hypothesis of their possible involvement in chemokine-receptor network regulation [73, 74].

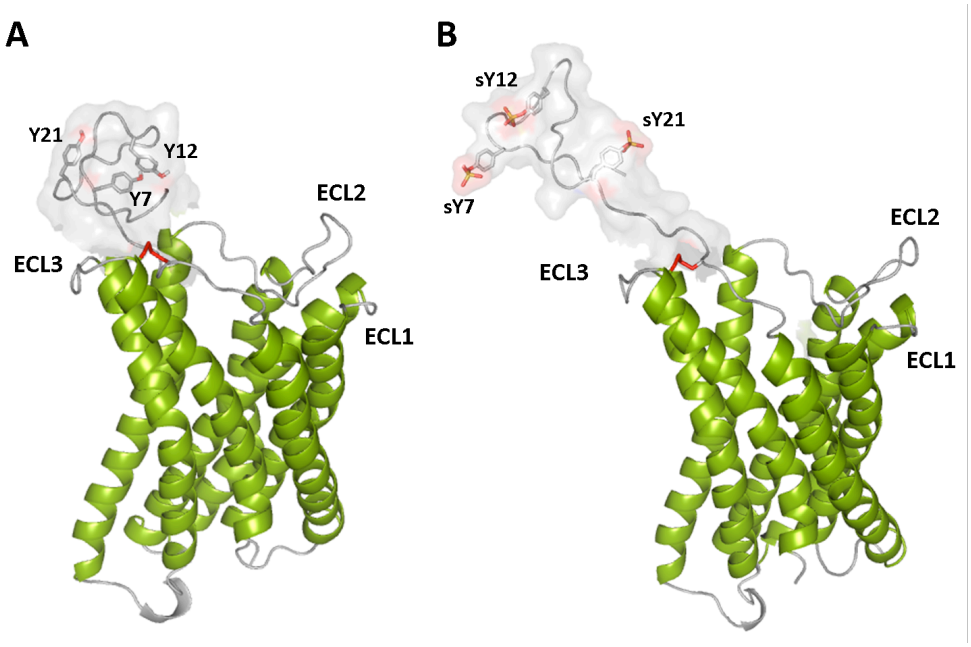

Fig. 2. Impact of tyrosine sulfation on CXCR4 N terminus conformation. CXCR4 N terminus with non-sulfated tyrosines **(A)** and CXCR4 N terminus with sulfotyrosines **(B)** derived from the last snapshot (20ns) of MD simulation carried out with the whole receptor (PDB ID: 3OE0 [16]). Receptor helical structures are shown in green; ECLs, ICLs and N terminus are represented in grey; tyrosine and sulfotyrosine residues are displayed as sticks and the disulphide bond between the N terminus and ECL3 is coloured in red. Guided MD simulations suggest that in absence of sulfate groups the N terminus tends to collapse forming a condensed structure, whereas tyrosine sulfation creates repulsive interactions promoting the adoption of a an extended structure largely accessible for chemokine binding.

2.2.3 Glycosylation

Like other transmembrane receptors, chemokine receptors may also be post-translationally modified by the addition of sugar moieties either to the amide group of asparagine residues

(N-glycosylation) or to hydroxyl groups of serine or threonine residues (O-glycosylation). N-glycosylation occurs at the consensus sequence N-X-S/T, where X is any amino acid except proline, while O-glycosylation sites are less well characterised and generally comprise serine/threonine-rich regions. These post-translational modifications occur in the Golgi and are catalysed by a series of glycosyltransferases and glycosidases that shape the carbohydrate chains. Most chemokine receptors bear one or two putative N-glycosylation sites as well as serine/threonine doublets or triplets within their M-C part. While no specific position or molecular signature can be defined for N-glycosylation, clusters of serine or/and threonine residues are generally found about two to four amino acids on either side of the conserved sulfated tyrosine (see section 2.2.2). Experimental data on human chemokine receptor glycosylation are however scarce and only five receptors have been shown to carry N-linked (CCR2B, CXCR2, CXCR4 and ACKR1 [54, 59, 75-77] or O-linked (CCR5 [78]) carbohydrate moieties in their N terminus (see table 1). The exact role of N-terminal domain glycosylation remains unclear. Similarly to other GPCRs, glycosylation of the extracellular domains of chemokine receptors has been proposed to increase their flexibility or to directly participate in ligand binding. Indeed, depending on the nature of the carbohydrate chains, glycosylation may provide additional negatively charged moieties for electrostatic interactions with the positively charged chemokines. While the presence of sialyted O-glycans in CCR5 N terminus (S6 and S7) was shown to be important for high-affinity binding to CCL3 and CCL4 [78], N-glycosylation of CXCR2 (N17), CXCR4 (N11) and ACKR1 (N16) appears to have no influence on CXCL7 (NAP2), CXCL12 and CXCL8 interactions, respectively [77, 79, 80]. CXCR2 glycosylation was, however, shown to be crucial for receptor maintenance on the cell surface, chiefly by protecting it against protease degradation. Furthermore, N-glycosylation patterns have been suggested to have an impact on the subcellular distribution of CXCR2 [77]. Additionally, although in the case of CCR5 it has been shown that O-glycosylation at S6 and S7 does not impair sulfation of Y10 [78], the vicinity of carbohydrate chains was proposed to negatively influence tyrosine sulfation [68]. It was also postulated that differential CXCR4 N-glycosylation may contribute to the presence of structurally and functionally distinct receptor isoforms [81]. Therefore, glycosylation of the receptor N terminus is likely to be of greater importance than initially

appreciated and in particular cell-dependent glycosylation patterns may represent an additional level in the finely tuned regulation of the chemokine-receptor network. In addition, glycosylation of the CXCR4 N terminus was shown to influence HIV-1 co-receptor usage (see section 3.2).

3. Ligand binding mode

3.1 Binding of chemokines to chemokine receptor N terminus

Numerous studies conducted with whole receptors [20-26, 39, 40] or receptor-derived synthetic peptides [27-36, 82] have demonstrated that the N-terminal domain of chemokine receptors holds an important role in ligand binding. Based on some of these results and the observation that chemokine binding and receptor activation are partly separable events driven by distinct molecular mechanisms and involving different structural determinants, a general two-site model was proposed by different authors to describe the interaction of chemokines with their cognate receptors [11, 12] (Fig. 3). According to this model, the receptor N terminus plays a crucial role in the initial recognition of the chemokine through the binding of its N-loop (site 1). This primary interaction is likely to contribute to correct chemokine orientation, promoting the binding of its flexible N terminus to the extracellular loops and the transmembrane segments of the receptor (site 2), triggering its activation.

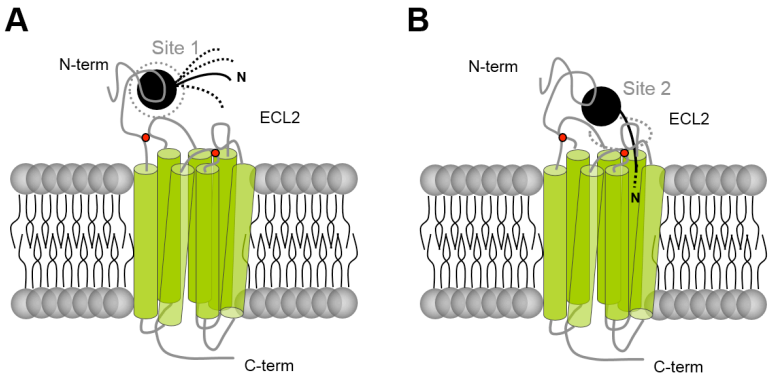

Fig. 3. Putative two-step/two-site mechanism for the interaction between chemokines and chemokine receptors. (A) First step: interactions between the N-loop (site 1) of the chemokine and the N-terminal domain of the receptor. **(B)** Step two: interactions between the flexible N terminus (site 2) of the chemokine and the extracellular loops as well as the transmembrane segments of the receptor. The disulphide bridges between N-term/ECL3 and ECL1/ECL2 are depicted as red dots.

To date, little information about the structure of chemokine receptor N termini is available. The N-terminal domains of chemokine receptors, especially their M-C part, are generally proposed to be highly flexible, showing an extended form when unbound and only adopting a fixed structure upon chemokine binding [30, 52]. This hypothesis is in line with the lack of clear electron density for the M-C part in the early small molecule bound crystal structures of CXCR4 and CCR5 [16, 17].

The N termini are the most variable extracellular domains of chemokine receptors in terms of sequence and length and this diversity is most probably an important determinant dictating the specificity of the receptor. The chemokine receptor N termini display net negative charges and their binding to chemokines is typically driven by electrostatic but also hydrophobic interactions. There exists a considerable amount of data on the importance of many individual residues within the N terminus, obtained mainly from binding studies with mutated receptors [20, 21, 45, 83, 84]. These residues are however rarely conserved among the receptors with the exception of a tyrosine found approximately nine residues before the C-TM part (see section 2.2.2). These observations suggest the existence of a common mechanism for N terminus binding involving the conserved sulfotyrosine but also relying on non-conserved residues that may determine the selectivity of the receptors. In accordance with this hypothesis, NMR studies conducted with labelled chemokines in the presence of receptor N terminus-derived peptides identified a groove delimited by the N-loop and the β-sheet as the receptor N terminus binding site. Although not identical, this binding site seems highly conserved among different chemokines [35, 36, 60].

In particular, for CXCL8, Skelton *et al.* demonstrated that a small modified peptide covering residues 9 to 29 (M_9WDFDD$_{14}$-linker-M_{20}PPADEDYSP$_{29}$) of the CXCR1 N terminus (K_i=13 μM) occupies a cleft between the N-loop and the third β-strand in an extended fashion and with only a limited number of contact residues (in bold: **P_{21}PA**DEDY**SP_{29}**) (Fig. 4A) [30]. In the complex, P21 and P22 formed hydrophobic interactions with L43^{CXCL8} and L49^{CXCL8} residues while P29 preceding the conserved cysteine wrapped around the chemokine β-sheet making hydrophobic contacts (I10^{CXCL8} and I40^{CXCL8}). Y27, conserved in almost all

chemokine receptors and most probably O-sulfated in the native CXCR1 receptor (see section 2.2.2), interacts with a pocket delimited by $I10^{CXCL8}$, $K11^{CXCL8}$, $Y13^{CXCL8}$ and $L49^{CXCL8}$. The binding is stabilised by an additional electrostatic interaction between D26 and chemokine $K11^{CXCL8}$. The importance of these residues was confirmed by site-directed mutagenesis of the complete CXCR1, indicating that the binding mode deduced from the NMR study most likely reflects the interaction of the chemokine with the complete receptor [46].

More recently, Veldkamp *et al.* reported the NMR structure of a strictly dimeric form of CXCL12 in complex with a full-length CXCR4 N-terminal domain peptide (1-38) bearing sulfotyrosines at positions 7, 12 and 21 [32]. This study provided the first structural evidence of the existence of sulfotyrosine recognition sites and demonstrated that the CXCR4 N-terminal peptides adopt an extended conformation with sulfotyrosines 12 (sY12) and 21 (sY21) binding to one chemokine monomer and sulfotyrosine 7 (sY7) interacting with the second monomer (Fig. 4B, 4C and 4D). Interestingly, in the complex, sY21 is orientated in the opposite direction compared to the equivalent sY27 in CXCR1 and interacts with a hydrophobic pocket defined by $V18^{CXCL12}$ and $V49^{CXCL12}$ and with the overhanging basic residue $R47^{CXCL12}$, which in CXCR1 is occupied by P21 (Fig. 3A and 3B). It is noteworthy that a residue equivalent to $R47^{CXCL12}$ is also present in CXCL8 ($R47^{CXCL8}$) but is involved in stabilising electrostatic interactions with E25 of CXCR1. Similarly, a positively charged residue equivalent to $K11^{CXCL8}$ is also present in CXCL12 ($R20^{CXCL12}$) but does not interact with any of the CXCR4 N terminus residues. Furthermore, this study also provided structural data on the binding mode of the two other CXCR4 sulfotyrosines, sY7 and sY12, that are not strictly conserved in other receptor N termini. In particular, sY12 was shown, just like sY21, to bind a hydrophobic pocket defined by $P10^{CXCL12}$, $L29^{CXCL12}$ and $K27^{CXCL12}$, whereas sY7 had no interacting partners on the first chemokine monomer and occupied a cleft delimited by the interface of the dimer forming an electrostatic interaction with $R20^{CXCL12}$ of the second monomer.

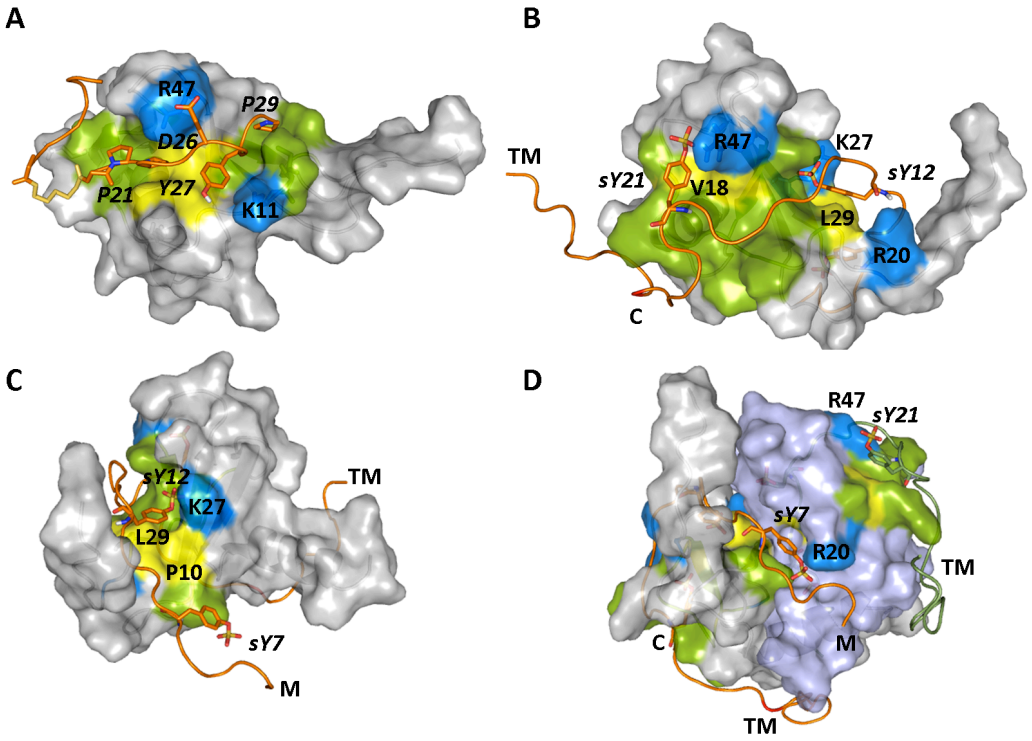

Fig. 4. Structures of chemokine/N terminus derived peptide complexes. Chemokines are represented as surface and coloured in gray. The hydrophobic N terminus-binding groove is coloured in green and yellow. N terminus-derived peptides are represented as cartoon, coloured in orange and annotated in italics. **(A)** NMR structure of the CXCL8-CXCR1 N terminus complex [30]. Tyrosine 27-binding site includes residues I10, Y13, L49 (yellow) and K11 (blue). D46 of CXCR1 forms electrostatic interactions with R47 of CXCL8. N terminus P21, P22 and P29 residues form hydrophobic interactions with the groove of the chemokine. (B, C and D) NMR structures of CXCL12 in complex with full-length CXCR4 N terminus bearing sulfotyrosines at positions 7, 12 and 21 [32]. **(B)** Recognition sites for sulfotyrosines sY12 and sY21. Conserved sY21 binds a pocket defined by V18 and V49 (yellow) and overhung by residue R47 (blue) while sY12 interacts with a similar pocket formed by residues L29, P10 (yellow) and K27 (blue). **(C)** Binding of sulfotyrosine sY7 and sY12 to a CXCL12 monomer (60° rotation relative to B). sY12 occupies a defined binding pocket while sY7 points in the opposite direction making no clear interaction with the chemokine momoner **(D)** Binding of sulfotyrosine sY7 to a dimer of CXCL12. sY7 occupies the cleft at the interface between two chemokine monomers and interacts with residues V24 and R20 of the second monomer. The second N terminus peptide binding to the second monomer is represented as cartoon and coloured in dark green.

Altogether these data demonstrated that sulfotyrosine recognition, critical for high affinity interactions with chemokines, occurs at particular binding sites sharing a similar architecture and that a given chemokine can display several sulfotyrosine-binding sites. Moreover, other interactions supported by non-conserved residues scattered along the N-terminal domains most probably also play essential roles in sulfotyrosine recognition and

in further stabilisation of the chemokine-receptor complexes, possibly providing the molecular basis for the differences in affinity and selectivity observed among the different receptors.

However, while the N terminus plays the predominant role in the initial chemokine binding, other extracellular parts have also been shown to participate in chemokine binding, in which case the combination of multiple low-affinity interactions provides high-affinity binding energy in chemokine-receptor interactions. Consistent with this assertion is the observation that by simultaneously grafting peptides corresponding to the CCR2 N terminus and ECL3 on a stabilised variant of the protein G B1 domain, the affinity for CCL2 is 100 times as high as when only the N terminus is present on the scaffold [38]. Similar results were obtained for CCR3 [37]. Other extracellular parts such as ECL2, which is involved in the formation of site 2, could be of importance for the overall affinity of the receptor.

Moreover, accumulating data suggest that the mechanism underlying chemokine binding to their receptors is likely to be more complex than a simple two-site model. It has been proposed that site 1 and site 2 interactions may be far from independent. Indeed, conformational changes in both the chemokine and the receptor that follow the initial chemokine binding to the N terminus of the receptor may energetically influence the subsequent interactions at site 2 [85]. This model may for instance explain why while CXCL8 binds CXCR1 with a significantly higher affinity than CXCL1, both chemokines bind the N terminus of the receptor with similar affinities [27]. Therefore, in contrast to CXCL8, the changes resulting from the binding of CXCL1 to site 1 would negatively affect further interactions of the chemokine at site 2. Such coupling between the two binding steps may thus have a major role in the regulation of chemokine affinity and selectivity for their receptors, providing yet another molecular basis for the complexity of chemokine-chemokine receptor network [86]. It is also conceivable that upon binding of the chemokine at site 1, the area for further interactions with site 2 increases, either as a result of conformational changes in the chemokine/receptor [86] or by complementation of sites that are partly present on the receptor N terminus and partly on the chemokine.

Recently, the possible role held by the vicinity of the cell membrane in the regulation of receptor N terminus interactions with the ligand has also been put forward. Indeed, it has been demonstrated that the CXCR1 N-terminal peptides interact with membranes or membrane-mimicking micelles in extended but constrained conformation that may energetically facilitate the interactions with the chemokine [27, 87]. NMR studies using a phospholipid bilayer-embedded CXCR1 receptor or an N terminus-TM1 construct suggested that the CXCR1 N-terminal domain may be anchored to the membrane via a tryptophan residue at position 10 (Fig. 5A). The release of the N terminus from the membrane upon strong interactions with the chemokine may thus be considered, at least for CXCR1, as the earliest step of the ligand binding mechanism [39]. Such hydrophobic association of the N terminus with the cell membrane may have a great impact on its binding properties. Indeed, the affinity of the CXCR1 N-terminal fragment for CXCL8 was shown to be 20 fold higher in detergent micelles than in solution ($K_D \approx 1$ μM versus 20 μM). Moreover, membrane-like environment has been reported to influence the binding selectivity of the receptor N-terminal domains [27].

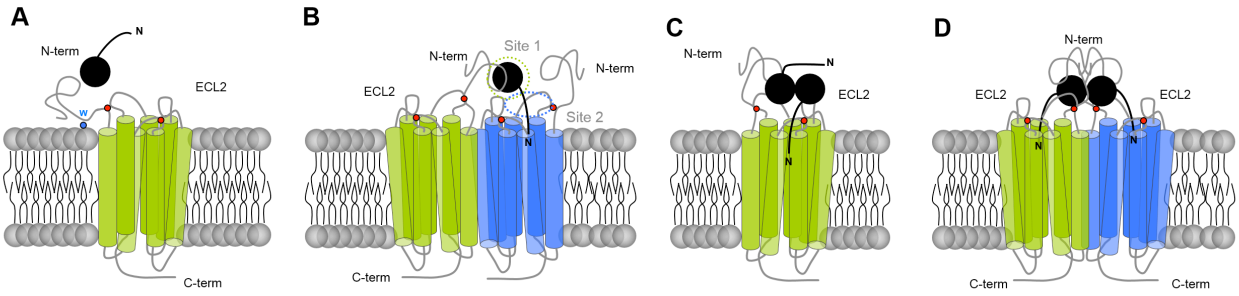

Fig. 5. Interaction models for chemokine receptors. (A) Anchoring of the N-terminal domain of CXCR1 into the membrane through hydrophobic contacts mediated by an aromatic residue (W) (blue dot). **(B)** Receptor trans-activation. Chemokine binds the N-terminal domain (site 1) of receptor 1 (green) and trans-activates receptor 2 (blue) by binding at its site 2. **(C and D)** Possible stoichiometries of CXCR4 interactions with CXCL12 dimer **(C)** Monomeric CXCR4 binds a dimer of CXCL12. **(D)** Dimeric CXCR4 binds a dimer of CXCL12. The disulphide bridges between N-term/ECL3 and ECL1/ECL2 are depicted as red dots.

Another poorly understood aspect is the stoichiometry of chemokine-receptor interactions (Fig. 5A-C). Since many chemokine receptors are known to form homo- or heterodimers, the possibility of a crosstalk, in which site 1 and 2 interactions would take place on separate receptors, should not be excluded (Fig. 5B). In accordance with this hypothesis, Monteclaro

et al. demonstrated that CCL2 binding to the CCR2 N terminus fused to CD8 can activate in trans a chimeric CCR2 that carries an irrelevant N terminus [40]. Besides the receptors, many chemokines as well have been shown to exist in different oligomeric states. Furthermore, in the case of CXCL12, it has been demonstrated that binding to the N terminus of CXCR4 induces its dimerisation in a symmetric 2:2 complex in which the dimerisation interface is shared by both the residues from the N-terminal domain and the chemokine [33] (Fig. 4D). While CXCL12 dimerisation has been suggested to be physiologically irrelevant [36], recent data recorded with strictly dimeric chemokine demonstrated distinct signalling pathways and differential chemotactic effect depending on the oligomeric state of CXCL12 [88]. Moreover, structural data showed that the CXCR4 N terminus binds differentially to CXCL12 monomers and dimers [88]. In particular, while residues 4-9 of the CXCR4 N-terminal peptide make strong interactions with CXCL12 monomers, they are only weakly associated with the chemokine in its dimeric form. Similar 2:2 interactions were proposed for CXCL8 and CXCR1 N terminus but remain controversial [29, 85]. One cannot rule out the possibility that such dimerisation may reflect the experimental setup, where in the absence of other possibilities of interactions with the receptor, chemokine dimerisation is energetically favoured. On the contrary, it has been proposed for CXCL8 that the binding of the N-terminal domain of CXCR1 to the chemokine dimer could promote its dissociation [85].

Chemokine receptor response specificities may also underlie the differences in receptor trafficking. Particularly, it has recently been proposed that the determinants of receptor internalisation rates following ligand binding may be harboured by the N terminus of chemokine receptors [26]. By swapping the N termini of CXCR1 and CXCR2, two chemokine receptors that share 77% of sequence identity but show different binding and signalling profiles towards CXCL8, it was demonstrated that the trafficking profiles of the chimeric receptors were defined by the N terminus and translated in temporal differences in activation of ERK1/2 signalling pathways, which are important for different signalling specificities. However, these determinants remain hitherto unidentified.

3.2 Binding of pathogen proteins to chemokine receptor N terminus

To subvert the host immune system and promote their pathogenesis, viruses such as herpesviruses, poxviruses and retroviruses have evolved various strategies to interfere with the host chemokine network, for instance by expressing chemokine analogues (reviewed in [89] and [90]).

The Human herpesvirus 8 (HHV-8), also named Kaposi's sarcoma-associated herpesvirus (KSHV), encodes three viral macrophage inflammatory proteins (vCCL1, vCCL2 and vCCL3) that share homologies with the human CC chemokines [91]. The characterisation of these proteins revealed that vCCL2 has the unique ability to cross-bind the four chemokine receptor families [92, 93] (see Chapter 4). In particular, vCCL2 binds to CXCR4 and CCR5 and is capable of inhibiting the interaction with their cognate chemokine ligands as well as infection of host cells by HIV-1 [92, 93].

Chemokine receptors can also be hijacked by pathogens to allow their entry into specific cell types. Two striking examples of such piracy are the malaria parasites (*Plasmodium vivax and Plasmodium knowlesi*) and the human immunodeficiency virus (HIV-1).

Plasmodium vivax and *Plasmodium knowlesi* belong to the five Plasmodium species responsible for human malaria. *P. vivax* and P. *Knowlesi* infect human erythrocytes by using the decoy chemokine receptor ACKR1 (previously known as DARC, Duffy blood group antigen) [94-96] that binds various CC and CXC chemokines [97]. Plasmodium interaction with ACKR1 was shown to be mainly mediated by a conserved cysteine-rich domain present in the parasite Duffy binding proteins (PvDBP and PkDaBP) [98] and by a modified 35-amino acid fragment (residues 8-42) of the receptor N terminus [99]. The tyrosine residues at position 30 and 41 of the N terminus of ACKR1 are sulfated although only the second one was reported as critical for PvDBP and PkDaBP binding [59]. Interestingly, erythrocytes interaction with PvDBP-expressing cells can be inhibited by a peptide derived from ACKR1 N terminus (IC_{50} = 1 μM) [99] while sulfation of tyrosine 30 and 41 in the peptide results in a more efficient inhibition (IC_{50} = 5 nM) [59]. Recent data point to the existence of a sulfotyrosine-binding pocket for ACKR1 N terminus on the interface of DBP dimer of *Plasmodium vivax* [100].

The human immunodeficiency virus (HIV-1), the causative agent of AIDS, uses CCR5 and CXCR4 as co-receptors for specific entry into host cells [9, 101-104]. This multi-step process is mainly mediated by envelope glycoproteins gp120 and gp41 organised in heterotrimer spikes on the outer surface of the viral membrane [105, 106]. Gp120 is constituted of an alternation of five constant domains (C1-C5) and five variable loops (V1-V5). The domains C1, C2 and C4 form a four-stranded antiparallel β-sheet called the bridging sheet. Upon binding to CD4, its primary receptor, HIV-1 envelope glycoprotein gp120 undergoes conformational changes resulting in the spatial reorientation of the bridging sheet and the variable V1/V2 and V3 loops exposing specific binding sites for the co-receptors [107-110]. Binding of gp120 to CXCR4/CCR5 leads to a rearrangement of gp41, bringing together the cellular and the viral membranes and allowing their fusion [111].

Interactions between gp120 and chemokine receptors CCR5 and CXCR4 have been investigated using different approaches including chimeric receptors [112-118], site-directed mutagenesis [44, 83, 119-123] and other biochemical and immunological methods [9, 124-130]. All these studies point to the importance of the co-receptor extracellular domains in gp120 binding, especially the receptor N terminus and ECL2, although their relative contribution depends on the HIV-1 strain [131]. Discrimination between CCR5 and CXCR4 has been shown to mainly depend on the determinants present in the V3 loop (± 35 aa) of gp120 such as positively charged amino acids at positions 11, 24 and 25, the overall charge and the distribution of the electrostatic potential [132, 133]. The co-receptor usage has also been shown to be affected by amino acid composition and glycosylation of the V1/V2 stem [134, 135].

In particular, the interaction between the V3 loop and the N terminus of CCR5 has been shown to depend on a cluster of negatively charged and tyrosine residues (D2, Y3, Y10, D11, Y14, Y15, E18) and suggested to be driven by electrostatic interactions [21, 116, 119, 120, 122]. Besides, the co-receptor function of CCR5 was also associated with other determinants such as S6, S7, I9, N13, Q21 and K22 [21, 120, 122]. Like for chemokines, sulfation of tyrosine residues, in particular Y10 and Y14, was shown to critically affect the binding of gp120 while O-glycosylation of serine residues had little effect [55, 78, 122, 128].

Together with structural analyses of the V3 loop [109, 110, 136, 137], these studies revealed the role of spatially distinct domains of gp120 in CCR5 interaction and led to the development of a two-site binding model, similar to that proposed for chemokines [138]. In this model, the conserved four-stranded bridging sheet (C4) and the base of the V3 loop bind to the CCR5 N terminus (residues 2-15) (site 1) through electrostatic interactions, while the crown of the V3 loop interacts with the co-receptor ECL2 (site 2) (Fig. 6).

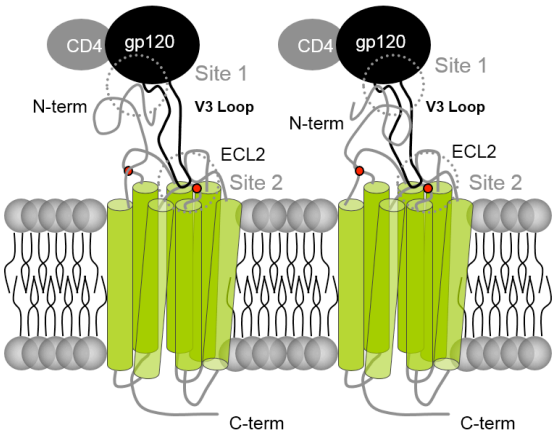

Fig. 6. Putative two-site binding mode describing the interactions between the gp120 protein and CCR5 [138]. Site 1: the N-terminal domain of the receptor binds to the bridging sheet and the base of the V3 loop of the gp120 protein trough electrostatic interactions. Site 2: the crown of the V3-loop interacts with the second extracellular loop of the receptor. Representation of the putative orientation of the N terminus with respect to two different docking models based on NMR studies of gp120 associated to synthetic peptides derived from CCR5 N terminus [139, 140].

In the absence of high-resolution structures, new insight into the molecular details of gp120-coreceptor interactions arose from NMR studies of gp120 bound to synthetic peptides derived from specific co-receptor domains [139, 140]. NMR study of a sulfated CCR5 N terminus peptide (sY10-sY14 CCR5 2-15) in complex with gp120 revealed a well-defined structure for residues 7 to 15. The docking of this peptide into the crystal structure of gp120-CD4 suggested that CCR5 N terminus binds to gp120 at the intersection of the bridging sheet and the V3 loop (Fig. 6 left panel) [139]. Residues S7 and P8 bind to the V3 stem while sY10, N11, Y15 interact with R327^{gp120}, R440^{gp120}, I439^{gp120}, respectively. The pocket between the bridging sheet and V3 encircles sY14 and rigidifies the V3 stem into a β-hairpin structure. A more recent study performed with a longer sulfated peptide (sY10-sY14 CCR5 1-27) showed that residues 7-23 bind to gp120-CD4 with P8-S17 and A20-I23 forming helical structures [140]. This study also provided a clearer picture of the main CCR5 binding determinants, emphasising the importance of the previously identified

residues D2, Y3, sY10, D11, sY14, Y15, E18 while contradicting the results regarding V5, I9, I12 and T16. The integration of these data in a gp120 structural model suggested the interaction of residues 2-22 with the fourth constant domain as well as the stem of the V3 loop (site 1). In contrast to the previous docking model, here, the peptide is flipped by 180° with sY14 fitting into a binding pocket and strongly interacting with R440 gp120 while sY10 binds to R323 ^{gp120} (Fig. 6 right panel).

The binding of gp120 to CXCR4 probably occurs via a similar mechanism, although the N terminus seems less important for infection by certain isolates [117, 141, 142]. In contrast to CCR5, no precise cluster of residues critically affected virus entry. Mutagenesis studies however revealed the role of individual residues (Y7, N10, Y12, N20, Y21, N22, S23 and E26) for the co-receptor function of CXCR4, although the extent of their contribution was strain-dependent [50, 83, 143]. The sulfation of tyrosine residues, in particular Y21, only had a minor effect on the entry of X4 viruses [56], while controversial data were obtained regarding the impact of glycosylation. Mutation of the N-glycosylation site of CXCR4 N terminus (N11) was initially shown to slightly facilitate R5 [144] or R5X4 [145] virus entry while having no effect on X4 viruses [83, 121, 145]. In another study however, the replacement of N11 with Q11 enhanced the binding and entry of X4 and R5 viruses [146].

4. Therapeutic discoveries targeting N terminus interactions

Since their discovery twenty years ago, chemokines and their receptors have emerged as fundamental regulators of human physiology. The interest in chemokine biology also arises from their key roles in such pathologies as cancer, inflammatory and autoimmune diseases as well as HIV-1 infection (see tables 1 to 5). Therefore much effort has been put into exploring ways to interfere with these processes, by either targeting the receptors or their ligands.

Therapeutic strategies directed against chemokine receptors have already proven efficacious in clinic. Two small molecules are currently on the market, namely the CXCR4 antagonist, AMD3100 (plerixafor, trade name Mozobil) used for hematopoietic stem cell mobilisation prior to autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma and Maraviroc (trade name Selzentry) for the treatment of R5 HIV-1

infection [147, 148]. These compounds, like the vast majority of chemokine receptor inhibitors, bind pockets in the transmembrane regions and do not interact with the N-terminal domain. However, because of their key roles in ligand recognition, the N termini of chemokine receptors may also represent highly relevant targets for drug discovery. To the best of our knowledge, small molecules specific to the N terminus have never been reported and this is certainly due to the unstructured, highly flexible nature of this domain. These characteristics however can also be regarded as advantageous for the generation of therapeutic antibodies able to block the initial site 1 interaction of intact receptors. Immunisation with synthetic N terminus derivatives allows for instance to circumvent the need for receptor purification or avoid eliciting antibodies against irrelevant epitopes in whole-cell antigens but may overlook the post-translational modifications often present in the extracellular domains [149, 150]. Antibodies recognising linear or conformational epitopes exclusively or partly present in the N terminus are commonly used in research and may also be exploited for therapeutic applications. Indeed, given that receptors which share ligands can at the same time have very distinct N termini (see tables 1 to 5) it is conceivable that highly specific, clinically-relevant antibodies can be raised against these fragments.

To date, there are no anti-chemokine receptor antibodies approved for clinical use. However, clinical trials for at least two anti-N terminus mAbs are in progress. This includes the anti-CCR5 mAb PRO140 currently evaluated against HIV infection as well as the CCR4-specific mAb KW-0761 for the treatment of adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma [151, 152].

Other rather encouraging results from studies with antibodies recognising the chemokine receptor N terminus have been published. Recently, a dromedary-derived VHH, CA52, directed against the N terminus of ACKR1, efficiently inhibiting *P. vivax* invasion and able to displace CXCL8 from the receptor was described [153]. Similarly, sera from rabbits immunised with the first seven CCR5 N terminus amino acids fused to T-helper cell epitope from tetanus toxoid were shown to inhibit HIV-1 infection of primary macrophages [150].

An attractive alternative to receptor inhibition consists of neutralising the ligand, in particular by blocking the N terminus-recognition site [154]. The report on the human mAb 10F8 whose epitope overlaps with the binding pocket of CXCR1 N-terminal domain (see section 3.1, [30]), illustrates well the feasibility of such a strategy [155]. This antibody was shown to interact with CXCL8 with picomolar affinity and to inhibit its binding to neutrophils (IC_{50} 0.3 nM) as well as chemokine-induced neutrophil activation and chemotaxis. In addition, it proved relatively efficient in treating palmoplantar pustulosis, an inflammatory disease in which CXCL8 plays a predominant role.

Moreover, a considerable therapeutic potential can be expected from approaches targeting specifically sulfotyrosine-binding pockets, as sulfotyrosine-mediated interactions seem widely exploited not only in chemokine biology but also by pathogen proteins. In line with this assumption is the report of a sulfated peptide corresponding to the first 60 residues of ACKR1 N terminus and blocking at low nanomolar concentration the association of plasmodium PvDBP and PkDaBP with the receptor [59]. Attempts to neutralise chemokines or the HIV-1 envelope protein gp120 using N terminus-derived peptides have so far proven unsuccessful mainly due to their low affinity and poor stability. It may however be possible to improve the affinity and pharmacokinetic properties of these peptides for example by incorporating in the sequence non-natural residues such as D-amino acids or chemical derivatives like the acid-stable sulfotyrosine mimic, (p-sulfomethyl)-phenylalanine [156, 157]. Interestingly, recent high-throughput in silico screening of small molecules targeting the $sY21^{CXCR4}$ sulfotyrosine-binding pocket on CXCL12 identified several lead compounds of which one (ZINC 310454) bound CXCL12 with an affinity of 64 μM [158]. Extending the screening target to larger parts of the chemokine-N terminus interaction surface may provide molecules of higher specificity and/or affinity.

5. Discussion

Chemokines are a family of small highly basic proteins that display a common fold but share little sequence similarities. By binding to chemokine receptors, they participate in many vital processes. The chemokine-receptor network is characterised by an apparent redundancy and many chemokines can bind to several receptors, while a chemokine

receptor usually has multiple ligands. This overlapping selectivity reflects however sophisticated regulation mechanisms that are still not fully elucidated.

The N terminus of chemokine receptors has a critical role in the initial step of chemokine binding as well as in determining the specificity and affinity of this interaction. At first sight, the N termini vary remarkably between different receptors in terms of length and amino acid sequence. However, on closer examination several common characteristics and signatures can be discerned.

One such feature is the conserved cysteine residue involved in a disulphide bond that links the N terminus and ECL3 and delimits two distinct regions within the N terminus, the M-C and C-TM parts. While this disulphide bridge has been shown to be important for chemokine receptor biology, the exact way of how it exerts its function remains unclear [40, 44-47]. It is likely that by linking the N terminus to ECL3, this disulphide bridge participates in the positioning of the M-C part above TM7 in an arrangement favouring the presentation of the chemokine to site 2. Moreover, as a large part of chemokine binding relies on the receptor extracellular domains, the C-TM "pseudo-extracellular loop" is perhaps an important additional feature shaping the ligand interaction interface in receptors having a relatively short N terminus, compared to other protein–binding GPCRs. This supposition may be substantiated by the observation that CXCR4 structure differs from other GPCRs in the location and the form of the ligand-binding pocket, which is situated closer to the extracellular surface [16]. In this context, the existence of different C-TM sizes (11 or 18 residues) identified here that bear distinct signatures (K-X_{3}-K/R-X_{7}-PPLYS/W and E/$D_{+3/+4}$-K/$R_{+9/+10}$ respectively) is rather intriguing and the potential impact of these elements on the receptor functionality should be addressed in the near future. Furthermore, the conservation of these motifs may open new perspectives for phylogenetic studies of chemokine receptors and allow their alternative classification that, in contrast to the current system, would not be merely based on the recognised ligands.

More information is available on the flexible M-C part of the N terminus since it had been early demonstrated to be directly involved in ligand binding. There has been growing interest in the post-translational modifications present in this region and one of the current central areas of concern in chemokine receptor interactions with ligands is sulfation of their

N-terminal domains. For several receptors this post-translational modification has been demonstrated to be important for high-affinity binding to chemokines. Most chemokine receptors bear a potentially sulfated tyrosine about nine residues before the conserved cysteine, which may therefore interact with the sulfotyrosine-binding pocket suggested to be present on the surface of all chemokines [62]. Complementation between the negatively-charged receptor N terminus and the positive charges within this conserved binding site as well as hydrophobic interactions were proposed to facilitate the binding by proper positioning of the sulfotyrosine-bearing N terminus and stabilisation of the interaction. Although sulfotyrosine-driven binding mechanism seems to be shared by many chemokine-receptor pairs, the auxiliary residues involved in this interaction are highly variable and might have co-evolved in the binding partners determining, at least in part, their specificity. In several chemokine receptors, many other potentially sulfated tyrosines are present in the N termini and appear to participate in high-affinity interactions with ligands. For CXCR4, their binding was shown to follow a mechanism similar to that proposed for the conserved sulfotyrosine indicating that other sulfotyrosine binding sites may exist at the surface of chemokines [32]. Sulfotyrosine-mediated recognition appears to be exploited not only in chemokine interactions but also by pathogen proteins and therefore sulfotyrosine-binding pockets represent valuable targets for drug development.

Furthermore, we propose that the presence of the multiple sulfate groups may provide repulsion forces that energetically favour an extended conformation of the N terminus, exposing the residues that are critical for ligand binding.

Other features commonly found in the M-C part are the putative N-glycosylation sites. The presence of sugar chains has been experimentally determined in only a few receptors and their exact role has yet to be further investigated. It is nevertheless highly plausible that similarly to tyrosine sulfation, cell-dependent glycosylation patterns result in structurally and functionally different receptor isoforms, like those observed for CXCR4 [81]. Such differences in the post-translational modifications may thus represent an additional level in the fine-tuning of the complex chemokine-receptor network.

Unfortunately, the recent resolution of the x-ray structure of CXCR4 failed to provide details on the flexible M-C part [16]. Nevertheless, alternative approaches exploiting

chimeric, mutated or truncated receptors produced a compelling set of information on the critical roles of these N terminus parts in ligand binding and receptor function [20-26]. In particular, NMR analyses of chemokines or viral proteins in complex with synthetic peptides derived from the receptor N termini were a considerable steppingstone in the understanding of the receptor N terminus biology and provided the first insights on the structural basis for site 1 interactions [30, 32]. However, data from these studies should be interpreted with some caution. Among the problems to be taken into consideration is the fact that the peptides used do not always cover the full N terminus sequence and often bear no post-translational modifications that are normally present in this receptor domain. Although studies with sulfated N-terminal fragments have been reported (mainly for CXCR4 and CCR5) the addition of this group is not a straightforward task [33, 65, 159, 160].

To date, many questions on chemokine receptors remain under debate. It has become clear that post-translational modifications of the N terminus should not be underrated in the role they play in receptor function but investigating it is somewhat challenging. The exact stoichiometry of chemokine-receptor interactions, including such aspects as receptor-receptor cross-talk, chemokine oligomerisation and the biological relevance of receptor N terminus-induced chemokine dimerisation as observed for CXCR4-CXCL12 couple, also need to be further examined. Although, the development of chemokine receptor antagonist still remains a major challenge, the efforts made to unravel and characterise the structural and functional properties of chemokine binding mode will probably, in the future, enable the development of new specific chemokine-neutralising molecules or N terminus-targeting antibodies with high therapeutic potential [154, 158, 161].

Table 1. Sequence, length, charge and post-translational modifications of C chemokine receptor N terminus

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
XCR1	XCL1 XCL2	RA	M**E**SSGNP**E**STTFFYY**D**LQSQPC-**E**NQAWVFAT	31 (-4)	22 (-3)	9 (-1)	2	0	

Table presents the length and (charge) of the complete N-terminal domain (N-term), M-C and C-TM parts. M-C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulphide bridge with the third extracellular loop (ECL3). C-TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimits the M-C part from the C-TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M-C part that are potentially sulfated ($_{p}$sY) are highlighted in gray. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. RA: Rheumatoid Arthritis. [a]: based on [162]. [b]: adapted from [163]

Table 2. Sequences, lengths, charges and post-translational modifications of CC chemokine receptors N termini

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_psY$	N-Glyco	Ref
CCR1	CCL3 CCL5 CCL7 CCL8 CCL13 CCL14 **CCL15** CCL16 **CCL23**	AR AS AT CA COPD HIV MS PS RA	ME**T**P<u>N</u>TT**ED**<u>Y</u>**D**TTT**E**F**D**<u>Y</u>G**D**ATPC-Q<u>*K*</u>VN**E**<u>*R*</u>AFGAQLL<u>*PPLYS*</u>	42 (-6)	24 (-7)	18 (+1)	2	1	
CCR2	**CCL2** CCL7 CCL8 CCL11 CCL13 CCL16	AS CA COPD HIV LP MS RA	MLSTSRSRFIRNT<u>N</u>*ESG**E**<u>**E**</u>VTTFF**D**<u>Y</u>*<u>**D**</u>Y<u>G</u>APC-H<u>*K*</u>F**D**V<u>*K*</u>QIGAQLL<u>*PPLYS*</u>	50 (-0)	32 (-2)	18 (+2)	2	1	[54]
CCR3	CCL2 CCL5 CCL7 CCL8 **CCL11** CCL13 CCL15 **CCL24** **CCL26** CCL28	AS CA COPD HIV	MTTSL**D**TV**E**TFGTTS<u>YY</u>**DD**VGLLC-**E**<u>*K*</u>**A**DT<u>*R*</u>ALMAQFV<u>*PPLYS*</u>	42 (-4)	24 (-4)	18 (0)	2	0	[63, 64]
CCR4	CCL17 CCL22	AD AS CA DI IBD PS	MNPT**D**IA**D**TTL**DE**SI<u>Y</u>SN<u>YY</u>L<u>Y</u>**E**SIPKPC-T<u>*K*</u>**E**GI<u>*K*</u>AFG**E**LFL<u>*PPLYS*</u>	47 (-4)	29 (-4)	18 (0)	4	0	
CCR5	CCL3 to CCL5 CCL8 CCL11 CCL14 CCL16	AR AS AT CA CH COPD HIV IBD MS PS RA	M**D**Y*QVS*S*PI<u>Y</u>*<u>**D**</u>IN<u>Y</u>*<u>Y</u>*TS**E**PC-Q<u>*K*</u>INV<u>*K*</u>QIAARLL<u>*PPLYS*</u>	38 (0)	20 (-3)	18 (+3)	4	0	[55, 78, 164]
CCR6	CCL20	CA IBD PS	MSG**E**SM<u>N</u>FS**D**VF**D**SS**ED**<u>Y</u>FVSV<u>N</u>TS<u>YY</u>SV**D**S**E**MLLC-SLQ<u>***E***</u>VRQFS<u>*R*</u>L	47 (-6)	36 (-7)	11 (+1)	3	2	
CCR7[ψ]	CCL19 CCL21	CA IBD MS	Q**DE**VT**DD**<u>Y</u>IG**D**<u>N</u>TTV**D**<u>Y</u>TLF**E**SLC-SKK<u>***D***</u>VRNF<u>*K*</u>A	34 (-4)	24 (-7)	10 (+3)	2	1	
CCR8	**CCL1** CCL4 CCL16 CCL17	AD AS	M**D**<u>Y</u>TL**D**LSVTTVT**D**<u>YYY</u>P**D**IFSSPC-**DA**<u>***E***</u>LIQTNG<u>*K*</u>L	36 (-5)	25 (-4)	11 (-1)	4	0	
CCR9	CCL25	CA IBD	MTPT**D**FTSPIPNMA**DD**<u>Y</u>GS**E**STSSM**ED**<u>Y</u>VNF<u>N</u>FT**D**F<u>Y</u>C-**E**<u>*K*</u>NNV<u>*R*</u>QFASHFL<u>*PPLYW*</u>	56 (-5)	38 (-7)	18 (+2)	3	1	
CCR10	CCL27 CCL28	AD CA PS	MGT**E**AT**E**QVSWGH<u>Y</u>SG**DEED**A<u>Y</u>SA**E**PLP**E**LC-YKA<u>***D***</u>VQAFS<u>*R*</u>A	42 (-6)	31 (-7)	11 (+1)	2	0	

The table presents the length and (charge) for the complete N-terminal domain (N-term), M-C and C-TM parts. M-C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulphide bridge with the third extracellular loop (ECL3). C-TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimits the M-C part from the C-TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M-C part that are potentially sulfated ($_psY$) are highlighted in gray. Potential N-glycosylation sites (NxS/T) are underlined.* denotes post-translational modifications that were experimentally demonstrated. Double underlined italic residues highlight (1) the conserved K-K/R-PPLYS/W motif located in the C-TM parts at positions C+2, C+6 and C+13 respectively or (2) the negatively and positively charged residues conserved at positions +3/4 and +10/11. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. [ψ]CCR7 N-terminal sequence presents a signal peptide of 24 residues. Processing prediction indicates Gln25 as the amino terminal residue of CCR7 N terminus. AD: Atopic Dermatitis, AR: Allograft Rejection, AS: Asthma, AT: Atherosclerosis, CA: Cancers, CH: Chronic Hepatitis, COPD: Chronic Obstructive pulmonary disease, DI: Type I Diabetes, HIV: Human Immunodeficiency Virus Infection, IBD: Inflammatory Bowel Disease, LP: Lupus, MS: Multiple Sclerosis, PL: Plasmodium infection, PS: Psoriasis, RA: Rheumatoid Arthritis. [a] based on [162], the principal endogenous agonists are represented in bold. [b]: adapted from [163].

Table 3. Sequences, lengths, charges and post-translational modifications of CXC chemokine receptors N termini

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CXCR1	CXCL1 CXCL6 **CXCL8**	AS CA COPD IBD PPP RA	MSNITDPQMWDFDDLNFTGMPPADEDYSPC-ML***E***T**E**TLN*K*Y	40 (-8)	30 (-7)	10 (-1)	1	2	[30]
CXCR2	CXCL1 to CXCL3 CXCL5 to CXCL8	AS AT CA COPD IBD PS RA	M**ED**FNM**ES**D**S**F**ED**FWKG**ED**LSN*YSYSSTLPPFLL**D**AAPC-**E**P***E***SL**E**IN*K*Y	49 (-10)	39 (-8)	10 (-2)	2	1	[54]
CXCR3	CXCL9 to CXCL11	AR AS AT CA CH COPD DI IBD LP MS PS RA	MVL**E**VS**D**HQVLN**D**A**E**VAALLENFSSSY*DY*GENESDSCCT SPPC-PQ***D***FSLNFD*R*A	54 (-9)	43 (-8)	11 (-1)	2	2	[25, 58]
CXCR4	CXCL12	AS AT CA HIV RA	M**E**GISIY*TS**D**N*Y*T**EE**MGSGD**Y***D**SMK**E**PC-FR**EE**NANFN*K*I	39 (-6)	28 (-6)	11 (0)	3	1	[32, 56, 65]
CXCR5	CXCL13	CA LP	MNYPLTL**E**M**D**L**E**NL**ED**LFW**E**L**D**RLD**D**NYNDTSLV**E**NHLC-PAT***E***GPLMASF*K*A	51 (-8)	38 (-8)	13 (0)	2	1	
CXCR6$^{\Psi}$	CXCL16	CA MS	MA**E**H**D**YH**ED**YGFSSFN**D**SSQ**EE**HQ**D**FLQFS*K*V	32 (-4)	32 (-4)	-	2	1	
CXCR7 (ACKR3)	CXCL11 CXCL12	CA	M**D**LHLF**D**YS**E**PGNFS**D**ISWPC-NSS***D***CIVV**D**TVMCPNMPN*K*S	41 (-4)	21 (-3)	20 (-1)	1	3	

The table presents the length and (charge) for the complete N-terminal domain (N-term), M-C and C-TM parts. M-C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulphide bridge with the third extracellular loop (ECL3). C-TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimit the M-C part from the C-TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M-C part that are potentially sulfated ($_{p}$sY) are highlighted in gray. Potential N-glycosylation sites (NxS/T) are underlined.* denotes post-translational modifications that were experimentally demonstrated. Double underlined italic residues highlight the negatively and positively charged residues conserved at positions +3/4 and +10/11. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. $^{\Psi}$CXCR6 does not present a cysteine in its N-terminal domain. AD: Atopic Dermatitis, AR: Allograft Rejection, AS: Asthma, AT: Atherosclerosis, CA: Cancers, CH: Chronic Hepatitis, COPD: Chronic Obstructive pulmonary disease, DI: Type I Diabetes, HIV: Human Immunodeficiency Virus Infection, IBD: Inflammatory Bowel Disease, LP: Lupus, MS: Multiple Sclerosis, PL: Plasmodium infection, PPP: palmoplantar pustulosis, PS: Psoriasis, RA: Rheumatoid Arthritis. [a]: based on [162], the principal endogenous agonists are represented in bold. [b]: adapted from [163].

Table 4. Sequence, length, charge and post-translational modifications of CX3C chemokine receptors N terminus

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CX3CR1	CX3CL1	AT CA IBD PS	M**D**QFP**E**SVT**E**NFE**Y*** **DD**LA**E**AC-YIG**DI**	26 (-8)	21 (-7)	5 (-1)	1	0	[57]

The table presents the length and (charge) for the complete N-terminal domain (N-term), M-C and C-TM parts. M-C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulphide bridge with the third extracellular loop (ECL3). C-TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimit the M-C part from the C-TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M-C part that are potentially sulfated ($_{p}$sY) are highlighted in gray. Potential N-glycosylation sites (NxS/T) are underlined.* denotes post-translational modifications that were experimentally demonstrated. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. AT: Atherosclerosis, CA: Cancers, IBD: Inflammatory Bowel Disease, PS: Psoriasis. [a]: based on [162]. [b]: adapted from [163].

Table 5. Sequences, lengths, charges and post-translational modifications of decoy receptors N termini

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
ACKR1	CCL2 CCL5 CCL7 CCL11 CCL13 CCL14 CCL17 CXCL1 CXCL3 CXCL5 CXCL6 CXCL8 CXCL11	PL PS	MGNCLHRA**E**LSPST**E**N*SSQL**D**F**ED**VWN*SS**Y**GVN***D**SFP **D**G**D**YGANL**E**AAAPC-HSCNLL**DD**SALPFF	65 (-9)	51 (-8)	14 (-1)	2	2	[48, 59, 75, 76, 79]
ACKR2	CCL2 to CCL8, CCL11 to CCL14 CCL17 CCL22	CA	MAATASPQPLAT**ED**A**D**A**E**NSSF**Y**Y**Y****D**Y**D****DE**VAFMLC-RK***D***AVVSFG***K***V	47 (-6)	36 (-7)	11 (+1)	4	1	
ACKR4	CCL19 CCL21 CCL25 CXCL13	CA	MAL**E**QNQST**D**Y**Y**Y**EE**N**E**MNGT**Y****D****Y**SQ**Y****E**LIC-IK**E*****D***VR**E**FA***K***V	42 (-7)	31 (-7)	11 (0)	6	2	

The table presents the length and (charge) for the complete N-terminal domain (N-term), M-C and C-TM parts. M-C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulphide bridge with the third extracellular loop (ECL3). C-TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimit the M-C part from the C-TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M-C part that are potentially sulfated ($_{p}$sY) are highlighted in gray. Potential N-glycosylation sites (NxS/T) are underlined. * denotes post-translational modifications that were experimentally demonstrated. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. Double underlined italic residues highlight the negatively and positively charged residues conserved at positions +3/4 and +10/11. CA: Cancers, PL: Plasmodium infection, PS: Psoriasis. [a]: based on [162]. [b]: adapted from [163].

Highlights

- Chemokine receptor N termini strongly contribute to the binding of chemokines as well as pathogen proteins.
- Despite their variability, chemokine receptor N termini harbour various conserved features or post-translational modifications, which are proposed to play important roles in receptor biology and ligand recognition.
    - Tyrosine sulfation may increase the affinity of the receptor for its ligand. All chemokine receptors bear at least one potentially sulfated tyrosine in their N terminus, which may be involved in a common mechanism of chemokine recognition through a conserved sulfotyrosine-binding pocket.
    - Disulphide bridge between the receptor N terminus and ECL3 (top of TM7) leads to formation of a pseudo-loop (ECL4) and may play a structure-stabilising role, shape the ligand interaction interface, and participate in the positioning of the N terminus for chemokine interactions.
    - Glycosylation is proposed to increase N terminus flexibility or to directly participate in ligand binding by providing additional negatively charged moieties for electrostatic interactions with the positively charged chemokines. It may also be important for protecting the receptor against protease degradation.
- We were also able to identify molecular signatures within the pseudo-loop regions of chemokine receptors, which allow their classification based on their length and conserved negatively or positively charged residues.

By writing the review on which the above chapter is based, I was initiated at the very beginning of the project into the complex field of chemokine receptors and their ligands. Not only did it allow me to have a broader view on the various aspects of this area of research, but also to appreciate the differences and similarities between CXCR7 and other chemokine receptors. We could indeed pinpoint some unique characteristics that distinguish the N terminus of CXCR7 from that of other receptors. These included (1) the single tyrosine residue at a non-conserved position, contrasting with the multiple tyrosines commonly found in other receptors, (2) the N-glycosylation site in its ECL4, the region which usually does not carry any post-translational modifications or (3) the two additional cysteine residues, which we hypothesised could be linked through a disulphide bridge forming an intra-N terminus loop. These uncommon features were to be further investigated later on in the project (see Chapter 3).

In only four years since the publication of the review, much progress has been made in the field. Some sections of this chapter, therefore, had to be rewritten or updated to include the recent knowledge, for instance offered by the several newly resolved three-dimensional structures of chemokine receptors. The growing body of data, both structural and functional is also gradually leading to changes in paradigms around chemokines and their receptors and this will be elaborated on in the last chapter of this thesis.
We had also identified conserved signatures within the N-terminal domains of chemokine receptors, within the so-called pseudo-loop or ECL4, which we were later able to refine based on the new chemokine receptor structures. These signatures and the pseudo-loops of chemokine receptors will be further discussed in the following chapter.

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Chapter 2

Closing the ring: a fourth extracellular loop in chemokine receptors

Adapted from Szpakowska M, Perez Bercoff D and Chevigné A, Closing the ring: a fourth extracellular loop in chemokine receptors, Science Signaling 2014 Sep 2;7(341):pe21.

Shortly after the publication of the review on the chemokine receptor N terminus, the resolution of the three-dimensional structures of CXCR1 and the first CC receptor, CCR5, confirmed the existence of a disulphide bridge linking the N terminus to the top of TM7. Importantly, it also brought new details allowing a better prediction of the boundary between the N terminus and TM1, especially in the CC receptors, and hence of the size of the pseudo-loop ECL4. We felt that the presence and the impact of the disulphide bridge and ECL4 on chemokine receptors had been understated by the scientific community, while they probably play critical roles in chemokine recognition and receptor activation. We therefore continued and refined our analysis of these regions.

This short chapter further discusses the features of these pseudo-loops, the structural requirements for their formation, and the effects they may have on receptor function.

1. ECL4 and chemokine receptor topology

Chemokine receptors are rhodopsin-like, guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) and are present at the surface of various cell types. By binding to their ligands, chemokine receptors regulate vital cellular mechanisms, including migration, adhesion, as well as growth and survival, but they are also involved in pathological processes, such as cancer and HIV-1 infection. Previously, knowledge about the structure of chemokine receptors was built on predictions based on other class A GPCRs and on functional studies. The resolution of the three-dimensional structures of three chemokine receptors by x-ray crystallography (for CXCR4 and CCR5) and nuclear magnetic resonance (NMR) analysis (for CXCR1) has provided more precise information on the conformations adopted by members of this receptor family (Fig. 1, A to D) [1-3].

Similar to other rhodopsin-like GPCRs, chemokine receptors consist of a flexible extracellular N terminus that is followed by a bundle of seven hydrophobic plasma membrane–spanning α-helices [known as transmembrane (TM) domains] that are connected by three hydrophilic extracellular loops (ECLs) and three intracellular loops (ICLs). In addition to the canonical disulphide bond that links the top of third TM domain (TM3, at the end of ECL1) to the middle of ECL2, all three of the currently available three-dimensional structures of chemokine receptors demonstrate the presence of a second disulphide bridge between the N terminus of the receptor and the top of TM7 (at the end of ECL3). As a consequence, the C-terminal residues of the N terminus of the receptor form an extracellular loop (which is termed "ECL4"), which connects TM1 and TM7 and closes the receptor into a ring-like conformation (Fig. 1A). This fourth loop consists of six (for CCR5) or eight amino acid residues (for CXCR1 and CXCR4) (see table Fig. 1), and is thus comparable to ECL1 and ECL3, which contain between four and eight residues each. With the exception of CXCR6, all of the chemokine receptors have a cysteine in the last third of their N-terminal regions, which suggests that the additional disulphide bridge is conserved. Although the formation of this disulphide bridge is critical for the function of several chemokine receptors, the role of the additional loop in ligand recognition and receptor activation mechanism has been given less attention.

The formation of ECL4 at the surface of chemokine receptors requires structural adaptations and possibly has consequences on receptor function. In CXCR1, CXCR4, and CCR5, the transmembrane helix that forms TM7 is two turns longer than that in other GPCRs. Elongation of the chemokine receptor helix seems to be required to position the conserved cysteine towards the inner face of TM7, which favours its engagement in the disulphide bridge with the N terminus of the receptor (Fig. 1, A and E). The ECL4 pseudo-loop may play an important role in chemokine recognition. Chemokine receptors are thought to bind to their ligands through a two-step mechanism that involves successive interactions between the chemokine and both the flexible N terminus of the receptor [chemokine recognition site 1 (CRS1)] and a pocket located in the vicinity of the transmembrane segments and the extracellular loops (CRS2) [4, 5]. Noteworthy, ECL4 and the disulphide bond between the N terminus and TM7 reposition the remaining flexible part of the N-terminal region of the receptor from the top of TM1 to the top of TM7 alongside ECL3 (Fig. 1E). Such a delocalisation is likely necessary for chemokine binding, and would provide an optimal orientation of the flexible N terminus of the receptor (CRS1) with respect to CRS2. This repositioning may be further facilitated by the proline residue that often directly precedes the conserved cysteine, which forms a kink in CRS1 and brings it in front of the β-hairpin of ECL2, a major determinant of CRS2 in CXCR4 (Fig. 1, A and E) [6]. ECL4 also influences the shape, size, and charge of the entrance of the transmembrane binding pocket for endogenous ligands (CRS2) and small pharmacological modulators (Fig. 1, B to D). Similarly to the canonical disulphide bridge between TM3 and ECL2, the bond between the N terminus and TM7 may also contribute to the overall stability and rigidity of the receptor, as well as to the conformational changes that occur upon chemokine binding. Finally, this loop may limit the diffusion of small molecules across the helix-bundle, and it may participate in receptor-receptor interactions, type I dimerisation, or both.

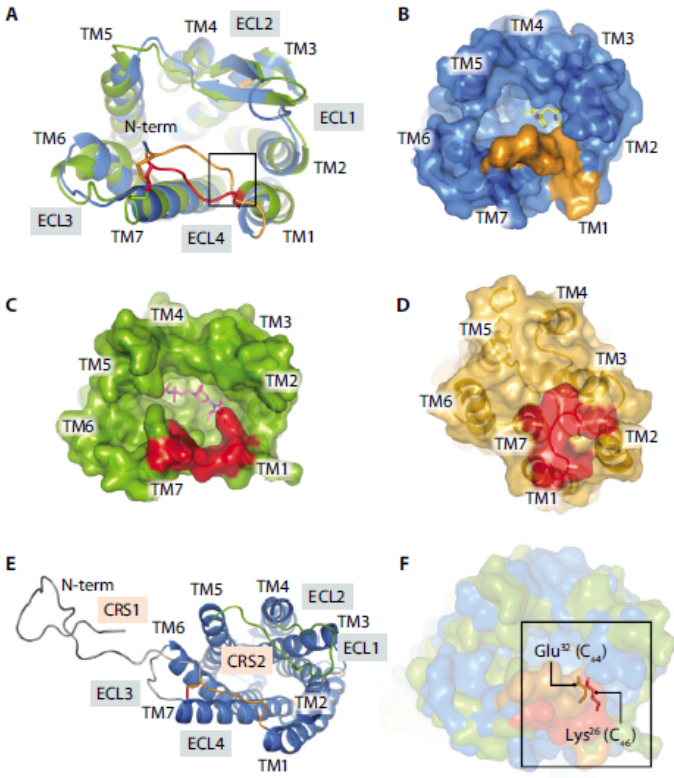
2. ECL4 molecular signatures

Despite difficulties in predicting the starting residue of TM1, and although there is little ECL4 sequence similarity among receptors, we identified three subfamilies of chemokine receptors that are characterised by different molecular signatures within their pseudo-loops (see table Fig. 1) [4, 7]. The receptors CCR1, CCR5, and CCR9 share conserved, positively charged residues at positions C_{+2} and C_{+6} (family A), whereas

CCR6, CCR8, CCR10, all of the CXC receptors, CX3CR1, and the atypical chemokine receptor ACKR2 (D6) have a negatively charged residue at position C_{+3} or C_{+4} (family B). The side chains of the residues that define family A (Lys^{26}, C_{+6}) and family B (Glu^{32}, C_{+4}) are well-aligned in the superposed x-ray structures of CXCR4 and CCR5 (Fig. 1F), and point towards the inner face of the receptors, suggesting that this position may be of importance for receptor function. This observation is consistent with data demonstrating that the Asp^{25} of CX3CR1 (C_{+4}) is critical for binding to its ligand CX3CL1 (fractalkine) [8], and that Glu^{32} of CXCR4 (C_{+4}) is predicted to interact with the N-terminal lysine of CXCL12 (also known as SDF1-α), which accounts for its agonist activity [9]. Other receptors, such as CCR2, CCR3, CCR4, CCR7, and ACKR4 (CCX-CKR) bear both types of signatures (family A/B). The receptors XCR1, ACKR1 (DARC), and CXCR7 (ACKR3) display no feature that enables their classification into one of the two families (family C). In contrast to sequences preceding the conserved cysteine, no posttranslational modifications are predicted among the different ECL4s, except for that of CXCR7, which displays a putative N-glycosylation site (NKS) at position C_{+5}.

3. ECL4 in other rhodopsin family receptors

The presence of a fourth ECL and its molecular signatures may not be restricted to chemokine receptors. Indeed, the additional cysteine residues in the N terminus and TM7 (ECL3) are also found in about 30 % of receptors belonging to the rhodopsin family, including receptors for lysophospholipid (LPA), bradykinin (B1-2), endothelin (ETA-B), melanocortin (MC1-5), serotonin (5-HT), angiotensin (AT1-2) as well as purinergic (P2Y) and orphan receptors (Fig. 2). The structure of the recently resolved rhodopsin-like receptors, $P2Y_{12}$, $P2Y_1$ and AT_1 revealed the presence of a pseudo-loop equivalent to that found in the chemokine receptors [10-12]; however, the conservation of these residues does not necessarily imply the formation of a pseudo-loop, as is shown by the structures of the dopamine D_3 receptor and the serotonin receptor $5\text{-}HT_{1B}$, which lack a disulphide bridge between the two conserved cysteines [13, 14] . Therefore, in the near future, the presence and the exact role of ECL4s in ligand-binding, signal transduction, and receptor interactions will need to be addressed in more detail, not only for chemokine receptors, but also for other receptor families.



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Receptor	Sequence	Family
XCR1	P-CENQAWVF	C
CCR1	P-CQKVNERA	A
CCR2	P-CHKFDVKQ	A/B
CCR3	L-CEKADTRA	A/B
CCR4	P-CTKEGIKA	A/B
CCR5	P-CQKINVKQ	A
CCR6	L-CSLQEVRQ	B
CCR7	L-CSKKDVRN	A/B
CCR8	P-CDAELIQT	B
CCR9	Y-CEKNNVRQ	A
CCR10	L-CYKADVQA	B
CXCR1	P-CMLETETL	B
CXCR2	P-CEPESLEI	B
CXCR3	P-CPQDFSLN	B
CXCR4	P-CFREENAN	B
CXCR5	L-CPATEGPL	B
CXCR6*	S-SQEEHQDF	B
CX3CR1	A-CYIGDIVV	B
ACKR1	P-CHSCNLLD	C
ACKR2	L-CRKDAVVS	B
ACKR3	M-CPNMPNKS	C
ACKR4	I-CIKEDVRE	A/B

Fig. 1. Overall structure of the extracellular surface of chemokine receptors and the location of ECL4 pseudo-loops. (**A**) Comparison between the x-ray structures of CXCR4 (blue) and CCR5 (green) showing the conserved overall arrangement of the extracellular features (ECL1, ECL2, and ECL3), as well as the location and shape of the ECL4 pseudo-loops of CXCR4 (orange) and CCR5 (red). Note that the ECL4 pseudo-loop of CXCR4 points more towards the inside of the ligand-binding pocket than does that of CCR5. The black square highlights the position of family-defining residues presented. (**B**) Ligand-binding pocket of CXCR4 (based on Protein Data Bank (PDB) structure 3ODU) in complex with IT1t (yellow). ECL4 is coloured in orange. (**C**) Ligand-binding pocket of CCR5 (PDB 4MBS) in complex with Maraviroc (pink). ECL4 is coloured in red. (**D**) NMR structure of CXCR1 in a liquid crystalline phospholipid bilayer (orange) (PDB 2NLN). ECL4 is coloured in red. In the absence of the small ligand, ECL2 lies on top of the ligand-binding pocket, blocking its access, and TM1 is off-centred from the TM circle in comparison with ECL2 of CXCR4 and CCR5. (**E**) Arrangement and position of CRS1 and CRS2 in CXCR4. The N terminus, ECL2, and ECL4 are coloured in grey, green and orange, respectively. The complete structure of CXCR4 was generated by molecular dynamic simulation after engraftment of the 28 N-terminal residues to the resolved x-ray structure (PDB 3ODU) [4]. (**F**) Positional conservation of residues at positions C_{+6} in CCR5 (family A, red) and C_{+4} in CXCR4 (family B, orange).

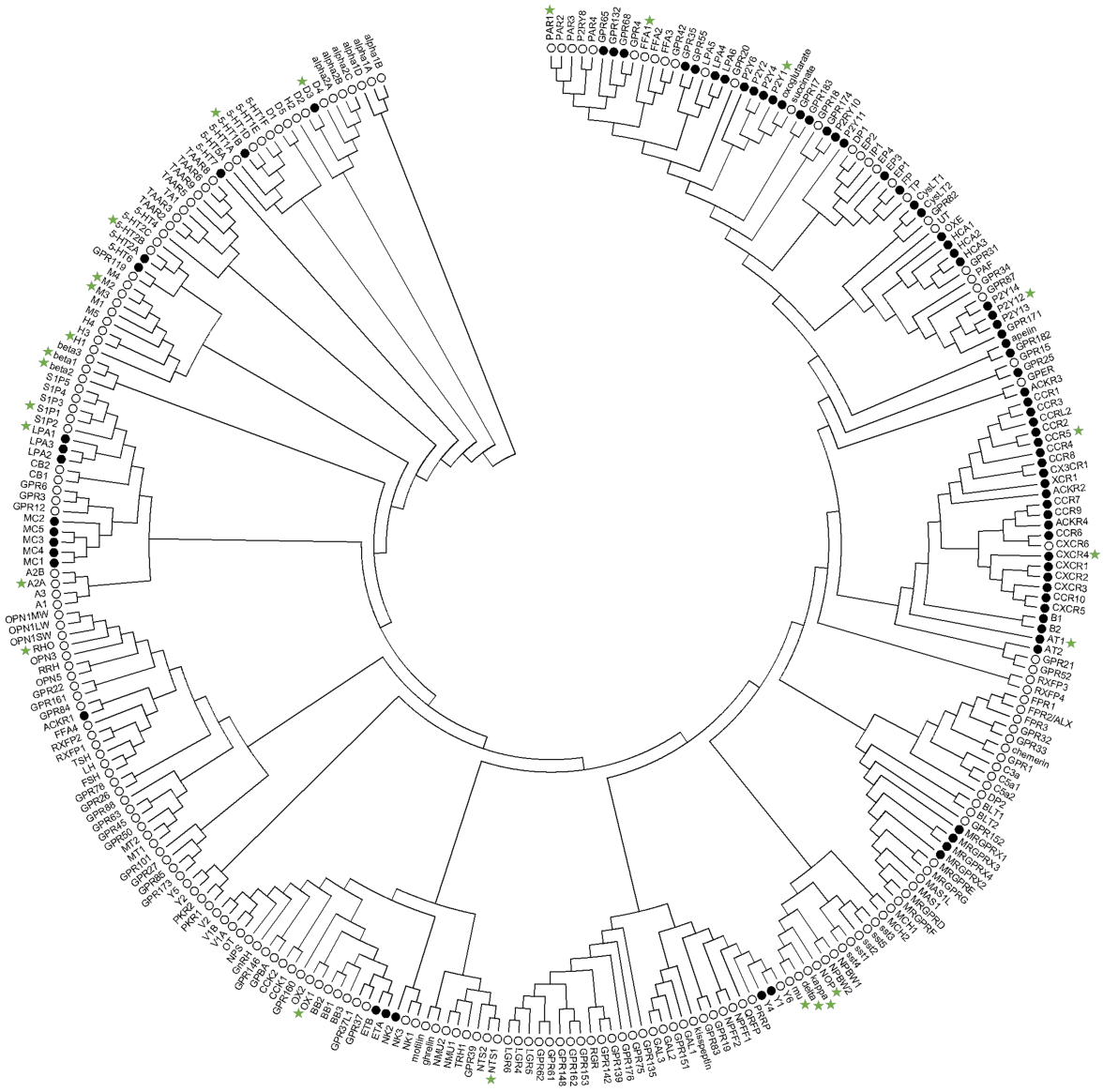

Fig. 2. Conservation of cysteine residues potentially involved in the formation of ECL4 among the human rhodopsin receptor family. The phylogenetic tree was constructed with MEGA 6 software using sequence similarity within the seven-transmembrane region (TM1-TM7) of human receptors of the rhodopsin family. Receptors are named according to the IUPHAR nomenclature. Black symbols highlight receptors presenting at least one cysteine in their N terminus and another at the top of their TM7 (ECL3), which are potentially involved in the formation of an ECL4 pseudo-loop. Apart from classical (C, CC, CXC, CX3C) and atypical chemokine receptors (ACKR), cysteine residues are also found in various receptors scattered among different rhodopsin receptor subfamilies (84 out of the 284 receptors represented here) including receptors for lysophospholipid (LPA), bradykinin ($B_{1\text{-}2}$), endothelin ($ET_{A\text{-}B}$), melanocortin ($MC_{1\text{-}5}$), serotonin (5-HT) as well as purinergic (P2Y) and orphan receptors. Receptors for which the three-dimensional structures are known are marked with a star. Among these, apart from CXCR1, CXCR4 and CCR5, the dopamine receptor 3 (D_3) (PDB: 3PBL), the 5-hydroxytryptamine receptor 1B ($5\text{-}HT_{1B}$) (PDB: 4IAR), the lysophosphatidic acid receptor (LPA_1) (4Z35), the angiotensin receptor 1 (AT_1) (PDB: 4YAY) and the purinergic receptors $P2Y_1$ (PDB: 4XNV) and $P2Y_{12}$ (PDB: 4NTJ) present additional cysteine residues in the N-terminal domain and ECL3, but these appear to be linked in a disulphide bridge forming a pseudo-loop only in AT_1, $P2Y_1$ and $P2Y_{12}$ [10-14].

Highlights

- Two highly conserved cysteine residues in the chemokine receptor N terminus and ECL3 (or top of TM7) are most likely linked by a disulphide bridge.
- The presence of this cysteine bridge was confirmed in all of the four chemokine receptors for which the three-dimensional structure has been solved.
- As a consequence of this disulphide bridge, an additional extracellular pseudo-loop is formed, which we called ECL4.
- ECL4 shapes the entrance of the ligand-binding pocket and adds rigidity to the overall receptor surface and may participate in ligand binding and receptor activation.
- Similar conserved cysteine residues are also found in various receptors of the rhodopsin receptor family but these do not appear to be always linked in a disulphide bridge.
- We identified new molecular signatures within ECL4 according to which the receptors can be classified in three subfamilies.

Although the two cysteines in the N terminus and ECL3 (or top of TM7) are conserved in all but one chemokine receptors, they are still rather poorly investigated at the functional level and their exact role remains obscure. It is generally accepted that these cysteines are linked by a disulphide bridge but the pseudo-loop ECL4, formed as a consequence, was somehow disregarded. The aim of this short perspective paper was to draw attention of the scientific community towards this structural characteristic of chemokine receptors. On a slightly different note, we were glad to see that there was still place, in a good quality journal, for an "old-school-science" paper based on simple sequence alignment analysis.

Importantly, the two very recently resolved structures of chemokine-bound receptors, CXCR4 and the HCMV-encoded US28, confirmed our hypothesis on the involvement of ECL4 in chemokine recognition. The region of CXCR4 partly covering its ECL4 was shown as an important interaction determinant in what was proposed as the intermediary chemokine recognition site CRS1.5 (see Chapter 8).

The sequence analysis that we performed here was also extremely helpful for proposing the most likely combination of disulphide bridges involving the three cysteines of CXCR7 N terminus. It suggested that most probably C34 is linked to C287 within ECL3, whereas C21 and C26 may in turn form an intra-N terminus disulphide bridge, creating a four-residue loop, which we named the tertapeptidyl arch or TPA.

The next chapter will summarise the preliminary results we accumulated so far in the study of CXCR7 N terminus, including what we learnt on the importance of its cysteine residues as well as its single tyrosine residue.

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Chapter 3

CXCR7 N terminus post-translational modifications: role in chemokine binding and receptor activation

1. Introduction

The atypical chemokine receptor CXCR7 binds two endogenous chemokines, CXCL12 and CXCL11, which are also the ligands for CXCR4 and CXCR3, respectively. CXCR7 has approximately a 10 times higher affinity for CXCL12 (K_D = 0.4 nM) compared with CXCR4 (K_D = 3.6 nM) whereas it binds CXCL11 with a 1à times lower affinity (K_D = 3 nM) compared with CXCR3 (K_D = 0.3 nM) [1-4]. Unlike CXCR4 and CXCR3 that signal via G proteins, CXCR7 functions rely exclusively on arrestin-dependent mechanisms. Moreover, due to its continuous cycling between the plasma membrane and the endosomal compartments, CXCR7 is proposed to act as a scavenging receptor regulating the availability of CXCL12 and CXCL11 for CXCR4 and CXCR3 [5-7].

As described in the previous chapters, the extracellular parts of chemokine receptors and particularly their N termini play a predominant role in ligand recognition. There exists, however, very little information about the exact molecular mechanisms driving CXCR7 interactions with CXCL12/CXCL11 or dictating its selectivity and affinity towards these chemokines.

This chapter presents a study on the importance of the post-translational modifications present in CXCR7 N terminus for chemokine binding and receptor activation. In addition to CXCL12 and CXCL11, the binding of vCCL2, a viral chemokine, which we identified as a third ligand for CXCR7 (see Chapter 5), will be investigated. CXCR7 displays about 25% of sequence identity with CXCR4 and CXCR3, and some motifs located in their extracellular parts are conserved, whereas others are not (Fig. 1).

CXCR4 1-MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANFNK-38
CXCR7 1-MDLHLFDYSEPGNFSDISWPCNSSDCIVVDTVMCPNMPNK-40

Fig. 1. Sequence comparison between the N terminus of CXCR4 and CXCR7 and putative sites of post-translational modifications. The two sequences were aligned with respect to the cysteine residues predicted to be involved in the disulphide bridge with (ECL3) the top of TM7. Potentially sulfated tyrosine residues are coloured blue, asparagine residues that are part of the potential N-glycosylation sites are coloured orange and cysteine residues are coloured green.

Remarkably, the N terminus of CXCR7 presents only one putative sulfotyrosine (tyrosine 8) that corresponds to the sulfotyrosine 7 or 12 in CXCR4. To date, no

information about the sulfation state of tyrosine 8 in CXCR7 and its possible contribution to CXCL12 and CXCL11 binding is available. Interestingly, sulfotyrosine 21, which is crucial for CXCR4 binding to CXCL12, is not conserved in CXCR7, suggesting that the binding of CXCL12 to the N terminus of CXCR7 may follow a different mechanism than observed for CXCL12-CXCR4 interactions [8, 9] (Fig. 1 and 2).

CXCR7 also displays three putative glycosylation sites in its N terminus at position 13, 22 and 39 that are not conserved in CXCR4. N-glycosylation site at position 39 is unusual as, according to our inventory of chemokine receptor N-terminal sequences, post-translational modifications are not found within ECL4 of other chemokine receptors.

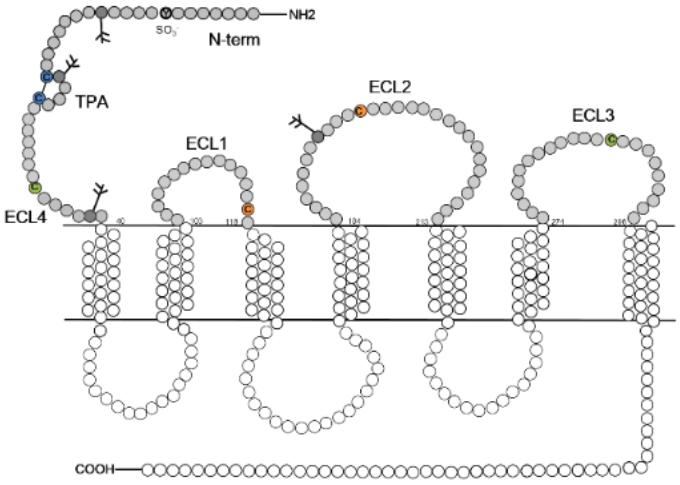

Fig. 2. Schematic representation of CXCR7. The putative post-translational modification sites identified in CXCR7 N terminus and other extracellular loops are shown. The combinations of the three cysteine pairs potentially linked by disulphide bridges are represented in orange, green and blue.

Remarkably, our sequence analysis of chemokine receptor N termini also revealed a striking particularity in the N-terminal domain of CXCR7. Besides a cysteine at position 34, which seems to correspond to the cysteine conserved in all chemokine receptors except CXCR6, the N terminus of CXCR7 bears two additional cysteine residues at positions 21 and 26 separated by a four-amino acid segment. Since no other unusual cysteines are found in the remaining extracellular parts of the receptor, it is plausible that these cysteines could be engaged in a disulphide bridge, giving rise to an intra-N terminus loop. We named this hypothetical loop TPA for TetraPetidyl Arch (Fig. 2). Because the N termini of other CXC receptors do not have analogous cysteine residues, this small arch could account for the existence of either a unique receptor structure or an unusual mode of interaction between CXCR7 and its ligands. However, the presence of

the TPA and the correct configuration of the different disulphide bridges in the N terminus of CXCR7 remain hypothetical and require further investigation.

Therefore, to examine the importance of the N terminus of CXCR7 and its post-translational modifications in chemokine-receptor interactions, we generated a set of cells lines stably expressing chimeric and mutated CXCR7 and assessed their binding and signalling properties by competition binding assay using fluorescently labelled CXCL12 and β-arrestin recruitment assay in comparison with the WT CXCR7.

2. Methods

2.1 Generation of U87 cell lines expressing WT and mutated CXCR7

U87 cells stably expressing N terminus-chimeric or mutated CXCR7 were established using pBABE vector. A chimeric CXCR7 was constructed, in which the first 33 residues corresponding to the flexible N terminus located upstream of the cysteine 34 were replaced with the corresponding sequence of CXCR4 (1-27). CXCR7 was also mutated and several positions, including the potentially sulfated tyrosine (Y8A and Y8F), the putative N-glycosylation sites (N13Q, N22Q and N39Q) as well as cysteines (C21S, C26S, C34S and C287S). U87 cells stably expressing the modified CXCR7 were obtained following puromycin selection and subsequent single cell sorting. The presence of the mutations and the surface expression level of the mutated receptors were verified by DNA sequencing and flow cytometry using an antibody recognising the proximal N terminal part of CXCR4 (anti-CXCR4 clone 4G10) or CXCR7 (anti-CXCR7 clones 11G8 and 9C4).

2.2 Binding competition with fluorescently labelled CXCL12

Alexa Fluor 647-labelled CXCL12 (40 ng/ml) (Almac) was mixed with unlabelled CXCL12, CXCL11, vCCL2, or CXCL10 (Peprotech) at concentrations ranging from 6 pM to 1 μM and incubated with U87.CXCR7 cells for 90 minutes at 4°C. CXCL10 was used as negative control. Non-specific binding of CXCL12-AF647 was evaluated by adding a 250-fold excess of unlabelled CXCL12. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Canto cytometer (BD Biosciences).

2.3 Arrestin recruitment

Chemokine-induced β-arrestin-2 recruitment to WT CXCR7 and mutant receptors, in which cysteines were replaced by serines, was monitored by firefly luciferase complementation assay. HEK cells stably expressing β-arrestin-2 fused at its N terminus to the first 415 amino acids of firefly luciferase were transfected with a pIRES vector (Addgene) encoding the 413-549 amino acids of the firefly luciferase fused to the C terminus of WT or mutated CXCR7. 24 hours post-transfection β-arrestin-2 recruitment was evaluated after 10-minute incubation with chemokines at concentrations from 1 μM to 0.1 nM. Luciferin (Synchem) was then added and luminescence was measured with a luminometer Centro XS3 LB 960. Experiments were carried out in duplicate.

3. Results and discussion

3.1 Importance of the flexible N terminus for CXCL12 binding

The conservation of the CXCL12 binding properties of the chimeric CXCR7 receptor, in which the flexible N terminus of the receptor was swapped with that of CXCR4, was assessed using fluorescently labelled CXCL12. The expression of this chimeric receptor at the surface of U87 cells was first confirmed by flow cytometry using the 4G10 antibody. Binding experiments with fluorescently labelled CXCL12 revealed that this chimera was unable to bind the chemokine even at a concentration as high as 300 ng/ml. In contrast, WT CXCR7 bound labelled-CXCL12 with an EC_{50} value of 23 ng/ml indicating that the N terminus is crucial for CXCL12 binding and that CXCR7 and CXCR4 N termini are not exchangeable.

3.2 Importance of the tyrosine 8 for chemokine binding

The presence and the importance of the potential sulfation of the tyrosine 8 of CXCR7 was assessed by creating two mutants, Y8A and Y8F, in which the possibility of sulfation was abolished while maintaining (Y8F) or not (Y8A) the aromatic properties of the tyrosine side chain. Surprisingly, these two mutants retained WT CXCR7 chemokine binding properties. CXCL12, CXCL11 and vCCL2 bound to the mutants Y8F and Y8A with IC_{50} values comparable to WT CXCR7 receptor (Table 1, Fig. 3).

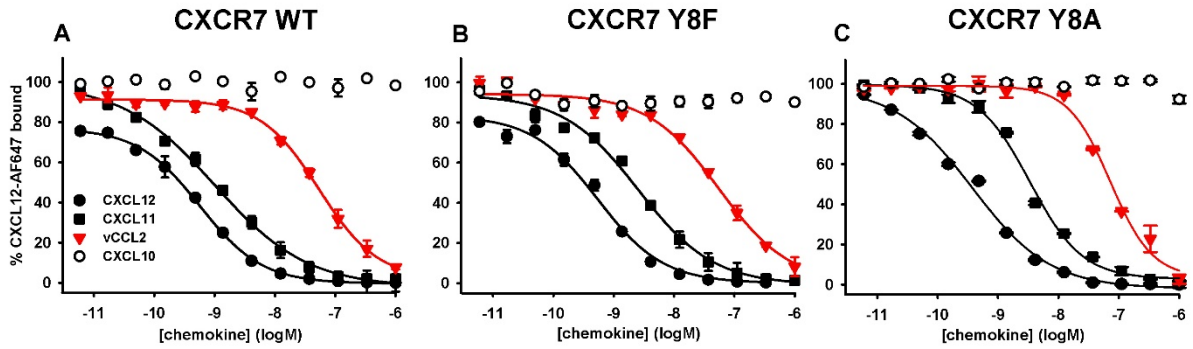

Fig. 3. Chemokine binding to CXCR7 tyrosine mutants. U87 cells stably expressing (A) WT CXCR7 or (B) Y8F mutant or (C) Y8A mutant were incubated 90 min on ice with increasing concentrations of non-labelled chemokines and Alexa Fluor 647-coupled CXCL12 (40 ng/ml). The binding of CXCL12-AF647 was analysed by flow cytometry. Concentration-response curves are shown for CXCL12, CXCL11 and vCCL2 and the negative control chemokine, CXCL10.

These results indicate that tyrosine 8 is not critical for the binding of the three chemokines and that if this tyrosine is sulphated, this post-translational modification is not important for chemokine binding which contrast to what is observed for CXCR4 [8].

Table 1. Chemokine binding properties of mutants Y8F and Y8A

Chemokine	CXCR7 WT	CXCR7 Y8F	CXCR7 Y8A
CXCL12	0.3 ± 0.5 nM	0.5 ± 1.2 nM	0.4 ± 0.1 nM
CXCL11	2.5 ± 1.2 nM	2.6 ± 1.6 nM	3.3 ± 0.4 nM
vCCL2	51.3 ± 11.6 nM	55.4 ± 10.0 nM	72.5 ± 11.3 nM
CXCL10	> 1000 nM	> 1000 nM	> 1000 nM

Although the absence of tyrosine sulfation has to be confirmed for instance by mass spectrometry or by Western blotting using anti-sulfotyrosine antibody, the possibility that CXCR7 does not require sulfotyrosine for tight chemokine binding may nevertheless be related to its scavenging function [5, 6]. Indeed, through its rapid and continuous cycling from the membrane to the endosomal compartments CXCR7 is proposed to act as a scavenger receptor for CXCL12 and CXCL11, internalising the chemokines and limiting their availability for CXCR4 and CXCR3. Therefore, the absence of sulfotyrosine, may be required to sustain the receptor binding capacity especially after passages in intracellular acidic compartments, where the chemokines are removed from the receptor and degraded, before the receptor is recycled to the membrane [5, 6].

3.3 Importance of disulphide bridges C21-C26 and C34-C287 for chemokine binding and receptor activation

In order to evaluate which cysteine in the N terminus of CXCR7 is linked to the cysteine 287 present in ECL3 and which other two may form an additional disulphide bridge, we established several U87-derived cell lines stably expressing CXCR7 bearing cysteine-to-serine mutation at either position 21 (C21S), 26 (C26S), 34 (C34S) or 287 (C287S). We evaluated their expression and compared their binding and their ability to recruit arrestin in response to chemokines.

All mutants were expressed at the cell surface at comparable levels and retained their ability to bind fluorescently labelled CXCL12. Their binding properties were further evaluated in competition assay and surprisingly all mutants conserved the ability to bind CXCL12, CXCL11 and vCCL2 albeit with somewhat reduction of affinity (Table 2).

Table 2. Chemokine binding properties of mutants C21S, C26S, C34S and C287S

Chemokine	CXCR7 WT	CXCR7 C21S	CXCR7 C26S	CXCR7 C34S	CXCR7 C287S
CXCL12	0.3 ± 0.5 nM	0.7 ± 1.2 nM	1.1 ± 0.1 nM	2.0± 0.4 nM	1,2 ± 02 nM
CXCL11	2.5 ± 1.2 nM	10.6 ± 1.3 nM	5.9 ± 1.1 nM	5.7 ± 0.6 nM	8.8 ± 1.9 nM
vCCL2	51.3 ± 11.6 nM	151.4 ± 1.6 nM	112.4 ± 7.7 nM	70.0 ± 39.1 nM	32.7 ± 16.7 nM
CXCL10	> 1000 nM	> 1000 nM	> 1000 nM	> 1000 nM	> 1000 nM

These data suggest that the disulphide bridges between the CXCR7 N terminus and ECL3 and those forming the hypothetical TPA, if present, are not critical for chemokine binding. It is impossible to pair the four cysteines based on chemokine binding potencies of the mutant receptors, as no clear differences could be observed. Only a slight decrease in vCCL2 binding suggests a similar impact for mutants C21S and C26S on one hand and C34S and C287S on the other hand.

The ability of these different mutants to recruit β-arrestine-2 in response to chemokines CXCL12, CXCL11, vCCL2 and CXCL10 was also analysed using a split luciferase assay. In agreement with the binding data, recent preliminary results showed that all mutants retained the ability to recruit arrestin following chemokine stimulation. This set of data needs now to be confirmed and the impact of each mutation on the potency and efficacy of arrestin recruitment induced by the different chemokines further characterised.

All these data suggest that the potential sulfotyrosine and disulphides found in the N terminus of CXCR7 are not critical for ligand binding and receptor activation. Although these results were somehow disappointing, we continued the investigations, generating cell lines expressing mutants of the different N-glycosylation sites and establishing cell lines overexpressing a HA epitope-tagged CXCR7, with the aim to purify the receptor and to analyse by mass spectrometry the presence of the potential disulphide bridges, the exact cysteine pairing as well as the tyrosine sulfation state.

The identification of vCCL2 as a third chemokine ligand for CXCR7 coincided with the study presented above. We therefore chose to focus on the more encouraging results and to characterise in depth this new interaction.

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Chapter 4

vCCL2/vMIP-II, the viral master KEYmokine

Adapted from Szpakowska M and Chevigné A, vCCL2/vMIP-II, the viral master KEYmokine, Journal of Leukocyte Biology 2015 Dec 23. pii: jlb.2MR0815-383R. [Epub ahead of print]

One of the most significant findings of the project was the identification of the human herpesvirus 8 (HHV-8)-encoded chemokine vCCL2 as a third high-affinity ligand for CXCR7 (described in chapter 4). By binding a broad range of chemokine receptors, this viral chemokine strongly contributes to the virus immune evasion and persistence. The most remarkable mechanism of action proposed for vCCL2 is the rewiring of the chemokine-receptor network through its selective agonist or antagonist activity, inducing or blocking the recruitment of specific immune cell subsets.

The following chapter arises from our wish to better understand the role of vCCL2 in HHV-8 infection in order to appreciate the potential biological relevance and the molecular basis of its newly identified interaction with CXCR7. The knowledge currently available on vCCL2 binding, signalling and structural mimicry will be summarised here, discussing its role and importance for the virus, the therapeutic potential and the open questions regarding the biology of this fascinating chemokine.

1. Human chemokine-receptor network

Chemokines are small (8-14 kDa) secreted proteins that play a central role in guiding directional migration (chemotaxis) of leukocytes in immunosurveillance and immune responses and are important mediators of cell adhesion, growth and survival [1]. They exert these functions by interacting with chemokine receptors that are seven-transmembrane domain G protein-coupled receptors (GPCRs). To date 47 chemokines and 19 receptors, forming a highly intricate and precisely regulated network, have been identified in humans. Based on conserved cysteine motifs, chemokines are divided into four subfamilies: CC, CXC, XC and CX_3C and the receptors are named according to the subfamily of chemokines they bind (CCR, CXCR, XCR and CX_3CR). In addition, other receptors referred to as Atypical Chemokine Receptors (ACKR1-4) can recognise chemokines and act as scavengers or signal through alternative pathways, further contributing to the complexity of the chemokine network [2].

Among a myriad of mechanisms that viruses use to evade the immune system or exploit various biological processes of the host cell to promote their survival, large DNA viruses, such as herpesviruses and poxviruses, have evolved strategies to interfere with the chemokine-receptor network by encoding their own chemokine and receptor homologues or chemokine-binding proteins capable of sequestering a broad range of chemokines [3-6].

2. vCCL2/vMIP-II, a viral chemokine homologue encoded by HHV-8

The viral macrophage inflammatory protein II (vMIP-II or vCCL2) is a viral CC chemokine encoded by the human herpesvirus 8 (HHV-8), also known as the Kaposi's sarcoma-associated herpesvirus (KSHV), the causative agent of Kaposi's sarcoma, a disease generally linked with immunodeficiency, but also two rare proliferative disorders, primary effusion lymphoma and multicentric Castelman disease [7-9].

vCCL2 was initially identified from a fragment of the HHV-8 genome isolated from a Kaposi's sarcoma biopsy [10]. Among the viral open reading frames present in the HHV-8 genome, three (K6, K4 and K4.1) were predicted to encode CC chemokine homologues, vCCL1/vMIP-I, vCCL2/vMIP-II and vCCL3/vMIP-III, respectively, and one (ORF74) a CXC chemokine receptor homologue [11]. vCCL2 is produced as a 94-

amino acid precursor with a 23-aa N-terminal signal peptide and a C-terminal arginine, which are cleaved to yield the mature 70-aa chemokine (7.9 kDa) (Fig. 1A) [11, 12].

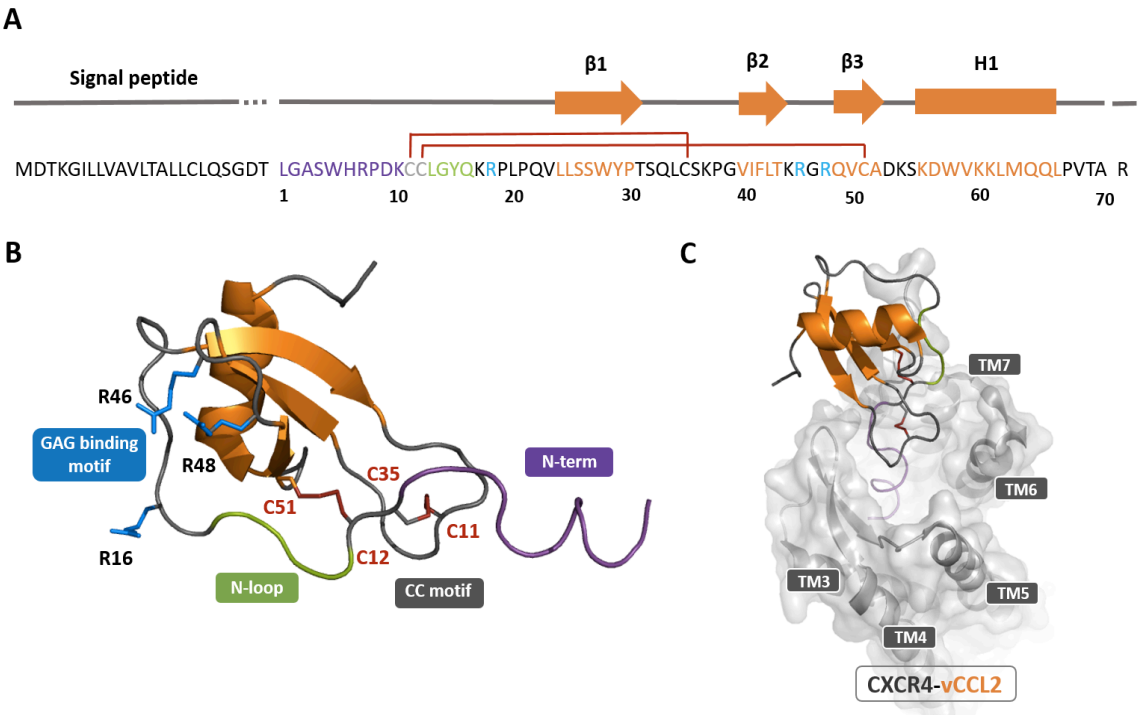

Figure 1. Structures and N-terminal features of vCCL2. (A) Primary sequence of the vCCL2 precursor encoded by HHV-8 ORF K4. vCCL2 precursor (94-amino acid) presents an N-terminal signal peptide and a C-terminal arginine (R), which are cleaved to yield the 70-aa mature chemokine. The flexible N terminus (residues 1-10), the cysteine motif (C_{11}-C_{12}) and the N-loop (residues 13 to 21) are coloured in purple, grey and green, respectively. The three β-strands and the C-terminal α-helix are represented in orange and the disulfide bridges connecting the cysteine motif with the core of the chemokine are represented by red lines. Arginine residues involved in GAG binding (R16, R46 and R48) are coloured blue. **(B)** Three-dimensional structure of vCCL2 resolved by X-ray crystallography (PDB 4RWS) showing the structural arrangement of vCCL2 features including the highly disordered N terminus (purple), the N-loop (green) and the core of the chemokine with the three β-strands and the C-terminal α-helix (orange) and the conserved cysteines (red). **(C)** Three-dimensional structure of the complex between CXCR4 and vCCL2 (PDB 4RWS). The receptor is shown as cartoon/surface and coloured grey. The chemokine is represented as cartoon and coloured purple (N-term), green (N-loop) and orange (core). The structure-stabilising disulfide bridges are coloured in red. vCCL2 makes substantial contacts with the receptor with the N terminus inserted deep in the transmembrane (TM) cavity.

vCCL2 was most likely captured from a cluster on the human chromosome 17 (17q11-32), which contains genes encoding most of the CC chemokines. Indeed, it shows high sequence identity with the human chemokines CCL3 (47.1 %), CCL18 (44.1 %), CCL15 (43.4 %) as well as CCL4 (40.6 %) which bind CCR1, CCR3, CCR5 and CCR8. vCCL2 has also a high sequence identity with vCCL1 (55.7 %) which acts as an agonist of CCR8 [13, 14] but much less identity with vCCL3 (24.5%) which activates CCR4 and XCR1 [15, 16].

The three-dimensional structure of vCCL2 was resolved by both x-ray crystallography [17] and NMR [18] and it was shown to adopt a fold typical of human chemokines characterised by a flexible and disordered N terminus of 10 residues followed by the cysteine motif (C_{11} and C_{12}), an N-loop, three anti-parallel β-strands and a C-terminal α-helix. The N terminus and the β-sheet of vCCL2 are connected by two disulfide bridges linking the cysteine residues 11 to 35 and 12 to 51 (Fig. 1B). However, in contrast to its closely related human CC chemokine homologues, in solution, vCCL2 exists exclusively as a monomer [18, 19] although an engineered dimeric variant (L13F) has been reported [20].

Ever since its discovery almost two decades ago, vCCL2 has been intensively investigated and demonstrated to have quite atypical binding and signaling properties reflecting the complex and sophisticated mechanisms that HHV-8 has evolved to control its life cycle and modulate the host immune response.

3. Binding and activity of vCCL2 towards viral and human chemokine receptors

vCCL2 binds with nanomolar affinity to a broad spectrum of viral and human chemokine receptors and, depending on the receptor, acts either as an antagonist or an agonist. It is the only chemokine identified so far capable of binding to chemokine receptors of the four families (CCR, CXCR, XCR and CX3CR) and may be seen as a "master KEYmokine", a master key for chemokine receptors (Fig. 2).

Being a CC chemokine, the majority of receptors recognised by vCCL2 belong to the CC family. vCCL2 was shown to act as an antagonist towards CCR1, CCR2, CCR5 [12] and CCR10 [21], competing with the endogenous chemokine binding and signaling but also as an agonist of CCR3 [22] and CCR8 [23] triggering G protein-mediated intracellular calcium release, activation of ERK and PI3K/AKT signalling pathways or chemotaxis [24]. Noteworthy, several ligands for these receptors including CCL3, CCL4, CCL5 and CCL15 have high sequence identity with vCCL2 (Fig. 2).

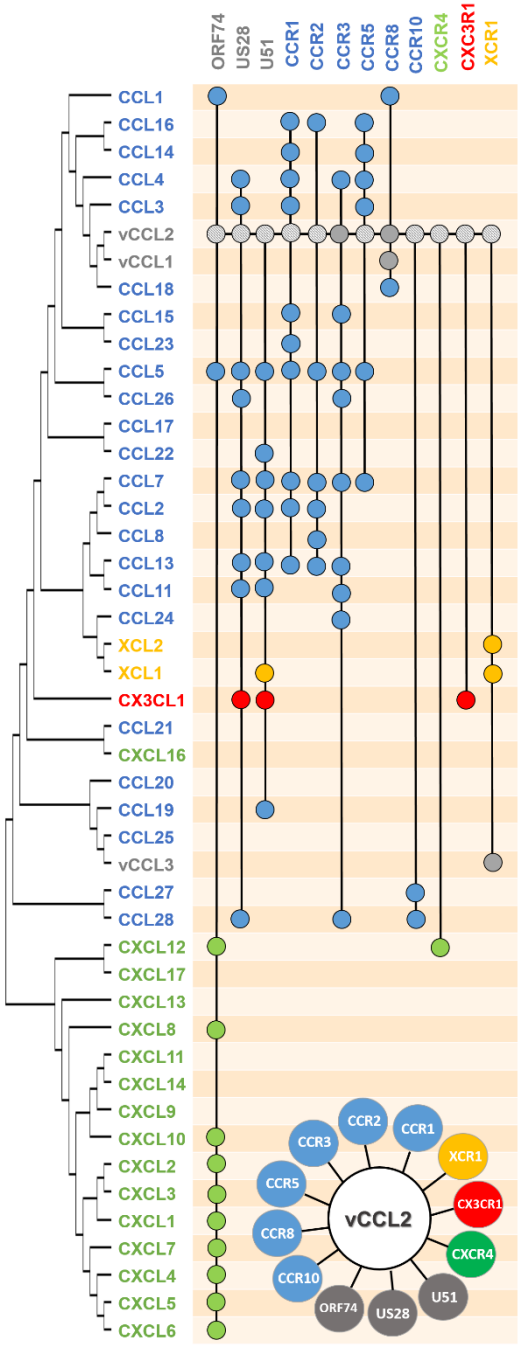

Figure 2. Similarity between HHV-8-encoded and human chemokines and overview of vCCL2-binding receptors. Amino acid sequence similarity of HHV-8 and human chemokines and their binding specificity. Only chemokine receptors targeted by vCCL2 are shown. Left side: Sequences were aligned with ClustalW and the similarity tree was built using Omega6. The names of chemokines are coloured according to their classes: CC (blue), CXC (green), XC (yellow) and CX3C (red). HHV-8 chemokines are coloured grey. Right side: Chemokine binding specificity of receptors targeted by vCCL2. Agonist and antagonist activities of viral chemokines towards the different receptors are represented by dark and light grey circles, respectively. **Inset.** Overview of the diversity of human and viral chemokine receptors targeted by vCCL2. Human receptors are colored according to the class of chemokine they bind: CCR (blue), CXCR (green), XCR (yellow) and CX3CR (red). Viral chemokine receptors are coloured grey.

Apart from CC receptors, vCCL2 is also an antagonist ligand for the only two representatives of the XCR and CX3CR families, XCR1 [25, 26] and CX3CR1 [27, 28], and for only one CXC receptor, CXCR4 [12] (Fig.2 Inset). The recently resolved crystal structure of vCCL2 in complex with CXCR4 [29] revealed a 1:1 stoichiometry interaction with extensive contact surface between the two partners. In addition, the study provided a molecular explanation for the specificity of CC and CXC chemokines towards their respective receptors as well as for the cross-family interaction for the vCCL2-CXCR4 pair. It suggested that on one hand, vCCL2 bears CXC chemokine-like

features, which are involved in the interaction with CXC receptor-conserved residues of CXCR4. On the other hand, some residues present in CXCR4 are signatures of CC rather than CXC receptors and therefore may contribute to the binding of vCCL2 [29].

vCCL2 also binds to several viral chemokine receptor homologues. It was shown to downregulate the constitutive activity of the HHV-8-encoded receptor ORF74, a viral homologue of the human CXCR2, binding to many CXC and CC chemokines [30, 31]. Similarly, vCCL2 is described to inhibit the constitutive activity of US28 [12] and U51 [32], which are homologues of CX3CR1 and CCR7 encoded by HCMV and HHV-6, respectively.

Besides its interactions with chemokine receptors, it is proposed that vCCL2 binding to glycosaminoglycans (GAGs) present on the surface of endothelial cells may also play an important role *in vivo*. Although their interaction modes are similar, vCCL2 binds to GAGs much more tightly than most endogenous chemokines [20]. Therefore, by binding to GAGs vCCL2 may build up its own chemokine gradient to interact more efficiently with leukocytes expressing its target receptors but also to compete with the binding of the endogenous chemokines to GAGs, interfering with the normal leukocyte recruitment. Additionally, binding to GAGs may protect vCCL2 against proteolysis [20] (Fig. 3).

4. Role of vCCL2 in HHV-8 biology

HHV-8 can establish life-long asymptomatic infections in immunocompetent individuals but it is most notorious for its association with Kaposi's sarcoma, often affecting AIDS patients, as well as two other proliferative diseases, primary effusion lymphoma and multicentric Castelman disease [7-9]. HHV-8 infects mainly endothelial and B cells but also monocytes and dendritic cells [33-35]. As in other herpesviruses, its life cycle consists of two stages characterised by different gene expression programs. During the latent or dormant phase only a limited number of proteins is expressed while in the course of the lytic or productive phase the majority of genes are transcribed and the replication with viral progeny production takes place [36, 37]. A fine regulation between the latent and the productive cycle allows the virus to propagate, to persist for a long period in the host and to avoid clearance by the immune system. HHV-8 has also evolved complex mechanisms including the use of viral chemokines and receptors as a

means of exploiting the host chemokine system to favour its own survival. vCCL2 is expressed as an early lytic gene [38, 39] and plays an important role in modulating the activity of HHV-8 and host chemokine receptors through autocrine and paracrine effects.

One of the strategies the virus employs is the skewing of the host immune system away from the Th1 cytotoxic response, undesirable for the virus-infected cells, towards a Th2 response, less effective against intracellular pathogens. vCCL2 is proposed to strongly contribute to this mechanism of immune evasion through its selective antagonist action on Th1-specific chemokine receptors (CCR1, CCR2, CCR5 and CX3CR1) and agonist activity on Th2-related CCR3 and CCR8 (Fig. 3).

Numerous studies report an antagonist effect of vCCL2 on CCR1, CCR2, CCR5, CXCR4 or CX3CR1 inhibiting endogenous ligand-induced calcium responses as well as cell migration in both cell lines overexpressing the receptor of interest or lymphocytes isolated from peripheral blood [12, 22, 23, 27, 28]. Interestingly, owing to its large spectrum of receptors, vCCL2 is also able to block the recruitment of immune cells at different stages of their activation. It has for instance been shown that despite the differences in the patterns of chemokine receptors expressed, vCCL2 can inhibit the migration of both naïve and activated NK cells through interaction with CX3CR1 and CCR5, respectively [40]. vCCL2 is often referred to as the broad-spectrum antagonist chemokine but its agonist activity towards CCR3 and CCR8 is also documented. vCCL2 was shown to induce CCR3-dependent calcium release and eosinophil chemotaxis [41] and arrest in shear flow [22]. Similarly, CCR8-transfected cells were reported to migrate in response to vCCL2 [23].

In addition to facilitating the evasion from cytotoxic immune responses, vCCL2 plays a role in blocking the defence mechanisms also from within the cells. It was shown to inhibit lytic cycle-induced pro-apoptotic signals in infected cells, thereby prolonging their survival and favouring the virus productive replication and propagation. This anti-apoptotic effect of vCCL2 is suggested to operate, at least in part, through CCR8 in both autocrine and paracrine manner contributing to viral persistence and latency maintenance [24].

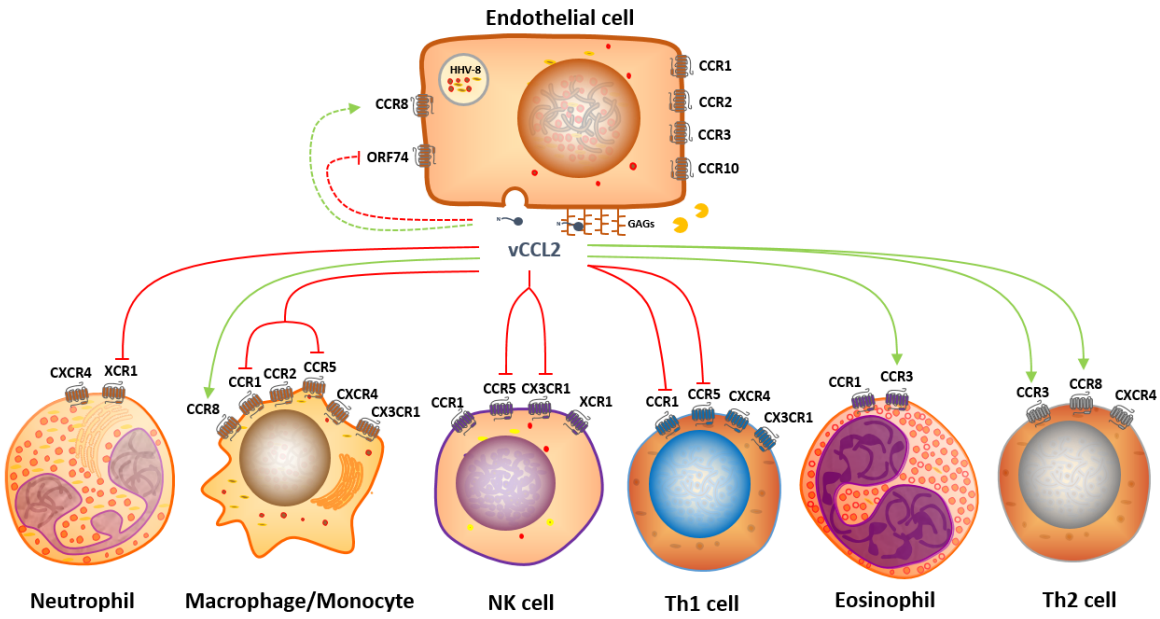

Figure 3. Presumed interplay between vCCL2 and chemokine receptors expressed by infected and immune cells. Receptor antagonist (red) and agonist (green) activity of vCCL2 is represented by solid (paracrine) and dashed (autocrine) lines. Only the interactions for which functional experimental data have been reported are presented. vCCL2 expressed by HHV-8 infected endothelial cells interacts with human chemokine receptor CCR8 and ORF74 to promote cell survival and limit ORF74-driven tumourigenesis (autocrine activity). vCCL2 targets chemokine receptors expressed by different immune cells to limit antiviral cytotoxic responses by blocking CCR1 and CCR5 expressed by Th1 cells and macrophages while it induces the recruitment of Th2 cells and eosinophils by acting on CCR3 and CCR8. For clarity, the chemokine receptor expression patterns of the different cell subsets include only vCCL2-binding receptors.

Moreover, vCCL2 may participate in controlling the two gene expression programs of HHV-8. vCCL2 acts as an inverse agonist towards the HHV-8-encoded GPCR, ORF74. This constitutively active receptor is expressed during the early lytic stage of the virus and shows a highly proliferative potential. The down-regulation of ORF74 by vCCL2 may temporally control the activity of HHV-8 by repressing its reactivation thereby helping to escape the host immune surveillance [30, 31].

vCCL2 is also proposed to facilitate virus dissemination through its pro-angiogenic effect. It has been shown by different approaches including *in ovo* chick embryo chorioallantoic membrane-based assay or *in vivo* using lentivirus-delivered vCCL2 that it has the potential to enhance blood vessel formation and survival and may therefore also contribute to Kaposi's sarcoma or PEL-linked pathogenesis [41, 42].

5. Therapeutic potential of vCCL2 and its derived peptides

Since the first description of its antagonist properties towards several receptors binding to pro-inflammatory chemokines and/or mediating HIV entry, vCCL2 and peptides derived from its N terminus have been explored for their immunomodulatory potential in acute and chronic inflammatory diseases or for their anti-HIV and anti-tumoral activity.

vCCL2 has been expressed as a full-length native recombinant protein or as a fusion protein [43, 44] in various eukaryotic [44, 45] and prokaryotic systems [18, 20] or using different gene delivery approaches [42, 46, 47]. Fully synthetic vCCL2 with or without non-natural amino acids has also been produced [17, 48, 49]. Because of its specific inhibitory properties towards many receptors for pro-inflammatory chemokines such as CCL2, CCL3, CCL4 and CCL5, vCCL2 has been tested *in vitro* and in mice and rats for its ability to block leukocyte recruitment and infiltration to reduce Th1-driven inflammation following ischemic brain and spinal cord injuries [50-52], to limit cardiac, corneal or renal allograft rejection [46, 47, 53], glomerulonephritis [27] and cutaneous hypersensitivity reaction [45] or to promote post-transplantation angiogenesis [42]. In addition, vCCL2 labelled with ^{64}Cu-DOTA has also been proven a sensitive probe to detect by PET imaging the upregulation of different chemokine receptors involved in atherosclerosis [54]. Owing to its unique ability to bind to CCR5, CXCR4 and CCR3, vCCL2 has also been evaluated as a HIV-1 inhibitor to block the viral entry through several co-receptors [12, 41, 55]. vCCL2 showed moderate inhibition of HIV entry through the main co-receptors, CXCR4 and CCR5 but seemed more potent in blocking CCR3, important for HIV infection of microglia [12]. This higher potency may be partly explained by the agonist activity of vCCL2 towards CCR3 demonstrated in other studies [22, 41], which could affect HIV infection by triggering specific cellular responses and/or subsequent receptor internalisation.

In parallel to studies carried out with the full-length chemokine, the possibility of reducing the size of vCCL2 to a peptide level, down to as few as nine residues, while maintaining the parental activity and selectivity, has also been investigated. Due to their small size, such peptides are easier to produce and modify and were proposed to hold a great potential for the design of novel therapeutics [56]. In accordance with the generally accepted chemokine-receptor two-step binding mode [57], peptides encompassing the

flexible N terminus, the cysteine motif and the N-loop of vCCL2 (1-LGASWHRPDKCCLGYQKRPLP-21) were shown to act as CXCR4 inhibitors blocking the entry of HIV X4-stains [58-61] or modulating inflammation [58-62] with potency in the micromolar range. Notably, binding of peptides derived from the first 21 residues of vCCL2 was shown to be maintained for its all-D-amino acid analogue, revealing the unsuspected high permissivity of CXCR4 to stereoisomer replacement and offering interesting possibility to design peptides with higher resistance to proteolysis [58, 63-65]. These studies also pointed out the importance of residues Leu1, Arg7 and Lys9 for CXCR4 binding and demonstrated that dimerisation improved the potency of the peptides to nanomolar range providing one of the first indications of the high propensity of CXCR4 to form homodimers [66]. Notably, the peptide corresponding to residues 1 to 21 did not show any HIV-1 inhibitory ability against R5-viruses suggesting that vCCL2 most likely interacts with CXCR4 and CCR5 according to a slightly different binding mode or using different determinants, with a higher contribution of the N-terminal fragment in CXCR4 binding. No information is available on the binding of vCCL2 peptide derivatives to other receptors targeted by the parental chemokine.

Cyclic peptides bearing homologies with the three-residue segment Trp5-His6-Arg7 present in vCCL2 N terminus have also been explored for their anti-tumoural activity in *in vivo* models of lung metastases and growth of renal cells xenograft. These peptides showed significant reduction of tumor spread and expansion with potency in the micromolar range [67].

Finally, D-peptides derived from the flexible N terminus of vCCL2 (residues 1-10) have also been explored as vehicle to specifically target and deliver molecules such as small drugs or DNA to CXCR4-overexpressing cancer cells [68].

6. Discussion

vCCL2 is an atypical and a fascinating chemokine. It binds to a broad spectrum of both viral and human chemokine receptors across the four families, showing antagonist or agonist activity at both autocrine and paracrine levels. These unique properties reflect a sophisticated strategy of molecular mimicry and receptor piracy that HHV-8 has evolved to turn the host chemokine receptor network to its own advantage.

Despite the number of receptors vCCL2 binds, it should not be regarded as a non-selective chemokine. Its broad-spectrum binding properties should be rather seen as tightly linked to its ability to precisely modulate multiple facets of host chemokine-mediated defences. vCCL2 was shown to favour the survival of infected cells by skewing the host immune response away from the deleterious Th1 type and towards Th2 type. Although vCCL2 has initially been described as an antagonist of numerous receptors, notably those acting as HIV entry co-receptors [12], it has since been demonstrated to also activate CCR3 and CCR8, triggering direct G protein signalling. This dual activity and tailored specificity makes the unique character of vCCL2.

The binding of vCCL2 to its receptors does not appear to be dictated by their degree of promiscuity. vCCL2 binds several multiple-ligand receptors such as CCR1, CCR2, CCR3 and CCR5 but also interacts with receptors having a narrow ligand spectrum such as CCR8, CCR10, XCR1, CX3CR1 and CXCR4 (Fig. 2). Moreover, the agonist or antagonist activity of vCCL2 towards a particular receptor cannot be easily predicted or explained based on vCCL2 sequence or its similarity with human chemokines. Among the CCR family, vCCL2 activates CCR3 and CCR8 but antagonises CCR1 and CCR5 the four receptors being activated by CCL3, CCL4, CCL15 or CCL18, the human chemokines closest to vCCL2. At the same time, vCCL2 also interacts with CCR2 and CCR10 that are activated by endogenous chemokines having poor sequence identity with vCCL2.

The ability of vCCL2 to bind receptors outside the CCR family is unusual but was probably acquired to achieve more efficient polarisation of the immune response by blocking XCR1, CX3CR1 and CXCR4. Reprogramming vCCL2 to target these receptors was most likely less difficult as XCL1, XCL2 and CX3CL1 cluster with the CC chemokines on the similarity tree and CXCL12 is one of the CXC chemokine displaying the highest sequence similarity with the CC family (Fig. 2). vCCL2 binding and signalling properties are even more remarkable given that it shares high sequence identity with vCCL1, which binds to only one receptor, and that the two chemokines are proposed to have evolved by gene duplication within the virus.

The atypical binding properties of vCCL2 appear to be the result of a multi-constraint compromise in molecular reshaping of both its N terminus (addressing and message) and its core (addressing). It has been shown, for instance, that specific modifications in vCCL2 can improve its affinity for a particular receptor class at the expenses however of

the binding to other receptors [28] illustrating the fragility of this compromise. It cannot be however excluded that vCCL2 binding to some receptors may simply be a side effect of its intrinsic promiscuity. The resolution of additional structures of human receptors in complex with vCCL2 and further studies on the role of its core and N terminus will nevertheless be required to unravel the molecular basis of its unusual behaviour.

It is uncertain whether all host receptors identified *in vitro* for vCCL2 are used *in vivo.* Considering the intricacy and the spatiotemporal expression variability of the chemokine-receptor network, the relevance of these interactions to HHV-8 in physiological conditions remains unclear. Moreover, although the functional consequences of the binding of vCCL2 are well described for several receptors, its activity towards others, such as CCR2 or CCR10, remains less documented.

Among the three chemokines encoded by HHV-8, vCCL2 is the only one able to bind and to reduce the constitutive activity of ORF74. Although blocking the activity of its own receptor may seem counterproductive for the virus, it probably plays an important role in controlling the potential deleterious tumourigenic effect of ORF74. vCCL2 was also shown to bind U51 and to display subnanomolar affinity towards US28, the chemokine receptors expressed by two other herpesviruses, HHV-6 and HCMV, respectively. The biological relevance of these observations remains to be investigated. However, because HHV-6 and HCMV can infect similar cell types as does HHV-8, including monocytes or endothelial cells, and because both viruses have also been found in Kaposi's sarcoma lesions, one could speculate that vCCL2 may be involved in some aspects of their infection [69-71]. In a similar manner, HHV-6 has been previously suggested to promote HHV-8 pathogenesis [39].

The signalling by human and viral chemokine receptors in response to vCCL2 has been mostly studied in the context of their canonical G protein pathways, mainly by monitoring the intracellular calcium mobilisation. However, in light of the recent observations that chemokine receptors can also trigger alternative pathways dependent on β-arrestin or other G protein subtypes, it cannot be excluded that vCCL2 signalling may also be more complex than initially thought [72, 73]. Furthermore, vCCL2 may activate different signalling pathways through the same receptor but depending on the cell type (tissue or cell bias) or on which receptors are co-expressed [40], adding a level of complexity to its interplay with chemokine receptors. These aspects together with the

use of different cellular models and assays may also explain some of the discordant results obtained for the agonist vs antagonist activity of vCCL2 towards several human receptors such as CCR5 [43], CCR8 [13, 74] and CCR10 [26].

In addition, the binding and activity of vCCL2 towards recently deorphanised receptors such as CXCR8 or the four representatives of the atypical chemokine receptor (ACKR) family considered as silent/scavenger or arrestin-signalling receptors have not been documented yet. Since some of these receptors are expressed either by cells susceptible to HHV-8 infection or cells of the immune system, their interaction with vCCL2 should be given more attention in the future.

Beyond the better comprehension of HHV-8 biology and the complex interplay that this virus has evolved to inflect the host immune response, the investigation conducted on vCCL2 has allowed to significantly improve our understanding of the human chemokine receptor network and especially of the molecular basis of human receptor recognition and activation. The atypical binding properties of this viral master KEYmokine have also been shown to be instrumental to take up several technical challenges and have opened some promising therapeutic avenues for immune modulation. Nevertheless, many aspects of vCCL2 biology and of its molecular mimicry remain to be uncovered.

Highlights

- vCCL2 is the only chemokine able to bind all the four families of chemokine receptors.
- vCCL2 through the highly specific and tightly regulated interactions with the host chemokine network can induce or block the recruitment of specific immune cell subsets, facilitating the virus immune evasion.
- vCCL2 is also proposed to promote virus survival by inhibiting pro-apoptotic signals in infected cells and to facilitate virus dissemination through its pro-angiogenic effect.
- The interactions between vCCL2 and the atypical chemokine receptors remain to be investigated.

This chapter is based on a review recently accepted for publication in the special issue of Journal of Leukocyte Biology dedicated to the European Chemokine and Cell Migration conference, which took place in June 2015 and during which I had the chance to give a short presentation on vCCL2-CXCR7 interactions. The writing of this review and the literature study it required was an invaluable occasion to better understand the properties and the role of vCCL2 and thus also to be able to judge on the biological relevance of its new interaction with CXCR7.

The study on the interaction between vCCL2 and CXCR7 will be presented in the following chapter.

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Chapter 5

Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II: new agonist of CXCR7

Adapted from Szpakowska M, Counson M, Dupuis N, Hanson J, Piette J and Chevigné A, Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7, submitted to Biochemical Pharmacology, February 2016

Soon after HHV-8-encoded chemokines were identified in the late 1990s, their potential agonist and antagonist activity was tested on the complete panel of then-known chemokine receptors. CXCR7, however, escaped this screening simply because back then it had not yet been identified as a chemokine receptor.

The idea to investigate the potential interaction between vCCL2 and CXCR7 came shortly after my project started, when in the frame of another ongoing project we were investigating vCCL2 for its inhibitory properties towards CXCR4. It was legitimate to think that since CXCR7 and CXCR4 share CXCL12 as ligand it may be the same for vCCL2. Our hypothesis was also supported by a study, dating back to 2002 when CXCR7 was still an orphan receptor, which demonstrated its upregulation upon infection by HHV-8 thereby indicating that this receptor may play a role in this virus biology.

Since our identification of vCCL2 as a ligand for CXCR7 we have thoroughly investigated its binding and activation properties towards this atypical receptor. Our data suggest that CXCR7 acts as a scavenger receptor for vCCL2, regulating its availability for human receptors and may control its function in HHV-8 infection. The following chapter will describe the characterisation of this newly discovered vCCL2-CXCR7 interaction and discuss the possible implications it may have in the HHV-8 biology.

1. Introduction

Chemokines are small (8-14 kDa) secreted proteins that play a central role in guiding directional migration (chemotaxis) of leukocytes in immunosurveillance and immune responses. They exert these functions by interacting and activating a family of seven-transmembrane domain G protein-coupled receptors (GPCRs). To date, 47 chemokines and 19 receptors have been identified in humans. Based on conserved cysteine motifs, chemokines are divided into four subfamilies: CC, CXC, XC, CX3C and the receptors are named according to the subfamily of chemokines they bind (CCR, CXCR, XCR and CX3CR) [1]. The chemokine-receptor network is highly intricate and a chemokine can bind one or many receptors, while a receptor usually recognizes several chemokines. In addition, other receptors referred to as atypical chemokine receptors (ACKR1-4) can recognize chemokines and act as scavengers or signal through alternative pathways, further contributing to the complexity of the chemokine network.

Various pathogens have evolved ways to subvert and exploit the immune processes regulated by chemokines and their receptors in order to promote their survival and propagation. Viruses from different families (e.g. poxviruses, herpesviruses or retroviruses) encode chemokine-binding proteins, chemokine receptors as well as chemokine analogues or envelope proteins that hijack cellular chemokine receptors [2, 3]. Human herpesvirus 8 (HHV-8), also known as the Kaposi's sarcoma-associated virus (KSHV), strikingly illustrates such molecular mimicry and its role in virus pathogenesis. HHV-8 causes Kaposi's sarcoma (KS), a disease generally linked with immunodeficiency, but also two rare proliferative disorders, primary effusion lymphoma (PEL) and multicentric Castelman disease (MCD). HHV-8 infects endothelial cells in KS and is found in B lymphocytes from PEL and MCD lesions and the peripheral blood of KS patients [4]. The HHV-8 genome encodes one chemokine receptor, ORF74, and three CC chemokines, vCCL1/vMIP-I, vCCL2/vMIP-II and vCCL3/vMIP-III. Unlike vCCL1 and vCCL3, vCCL2 interacts with a broad spectrum of receptors and is the only chemokine reported so far capable of binding to receptors of the four classes. Besides downregulating the activity of ORF74 [5], vCCL2 binds to host CC receptors (CCR1, CCR2, CCR3, CCR5, CCR8, CCR10) but also to the two single representatives of XC and CX3C receptor families (XCR1 and CX3CR1) and to only one CXC receptor (CXCR4) [6, 7]. Although vCCL2 is generally described as an antagonist chemokine, it

also acts as an agonist towards two CC receptors, CCR3 and CCR8 [8-10]. Through its selective antagonist action on Th1-specific chemokine receptors (CCR1, CCR2, CCR5 and CX3CR1) and agonist effect on Th2-related CCR3 and CCR8, vCCL2 is proposed to play an important role in the skewing of the host immune system away from the Th1 cytotoxic response towards a Th2 response, which is less effective against intracellular pathogens [8, 10]. Additionally, by activating CCR8, vCCL2 is proposed to contribute to virus pathogenesis through an autocrine pro-survival action or angiogenic effect on neighbouring cells [10-14]. Up to now, the interaction of vCCL2 with the atypical chemokine receptors and especially with the most recently deorphanised chemokine receptor, CXCR7/ACKR3, has not been reported.

CXCR7, recently renamed ACKR3, is expressed in various cells such as B and T lymphocytes, neurons and endothelial cells and plays a crucial role in many processes including cardiovascular and neuronal development as well as in migration and homing of hematopoietic stem/progenitor cells [15-21]. An increasing number of studies point to the involvement of CXCR7 in many cancers. CXCR7 is expressed in various cancer cell types as well as on tumour-associated vasculature and accumulating evidence demonstrates its involvement in metastasis development [22-25]. CXCR7 was also shown to be upregulated upon infection by several cancer-inducing viruses including HHV-8, EBV, HTLV-1 and to play an important role in cell transformation and proliferation [26, 27]. Due to its unusual biology, it has recently been classified as an atypical chemokine receptor [28, 29]. Indeed, CXCR7 binds two endogenous chemokines, CXCL12 and CXCL11, which are also recognized by CXCR4 and CXCR3, respectively but unlike conventional chemokine receptors, CXCR7 does not activate the canonical G-protein pathways and is proposed to trigger β-arrestin-dependent signalling. In addition, through its continuous cycling between the plasma membrane and endosomal compartments and its capacity to efficiently internalize and degrade chemokines, CXCR7 functions as a scavenger receptor regulating the availability of CXCL12 and CXCL11 for CXCR4 and CXCR3 [30-32]. However, the exact molecular basis of the interactions of CXCR7 with its ligands and its ability to signal is poorly understood and still a matter of debate. Besides, many questions remain unanswered regarding the physiological roles of CXCR7, how it partakes in the complex chemokine-

receptor network and whether it can also be exploited by virus-encoded chemokines or the opposite, thwart their action.

In this study, we identified vCCL2, a chemokine encoded by HHV-8, as a new ligand for CXCR7. We showed that in addition to its unexpected agonist effect towards this atypical receptor, vCCL2 may also be subject to CXCR7 scavenging, regulating its activity on other human chemokine receptors that may be important for virus pathogenesis/biology.

2. Methods

2.1 U87.CXCR7 cell line validation

U87 cells obtained through the NIH AIDS Reagent Program from Dr. Deng and Dr. Littman [33] were transfected using Lipofectamine (Life Technologies) with pBABE-puro vector containing the CXCR7 sequence (Uniprot: P25106) optimized for mammalian expression. A stable U87.CXCR7 cell line was obtained following puromycin selection and subsequent single cell sorting. Chemokine receptor expression at the surface of this cell line was analyzed by flow cytometry using mAbs specific for CXCR7 (clones 11G8 (R&D Systems) and 8F11 (BioLegend)), CXCR4 (clones 4G10 (Santa Cruz Biotechnology) and 12G5 (BD Biosciences)), anti-CXCR3 (clone 1C6 (BD Biosciences)), anti-CCR3 (clone 5E8, BioLegend), anti-CCR8 (clone L263G8, BioLegend) and allophycocyanin–conjugated $F(ab')_2$ fragment of anti-mouse IgG (Jackson ImmunoResearch).

2.2 Binding competition with fluorescently labelled CXCL12

Alexa Fluor 647-labelled CXCL12 (40 ng/ml) (Almac) was mixed with unlabelled CXCL12, CXCL11, vCCL2, vCCL1, CCL3, CCL4, CCL15, CCL18 or CXCL14 (Peprotech) at concentrations ranging from 6 pM to 1 μM and incubated with U87.CXCR7 cells for 90 minutes at 4°C. CXCL10 was used as negative control. Non-specific binding of CXCL12-AF647 was evaluated by adding a 250-fold excess of unlabelled CXCL12. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Canto cytometer (BD Biosciences).

2.3 Arrestin recruitment

Chemokine-induced β-arrestin recruitment to CXCR7 was monitored by β-galactosidase complementation assay, as previously described [34], using CHO cells stably expressing β-arrestin-2 fused to enzyme acceptor of β-galactosidase and CXCR7 fused to the β-galactosidase ProLink donor peptide (DiscoveRx). Cells were seeded 48h before the experiment in 96-well plates at a density of 5000 cells/well. Chemokines at concentrations ranging from 50 pM to 1 μM were then added and incubated for 90 minutes at 37°C. For kinetic analysis, reversible complementation of split firefly luciferase was used [35] in HEK cells expressing β-arrestin-2 fused at its N-terminus to residues 1-415 of firefly luciferase and CXCR7 fused at the C-terminus to residues 413-549 of firefly luciferase. Cells were treated for different times at room temperature with 100 nM chemokines prepared in phenol red-free DMEM. Chemiluminescent signal was generated through addition of β-galactosidase substrate (PathHunter Detection reagent) or D-luciferin (Synchem) and plates were read with POLARstar Omega or Centro XS3 LB 960 luminometer.

2.4 Chemokine-induced CXCR7 internalisation

U87.CXCR7 cells were incubated 30 minutes at 37°C in the presence of chemokines at concentrations ranging from 0.3 nM to 1 μM. Internalisation was stopped by placing cells on ice and surface-bound ligand was stripped by a brief glycine wash (150 mM NaCl, 50 mM, pH 2.7). Cell surface levels of CXCR7 were then measured by flow cytometry using receptor-saturating concentration of the mAb 11G8 (R&D Systems) and allophycocyanin–conjugated $F(ab')_2$ fragment anti-mouse IgG (Jackson ImmunoResearch). Mean fluorescence intensity was quantified by using BD FACS Diva software. Internalisation was expressed as the percentage of CXCR7 detected at the cell surface relative to untreated cells.

2.5 CXCR7 transport to endosomes

Arrestin-dependent CXCR7 delivery to endosomes following chemokine treatment was monitored by β-galactosidase complementation assay using U2OS cells stably expressing β-arrestin-2 fused to the enzyme acceptor of β-galactosidase and an endosome marker fused to the β-galactosidase ProLink donor peptide (DiscoveRx). Cells

were seeded 48h before the experiment in 96-well plates at a density of 5000 cells/well. Chemokines were then added at concentrations ranging from 50 pM to 1 μM and after 90-minute incubation at 37°C chemiluminescent signal was generated through addition of β-galactosidase substrate (PathHunter Detection reagent). After 1h-incubation at room temperature, plates were read with POLARstar Omega.

2.6 ERK1/2 and Akt phosophorylation

70% confluent serum-starved U87 and U87.CXCR7 cells preincubated 10 min with or without the mAbs 11G8 (10 μg/ml) or overnight with the pertussis toxin (100 μg/ml) were stimulated 20 min at 37°C with vCCL2 100 nM. Cells were lysed with buffer containing urea 7M, thiourea 2M and 2 % ASB and 20 μg of protein from fresh cell extracts were resolved on 12 % polyacrylamide gels and analyzed by Western blot using antibodies against phosphorylated and non-phosphorylated ERK 1/2 and Akt (#9101, #4696, #4060, #9272, Cell Signaling Technology) at recommended dilutions. After incubation with peroxidase-coupled secondary antibody (1:40000, 115-035-003 Jackson ImmunoResearch) proteins were detected using enhanced chemiluminescence substrate (PerkinElmer). Band intensity was quantified using ImageJ.

2.7 vCCL2 scavenging

U87 and U87.CXCR7 cells were incubated 8 hours at 37°C in the presence of vCCL2 at concentrations ranging from 0.1 to 100 nM. vCCL2 remaining in the supernatant was then quantified by sandwich ELISA using anti-vCCL2 mAb as capture antibody (5 μg/ml, clone 82206, R&D Systems) and biotinylated polyclonal goat anti-vCCL2 IgG (0.05 μg/ml, R&D Systems) and horseradish peroxidase streptavidine conjugate (Sigma) for detection. After addition of substrate (SureBlue Reserve TMB, KPL) absorbance at 450 nm was measured using POLARstar omega. CXCR7 dependence of vCCL2 depletion was assessed by competition with an excess of CXCL12, CXCL11 or CXCL10 (1 μM). Student's t test was applied for statistical analysis using Prism 5 (GraphPad). Statistical significance was set at $P< 0.05$.

3. Results

We first investigated the interaction between vCCL2 and CXCR7 by binding competition with fluorescently labelled CXCL12 using U87 cells stably expressing CXCR7 but no CXCR4 (Fig. 1).

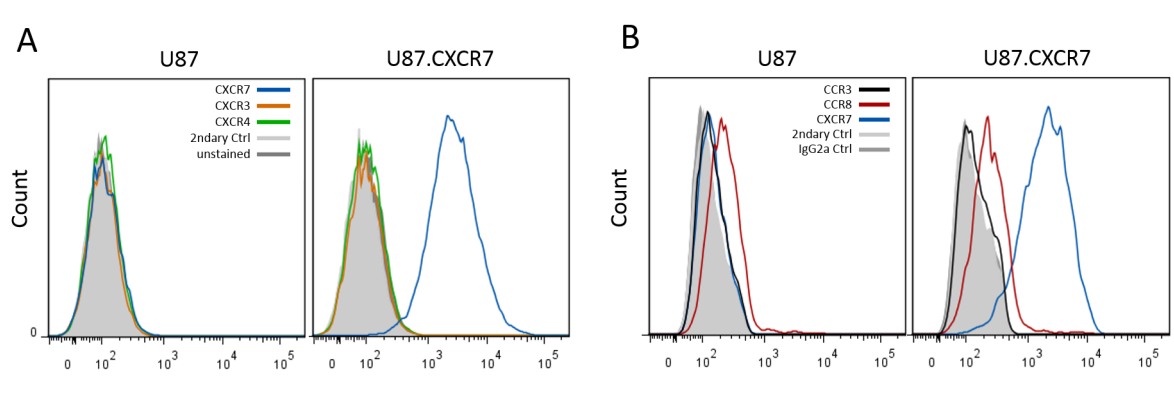

Figure 1. Chemokine receptor expression at the surface of U87.CXCR7 and untrasfected U87 and cells. (**A**) Flow cytometry analysis of receptors sharing ligands with CXCR7 using mAbs specific for CXCR7, clone 11G8 (R&D Systems) (blue), CXCR4, clone 12G5 (BD Biosciences) (green), CXCR3, clone 1C6 (BD Biosciences) (orange) and allophycocyanin–conjugated F(ab')2 fragment anti-mouse IgG (Jackson ImmunoResearch). Filled histograms correspond to unstained cells (dark grey) or cells stained with the secondary antibody only (light grey). (**B**) Flow cytometry analysis of the presence of CCR3 and CCR8 (the two chemokine receptors reported to be activated by vCCL2) using the mAbs anti-CCR3, clone 5E8 (BioLegend) (black), anti-CCR8, clone L263G8 (BioLegend) (red) and allophycocyanin–conjugated F(ab')2 fragment anti-mouse IgG. CXCR7 is shown in blue. Filled histograms correspond to cells stained with the secondary antibody only (light grey) or IgG2a isotype control (dark grey).

As shown in Fig 2A, vCCL2 interacted with CXCR7 with an IC_{50} of 53.6 ± 6.3 nM, while CXCL12 and CXCL11 bound the receptor with IC_{50} of 0.5 ± 0.1 and 1.0 ± 0.1 nM, respectively, in agreement with previously reported affinities [34]. CCL3, CCL18, CCL15 and CCL4, the human chemokines displaying the closest amino acid similarity to vCCL2 (above 40 % identity) did not bind CXCR7, while vCCL1 (55.7 % identity) displaced about 20 % of CXCL12 only at the highest concentration tested (1 μM), underscoring the specificity of vCCL2-CXCR7 interaction (Fig. 2B). As expected, CXCL10 used as negative control showed no binding to CXCR7 (Fig. 2A). No competition with labelled-CXCL12 was observed for CXCL14, an orphan chemokine recently proposed as a CXCR4 antagonist [36, 37].

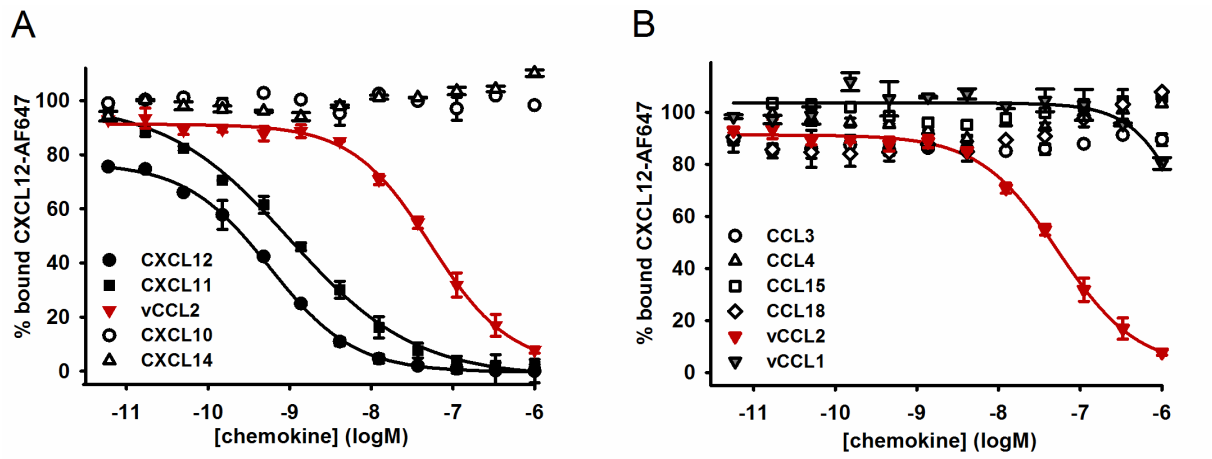

Figure 2. **vCCL2 binding to CXCR7.** U87 cells stably expressing CXCR7 but no CXCR4 or CXCR3 were incubated 90 min on ice with increasing concentrations of non-labeled chemokines and Alexa Fluor 647-coupled CXCL12 (40 ng/ml). The binding of CXCL12-AF647 was analyzed by flow cytometry. (**A**) Comparison of vCCL2 with CXCL12 and CXCL11, the endogenous CXCR7-binding chemokines, CXCL14 and the negative control chemokine, CXCL10. (**B**) Binding competition of the human chemokines with the highest sequence identity to vCCL2, CCL3 (47.1 %), CCL4 (40.6 %), CCL15 (43.4 %) and CCL18 (44.1 %), as well as the HHV-8 encoded vCCL1 (55.7 %), demonstrating the specificity of vCCL2-CXCR7 interaction. Each experiment was performed in duplicates and the values correspond to average ± standard deviation. The sigmoid curves were fitted to the data points using the four-parameter Hill equation.

Because CXCR7 activation is proposed to involve β-arrestin, we next investigated this question using luminescent protein-fragment complementation assays. vCCL2 acted as a partial agonist in β-arrestin recruitment to CXCR7 (EC_{50} of 35.4 ± 11.1 nM) inducing half of the maximal response observed with the endogenous chemokines CXCL12 and CXCL11 (EC_{50} = 1.2 ± 0.2 and 5.9 ± 1.0 nM) (Fig. 3A). Conflicting reports exist as for the profile of β-arrestin recruitment to CXCR7 for the two endogenous chemokines [28, 38, 39]. Our data showed, however, that vCCL2, just like CXCL12 and CXCL11, induced a delayed and prolonged recruitment of β-arrestin-2 to CXCR7, evocative of class B kinetics (Fig. 3B) [40].

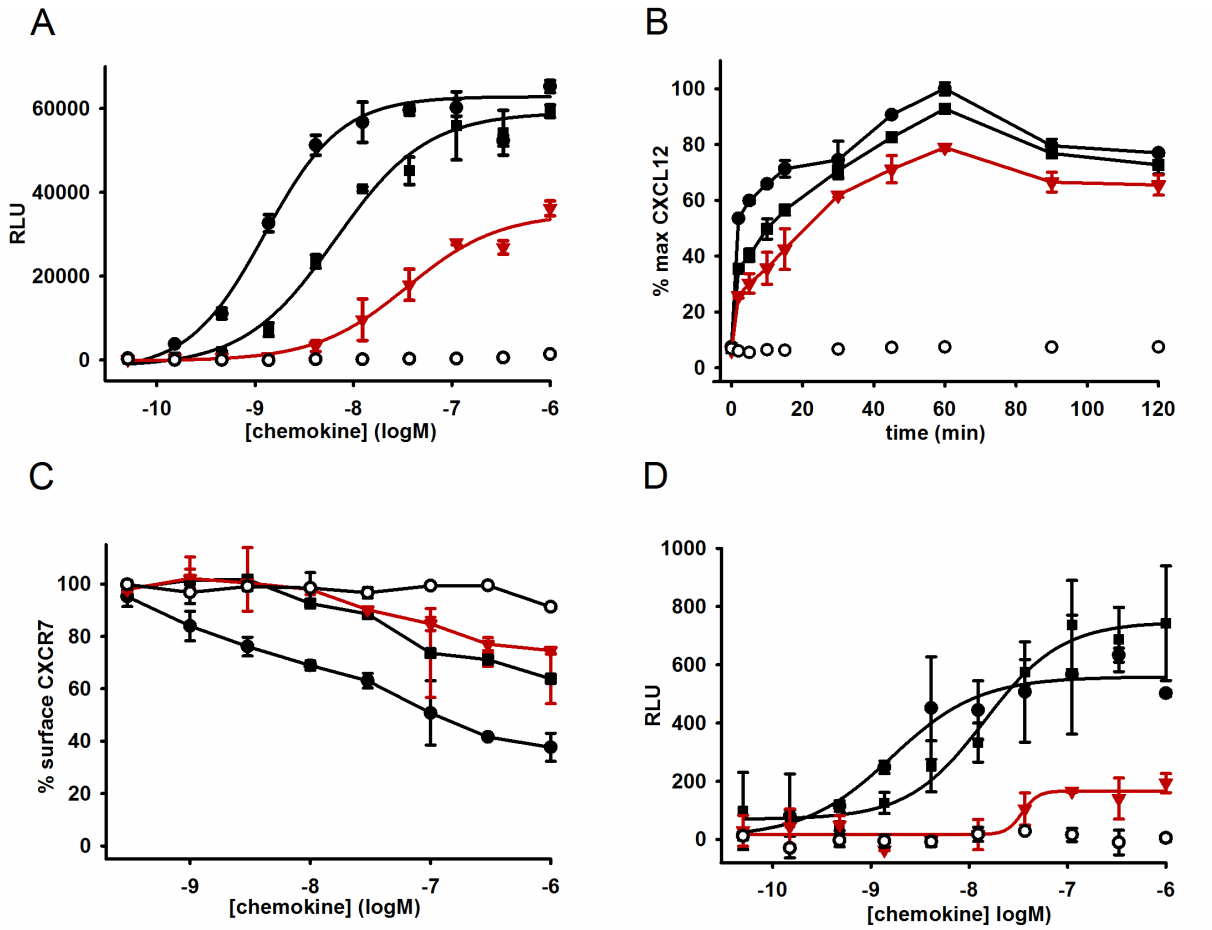

Figure 3. vCCL2 activity towards CXCR7. Chemokine-induced β-arrestin-2 recruitment to CXCR7 **(A)** in response to increasing concentrations of chemokines monitored by β-galactosidase complementation assay after 90-minute incubation at 37°C and **(B)** following treatment with chemokines (100 nM) at room temperature monitored at different time points by firefly luciferase complementation assay (**C**) Chemokine-induced CXCR7 internalisation. Cell surface levels of CXCR7 were monitored in U87.CXCR7 cells by flow cytometry using the mAb 11G8. Internalisation was expressed as percentage of CXCR7 detected at the cell surface of untreated cells. (**D**) Chemokine-induced CXCR7 delivery to endosomes monitored by β-galactosidase complementation assay. Each experiment was performed in duplicates and the values correspond to average ± standard deviation. The sigmoidal curves were fitted to the data points using the four-parameter Hill equation. CXCL12 (●), CXCL11 (■), vCCL2 (▼) and CXCL10 (○).

vCCL2 also reduced the levels of surface CXCR7 in a concentration-dependent manner with an efficiency similar to CXCL11 (Fig. 3C), suggesting that CXCR7 may act as a scavenger receptor also for this viral chemokine. This hypothesis is reinforced by the observation that internalized CXCR7 is targeted to the endosomes following exposure to vCCL2, although with lower efficacy (25 % of the maximal signal induced by CXCL11) and potency (EC_{50} = 34.7 ± 24.2 nM) than observed with CXCL12 and CXCL11 (EC_{50} = 1.6 ± 0.7 and 14.5 ± 3.2 nM, Fig. 3D), reminiscent of the β-arrestin-recruitment data (Fig. 3A). vCCL2 did not trigger intracellular calcium mobilisation or cAMP modulation in

U87 or U87.CXCR7 cells (data not shown), indicating that, similarly to the two endogenous ligands, the viral chemokine is unable to induce CXCR7-mediated G protein signalling. Treatment of U87 cells devoid of CXCR7 with vCCL2 resulted in MAP kinase- and PI3K/Akt-dependent signalling, (Fig. 4) and was most likely driven by the endogenously expressed CCR8 (Fig. 1), one of the two receptors previously shown to be activated by vCCL2 [9, 10]. Interestingly, the activation of these signalling pathways was independent of G_i protein as it was insensitive to pertussis toxin. vCCL2-induced ERK1/2 and Akt phosphorylation in U87 cells was reduced by one third in the presence of CXCR7 and was preserved upon addition of an CXCR7-specific antibody, endorsing the hypothesis that CXCR7 can function as a scavenger receptor for vCCL2 and regulate its availability for human receptors. This assumption was further supported by the significant and specific decrease of the extracellular vCCL2 concentration observed after 8 hours with U87.CXCR7 but not U87 cells (Fig. 4B).

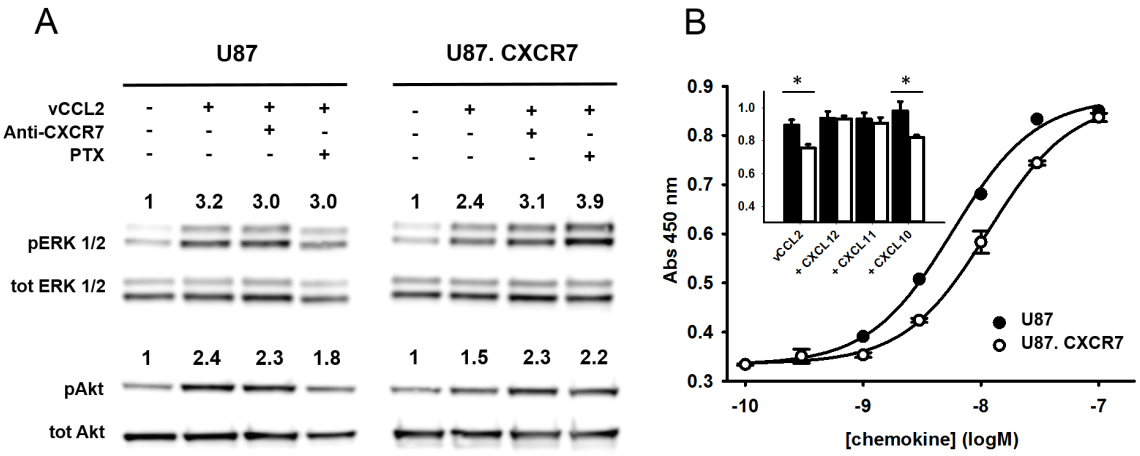

Figure 4. CXCR7-mediated vCCL2 scavenging. (**A)** Modulation by CXCR7 of vCCL2-induced activation of MAPK and PI3K/Akt pathways. Phosphorylation of ERK1/2 and Akt was evaluated after a 20-minute stimulation with vCCL2 (100 nM) in the presence or absence of anti-CXCR7 (11G8) (10 μg/ml) or pertussis toxin (100 ng/ml). Quantification of pERK and pAkt band intensity relative to total ERK or Akt intensity is shown. **(B)** vCCL2 depletion from U87 and U87.CXCR7 supernatant quantified by sandwich ELISA after 8-hour stimulation at 37°C. Measurements were performed in duplicates and the values correspond to average ± standard deviation. Inset: CXCR7-dependence of vCCL2 depletion was assessed by competition with excess of CXCL12, CXCL11 or CXCL10 (1 μM). * P< 0.05.

4. Discussion

The identification of vCCL2 as a ligand for CXCR7 may provide new insights into the regulation of HHV-8-related processes. However, because of the complex interplay between HHV-8 and the chemokine-receptor network and the numerous interactions of vCCL2 activating or blocking specific host receptors, the understanding of the impact of CXCR7 on vCCL2 functions and the viral life cycle is not straightforward. It remains to be investigated whether the ability of CXCR7 to bind and scavenge vCCL2 represents an advantage for the virus or a host defence mechanism or whether it is a secondary result of the pleiotropic vCCL2 binding. Nevertheless, our results showing highly specific and strong vCCL2 binding, inducing potent β-arrestin recruitment to CXCR7 suggest that this interaction is biologically relevant.

CXCR7 is expressed on various cells susceptible to HHV-8 infection [16, 32] and it was shown to be upregulated in dermal microvascular endothelial cell *in vitro* culture system and to play an essential role in cell transformation by the virus [27, 41, 42]. Therefore it is likely that CXCR7 as a scavenger receptor plays an important role in the virus life cycle by shaping the vCCL2 gradient. One of the possible actions of CXCR7 may be to participate in the regulation of the HHV-8-encoded chemokine receptor ORF74, whose constitutive activity conferring a highly proliferative potential to the cell is kept at low level during the latent stage of infection partly by the inverse agonist effect of vCCL2 as well as CXCL12. During the lytic stage, the control of ORF74 is released and CXCR7 by binding and internalizing vCCL2, and CXCL12, may contribute to this process.

In addition, it cannot be excluded that in certain cellular contexts the interaction between CXCR7 and vCCL2 may lead to β-arrestin-dependent signalling which would favour HHV-8 infection through direct cellular processes similar to those observed following the interaction with its endogenous chemokine ligands in various cancers [43-45]. For instance, vCCL2-CXCR7 interaction may delay the response to lytic cycle-induced pro-apoptotic signals in infected cells thereby favouring viral replication [9, 11]. Activation of CXCR7 by vCCL2 may also have an angiogenic effect as observed for the CXCL12-CXCR7 interaction [46, 47], which may increase vascular permeability and enhance migration of permissive cells into sites of productive replication or dissemination of newly infected cells [11].

Alternatively, in the context of HHV-8 infection, the presence of CXCR7 may instead be beneficial to the host. vCCL2 scavenging by CXCR7 may counteract its activity towards other host chemokine receptors and limit viral immune escape by restoring the Th1/Th2 balance or limit the CCR8 activation-dependent anti-apoptotic effect in infected cells. Similarly, by inducing CXCR7 internalisation, vCCL2 may decrease the ability of the receptor to scavenge CXCL11, which is known to shift the balance towards a Th1 antiviral response by activating CXCR3 and antagonizing CCR3 at high concentrations [48, 49].

Besides a possible new role in HHV-8 biology, the identification of vCCL2 as a third chemokine ligand for CXCR7 helps to better understand the biology and function of this atypical receptor. We showed that vCCL2 acts as partial agonist towards CXCR7, suggesting that it stabilizes a different active state of the receptor than do CXCL12 and CXCL11, which may be attributed to the different N-terminus orientation of CXC and CC chemokines [7, 50, 51]. Although vCCL2 can also bind to CXCR4, its cross-family agonist activity towards CXCR7 is a property that can more easily be attributed to atypical than conventional chemokine receptors. It extends thus the CXC chemokine selectivity of CXCR7, to which it owes its original name, to CC chemokines and further legitimizes its recent classification as an ACKR [52]. The interaction of vCCL2 with the three other representatives of the atypical chemokine receptor family (ACKR1, ACKR2 and ACKR4) has not been reported in the literature. Since these receptors are known to scavenge a broad range of CC chemokines and since they are also expressed either by cells susceptible to HHV-8 infection or cells of the immune system, it may be interesting to investigate their possible role in regulating the vCCL2 availability [52, 53]. Moreover, in light of our results suggesting that vCCL2 signalling through CCR8 is G_i-independent and the divergent reports showing a functional agonist effect of vCCL2 towards CCR3 and CCR8 [10, 54] but no direct G protein activation [6, 55], it should also be considered that vCCL2 can trigger alternative signalling dependent on β-arrestin or G protein subtypes other than G_i.

The biology of vCCL2 and its importance for HHV-8 is still not entirely understood. The finding that vCCL2, identified almost two decades ago, can bind and activate the atypical chemokine receptor CXCR7 illustrates how much of the chemokine-receptor network and the host-virus interactions remains to be unveiled.

Highlights

- The HHV-8-encoded chemokine vCCL2 is a third high-affinity ligand for CXCR7.
- vCCL2 acts as partial CXCR7 agonist, inducing β-arrestin recruitment, subsequent receptor internalisation and delivery to endosomes.
- CXCR7 reduces vCCL2-triggered MAP kinase and PI3K/Akt signaling through CCR8, a human receptor shown to play a role in HHV-8 immune evasion.
- CXCR7 acts as a scavenger receptor for vCCL2, regulating its availability for human receptors and potentially controlling its function in HHV-8 infection.

The study described in this chapter was challenging in several aspects and took a lot of time and effort. To be able to fully characterise the interaction between CXCR7 and its ligands, we first needed to optimise or develop various new functional assays, which had not been available in our laboratory. One of the difficulties we encountered was related to the signalling properties of CXCR7, proposed to activate β-arrestin-dependent pathways. Despite having tested multiple experimental conditions, we could not reproduce these results in neither of the cell lines we tested, be it in response to its endogenous ligands or vCCL2. CXCR7 signalling is indeed a controversial matter and some of the studies claiming that CXCR7 activates various pathways show weaknesses in their experimental design and sometimes lack appropriate controls. Nevertheless, CXCR7 may strongly depend on the cellular context, partly accounting for the confusion around this question.

Because in the beginning of the project our main objective was to study CXCR7 interactions with its endogenous ligands CXCL11 and CXCL12, we had established a U87 glioblastoma-derived CXCR7-positive cell line devoid of CXCR3 and CXCR4, the other receptors for these chemokines. In this respect, this cellular model is more appealing than some of the commonly used cell lines, like for instance HEK cells, which express both CXCR3 and CXCR4. However, U87 cells turned out not to be the most appropriate context to study the later-discovered viral ligand, as they endogenously express CCR8, which binds to vCCL2. Therefore, the affinity of vCCL2 for CXCR7

observed in our binding competition study as well as its potency in β-arrestin recruitment may be somewhat underestimated by the presence of this receptor, although such an imprecision is probably inevitable given the broad-range of chemokine receptors that vCCL2 binds.

The finding that CXCR7 is also susceptible to cross-family activation by a CC chemokine is probably another reason justifying its recent classification among the atypical chemokine receptors. Importantly for us, it extends the possibilities to further investigate the molecular determinants that dictate its chemokine binding and activation. The following chapter will present a comparative study on the contribution of various features of the N-terminal regions of CXCL12, CXCL11 and vCCL2 to their interactions with CXCR7 and CXCR4.

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Chapter 6

Different contribution of chemokine N-terminal features in binding and activation of CXCR7, CXCR4 and CXCR3

Adapted from Szpakowska M, Nevins A, Dupuis N, D'huys T, Gauthier P-A, Derj A, Girard M, Hanson J, Schols D, Volkman B, Heveker N and Chevigné A. Different contribution of chemokine N-terminal features in binding and activation of CXCR4, CXCR3 and the decoy receptor CXCR7/ACKR3. Manuscript in preparation.

The identification of vCCL2 as another ligand for the atypical receptor CXCR7 extends the possibilities of exploring the molecular basis of its functions. In addition, CXCR7 and the classical receptors CXCR4 and CXCR3 in conjunction with their ligands CXCL12, CXCL11, CXCL10, CXCL9 and vCCL2, of which some are shared or display opposite activities, may be regarded as a model of the intricate network of chemokines and their receptors.

In this chapter, a study will be presented in which we took advantage of these interconnected interacting partners. Using a panel of truncated, mutated, dimeric and D-stereoisomer peptides derived from the N-terminal regions of chemokines we compared the importance of their various features for binding and activation of CXCR7, CXCR4 and CXCR3. We were able to provide new insights into the plasticity of the receptor ligand-binding pocket, the activation mechanism of conventional vs. atypical receptors and the stoichiometry of chemokine-receptor interactions.

1. Introduction

Chemokines are a superfamily of small (7-14kDa) pro-inflammatory chemo-attractant cytokines, which regulate vital cellular mechanisms including migration, and adhesion, as well as growth and survival [1]. They play critical roles in many physiological and pathological processes such as immune response and surveillance, development, atherosclerosis, HIV infection or cancer [2, 3]. Despite their poor sequence similarities, all chemokines display a common fold consisting of a flexible N terminus, followed by a conserved cysteine motif, an N-loop, three anti-parallel β-strands and a C-terminal α-helix [4, 5] (Fig. 1A). The biological effects of chemokines are mediated through specific interactions with chemokine receptors, which are seven transmembrane domain G protein-coupled receptors (GPCRs). To date, 47 chemokines and 19 chemokine receptors have been identified in humans [6, 7]. The chemokine-receptor network is highly complex and a given chemokine may bind to several receptors, while a chemokine receptor usually has multiple ligands. Based on the conserved cysteine motifs present in their N terminus, chemokines are divided into four subfamilies (C, CC, CXC, CX3C) and the receptors are named according to the subfamily of chemokines they bind (XCR, CCR, CXCR and CX3CR) [6]. In addition to classical receptors, four receptors referred to as atypical chemokine receptors (ACKR1-4) can act as scavengers, regulating chemokine availability, or signal through alternative pathways, further contributing to the complexity of the chemokine network [7, 8].

CXCR4 is one of the best-studied receptors for chemokines, often considered the model for CXC chemokine receptors as a whole [9-13]. It binds a unique endogenous agonist chemokine, CXCL12, as well as the human herpesvirus 8 (HHV-8)-encoded broad-spectrum antagonist chemokine, vCCL2 [13-15]. Both CXCL12 and vCCL2 are also agonists of CXCR7, the lastly deorphanised chemokine receptor [16, 17]. In addition, CXCR7 shares one ligand, the chemokine CXCL11, with CXCR3, which also binds CXCL10 and CXCL9, albeit with lower affinity than it binds to CXCL11 [17-19]. Unlike CXCR4 and CXCR3, which signal via the canonical G protein pathways, modulating cAMP production and inducing intracellular calcium release, CXCR7 was proposed to trigger arrestin-dependent signalling [13, 20, 21]. CXCR7 was also shown to act as a decoy receptor for CXCL12 and CXCL11, thus regulating their availability for CXCR4 and CXCR3 [22-25]. Because of its unconventional properties, CXCR7 has recently been

renamed ACKR3 and classified among the atypical chemokine receptors [7, 8]. However, so far, the molecular basis accounting for its atypical functions and signalling remain unclear.

Based in part on the large amount of data on CXCL12 and CXCR4, the interaction between chemokines and their receptors is generally described as a multi-step process with extensive contacts between the two partners and 1:1 stoichiometry [12, 26-29]. In the initial step of the interaction, the extracellular N terminus of the receptor (chemokine recognition site 1, CRS1) binds the core of the chemokine, allowing for optimal orientation with respect to the top of the ligand-binding pocket (CRS1.5) [26]. This first step enables the insertion of the flexible chemokine N terminus into the transmembrane cavity (CRS2), which stabilises an active state of the receptor in turn triggering intracellular signalling [9, 12, 26, 30, 31]. Although chemokine receptors can function as monomeric signalling units, direct evidence suggests that they are able to form homo- and heterodimers in a ligand-independent manner [10, 25, 32]. Such oligomeric states were also observed for chemokines, and possibly add another level of fine-tuning to the already highly intricate chemokine-receptor interplay [33, 34].

The N-terminal portion of chemokines is a key determinant of their activity and selectivity and harbours several features important for the chemokine-receptor interaction including the N-loop, the cysteine motif, and the flexible N terminus (Fig. 1A) [4, 5, 30]. In addition, about one third of CC and CXC chemokines present a proline in their proximal N terminus, usually at position 2, which has been proposed to play an essential role not only in receptor activation, but also in the regulation of chemokine availability through degradation by extracellular proteases [29, 35]. Peptides derived from the N terminus of CXCL12 and vCCL2 were shown to be sufficient to specifically bind to CXCR4, while conserving the agonist or antagonist activity of the parental chemokine [30, 36, 37]. Notably, the introduction of further modifications in these peptides such as mutations, dimerisation or D-amino acid replacement emerged as a useful tool to assess the importance of specific residues to receptor binding and activation, the propensity of the receptor to dimerise, or the plasticity of the CRS2 [38, 39]. Moreover, amino-terminal deletions or P2G mutation were demonstrated to convert peptides derived from the N terminus of CXCL12 to CXCR4 antagonists, illustrating their therapeutic potential [30, 31, 40, 41]. So far however, except for CXCR4, little is

known about the importance of these proximal features of chemokines in receptor recognition and activation, or the conservation of their contributions across chemokine-receptor pairs. Therefore, CXCR4, CXCR7 and CXCR3 in conjunction with their ligands CXCL12, CXCL11, CXCL10, CXCL9 and vCCL2, of which some are shared or display opposite activities, offer an interesting opportunity to investigate these questions.

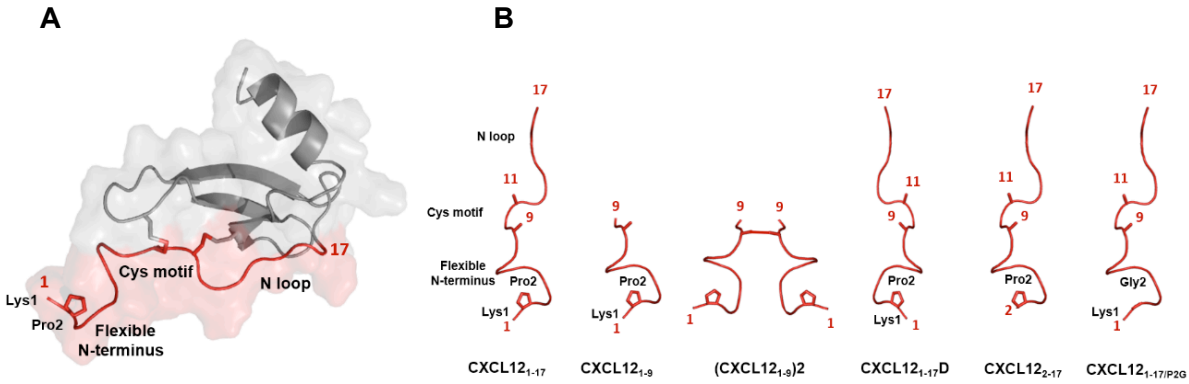

Fig. 1. Structure of the full-length chemokine CXCL12, location of the N-terminal features and representation of CXCL12 N-terminal peptides. **(A)** Fold and secondary structure of CXCL12. The N-terminal region (red) encompasses the flexible N terminus (residues 1-8), the CXC cysteine motif (residues 9-11) and the N-loop (residues 13-17). The core of the chemokine (grey) comprises the β-sheet, the connecting loops and the C-terminal α-helix. **(B)** Panel of CXCL12-derided peptides investigated in this study.

In this study, using peptides derived from the N terminus of chemokines (Fig. 1B), we compared the role of various chemokine N-terminal features in their interactions with CXCR3, CXCR4 and the atypical receptor CXCR7, providing insights into the plasticity of the ligand-binding pocket of the receptors, their activation mechanism and the stoichiometry of chemokine-receptor interactions.

2. Materials and methods

2.1 Cell lines and antibodies

U87 cells were obtained through the NIH AIDS program from Dr. Deng and Dr. Littman [42]. U87.CXCR3, U87.CXCR4 and U87.CXCR7 cell lines were obtained by Lipofectamine transfection (Life Technologies) of U87 cells with pBABE-puro vectors (Addgene) or pcDNA3.1-hygro (Invitrogen) encoding the different receptors, subsequent puromycin (1 or 0.5 µg/mL) or hygromycin selection (250 µg/mL) and single cell sorting. For each cell line, receptor surface expression was verified by flow

cytometry using mAbs specific for CXCR7 (clones 11G8 (R&D Systems) and 8F11 (BioLegend)), CXCR4 (clones 4G10 (Santa Cruz Biotechnology) and 12G5 (BD Biosciences)) and CXCR3 (clone 1C6 (BD Biosciences)).

2.2 Peptides and chemokines

All peptides were purchased from JPT and contain a free amine at the N terminus and an amide group at the C terminus to avoid additional negative charges. The sequences the chemokine N terminus-derived peptides are given in Table 1. Chemokines CXCL12 (SDF1α), CXCL11 (ITAC), vCCL2 (vMIP-II), CXCL10 (IP-10), CXCL9 (MIG) and CXCL4 (PF4) were purchased from PeproTech. Alexa Fluor 647-labelled CXCL12 (CXCL12-AF647) and CXCL11 (CXCL11-AF647) were purchased from Almac. Peptide and chemokine cytotoxicity was monitored using an amino-reactive cell viability dye (Life Technologies) as well as an ATP quantification-based cell viability assay (Promega).

2.3 Binding competition assays with labelled CXCL12

Binding of chemokines and peptides derived from chemokine N terminus to CXCR4 and CXCR7 expressed at the surface of U87 cells was evaluated by competition with Alexa Fluor 647 (AF-647)-labelled CXCL12. For CXCR7 binding, U87.CXCR7 cells were incubated with CXCL12-AF647 (40 ng/mL) and chemokines or peptides for 90 min at 4 °C. CXCR4 binding was evaluated in U87.CXCR4 cells by incubating CXCL12-AF647 (100 ng/mL) with chemokines or peptides for 45 minutes at 37 °C.

All binding experiments were performed in phosphate buffer saline containing 1 % BSA and 0.1% NaN_3 (FACS buffer). Nonspecific chemokine binding was evaluated by adding a 250-fold excess of unlabelled CXCL12 or CXCL11. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Canto or Fortessa cytometer (BD Biosciences).

2.4 cAMP modulation assay

U87 cells stably transfected with cAMP GloSensor 22F vector (Promega) (U87.Glo) were selected for hygromycin resistance (10 µg/mL) and tested for forskolin-induced luminescence response. U87.Glo cells were then transfected with pBABE or pIRES vectors encoding CXCR3, CXCR4 or CXCR7 and stably expressing cells were selected with puromycin (0.5 or 1 µg/mL) or hygromycin (250 µg/mL). Single clones were

isolated by cell sorting using the corresponding monoclonal antibodies and further validated by FACS. For cAMP modulation measurements, cells were incubated for 90 minutes in the dark at 37 °C in phenol red-free DMEM containing IBMX (500 μM) and 2% luciferin (GloSensor reagent, Promega). 15 x 10^4 cells per well were distributed into 96-well white LumitracTM plates (Greiner) containing chemokines or peptides at different concentrations in phenol red-free DMEM containing IBMX (500 μM) and 2% luciferin. Luminescence in forskolin-free conditions was recorded (40 measurements; 2 sec integration time) at different time points using POLARstar Omega.

2.5 Ligand-induced calcium mobilisation

Intracellular calcium mobilisation induced by chemokines or chemokine-derived peptides, was evaluated using a calcium-responsive fluorescent probe and a FLIPR system. U87.CXCR3, U87.CXCR4 and U87.CXCR7 cells were seeded in gelatine-coated black-wall 96-well plates at 20.000 cells per well and incubated for 12 hours. Cells were then loaded with the fluorescent calcium probe Fluo-2 acetoxymethyl (AM; TefLabs) or Fluo-3 AM (Molecular Probes) at a final concentration of 4 μM in assay buffer (Hanks' balanced salt solution (HBSS) with 20 mM HEPES buffer and 0.2% bovine serum albumin, pH 7.4) for 45 minutes at 37 °C. The intracellular calcium mobilisation induced by chemokines (2 to 1000 nM) or chemokine-derived peptides (200 nM to 100 μM) was then measured at 37 °C by monitoring the fluorescence as a function of time in all the wells simultaneously using a fluorometric imaging plate reader (FLIPR, Molecular Devices) as previously described [43].

2.6 Arrestin recruitment

Chemokines and peptides-induced β-arrestin-2 recruitment to CXCR3, CXCR4 and CXCR7 was monitored by reversible enzyme fragment complementation assay based on split firefly luciferase as previously described [44, 45]. HEK cells stably expressing β-arrestin-2 fused at its N terminus to the first 415 amino acids of firefly luciferase were transfected with a pIRES vector (Addgene) encoding the 413-549 amino acids of the firefly luciferase fused to the C terminus of CXCR3, CXCR4 or CXCR7. At 24 h post-transfection cells were seeded in 96-well plates at a density of 1.4 10^5 cells per well and incubated 10 min with serial dilutions of chemokines or peptides (concentration-response) or for different times with or without 100 nM chemokines or 100 μM peptides

(kinetic analysis). Luciferin (Synchem) was then added and luminescence was measured with a luminometer Centro XS3 LB 960. Experiments were carried out in duplicate.

2.7 Statistics

Concentration response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (Sigmaplot) to provide EC_{50} or IC_{50} values.

3. Results

3.1 Binding and activity of full-length chemokines towards CXCR4, CXCR3 and CXCR7

The binding and activity of full-length chemokines towards CXCR4, CXCR3 and CXCR7 were evaluated by competition studies with fluorescently labelled chemokines and various canonical G protein signalling or β-arrestin recruitment assays.

Full-length CXCL12 bound to CXCR4 with an IC_{50} of 20.2 ± 4.6 nM and acted as an agonist in both cAMP modulation and calcium mobilisation assays, with EC_{50} values of 0.22 ± 0.05 nM and 36.6 ± 3.8 nM, whereas vCCL2 bound to CXCR4 with a potency approximately 3 times lower (IC_{50} of 61.0 ± 9.7 nM) than CXCL12 and acted as an antagonist of CXCL12-induced signalling, with IC_{50} of 390.0 ± 70.0 nM in calcium release assay (Fig. 2A, Tables 1 and 2).

Strong non-specific binding of fluorescently labelled CXCL11, resistant to displacement by unlabelled chemokines, made it impossible to evaluate and compare CXCR3 interaction with full-length chemokines and peptides in binding competition studies. Nevertheless, in functional assays, CXCL11, CXCL10 and CXCL9 bound and activated CXCR3 expressed on U87 cells. CXCL11, CXCL10 and CXCL9 reduced cAMP production with EC_{50} values of 1.7 ± 0.3 nM, 8.3 ± 2.2 nM, and 128.5 ± 31.2 nM and induced intracellular calcium mobilisation with EC_{50} values of 27.4 ± 1.6 nM, 198.5 ± 1.8 nM and 760.5 ± 1.3 nM, respectively. No modulation of cAMP or calcium response was observed with vCCL2 in agonist or antagonist modes, confirming that CXCR3 is not a receptor for this viral chemokine (Table 2).

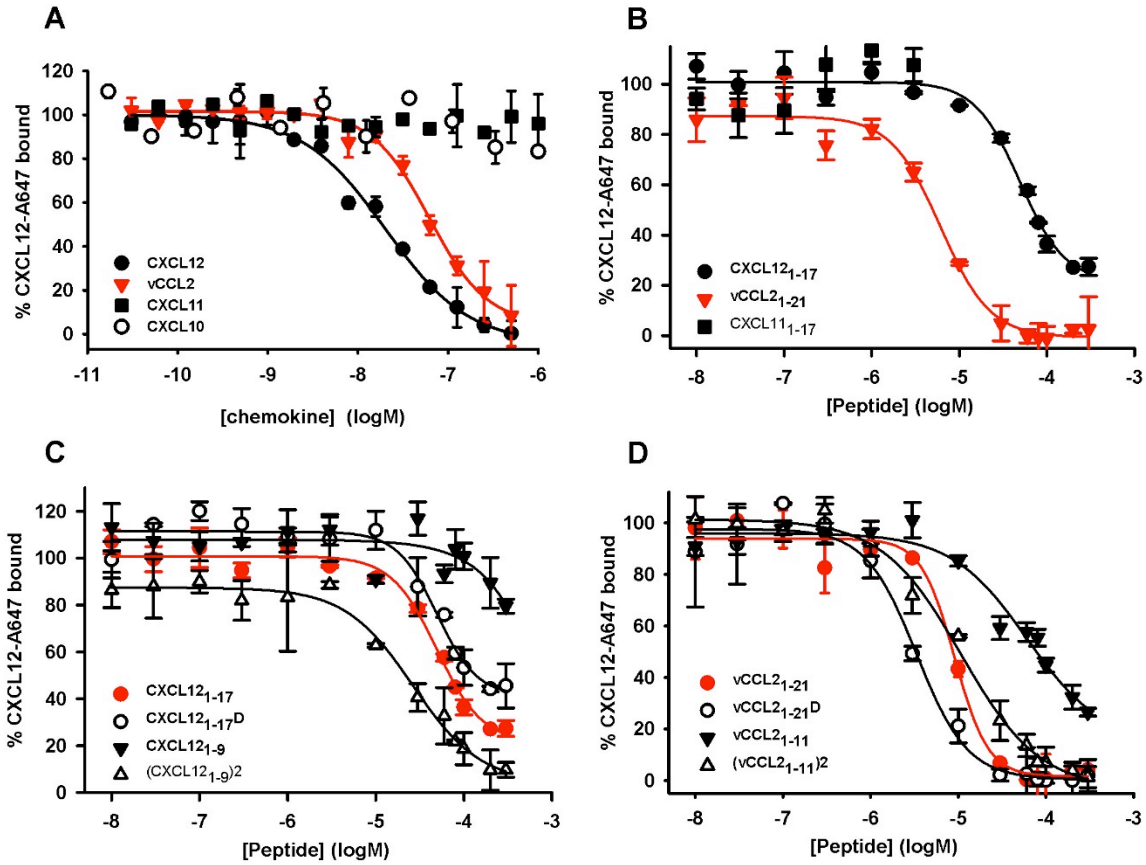

Fig. 2. Binding of full-length chemokines and chemokine N terminus-derived peptides to CXCR4. (A and B) Binding of full-length CXCL12, vCCL2, CXCL11, CXCL10 and peptides derived from their N termini to CXCR4, assessed by binding competition assay with fluorescently labelled CXCL12. **(C and D)** Binding of CXCL12 and vCCL2 N terminus-derived peptides to CXCR4. U87 cells stably expressing CXCR4 were incubated with increasing concentrations of peptides or non-labelled chemokines and Alexa Fluor 647-coupled CXCL12. The binding of CXCL12-AF647 was analysed by flow cytometry. Each experiment was performed in duplicates and the values correspond to average ± standard deviation. The sigmoidal curves were fitted to the data points using the four-parameter Hill equation.

CXCL12, CXCL11 and vCCL2 bound to CXCR7 with IC_{50} values of 1.3 ± 0.4 nM, 4.6 ± 0.4 nM and 32.5 ± 1.0 nM, respectively. No cAMP modulation or calcium release was detected in U87.CXCR7 cells in response to the three chemokines, confirming the inability of CXCR7 to signal via the canonical G protein pathways [20] (Fig. 3A, Tables 1 and 2).

The potency and efficacy of the chemokines in inducing arrestin recruitment to their cognate receptors was also evaluated and the kinetic profiles of each receptor were compared.

Arrestin recruitment to CXCR4 was only induced by CXCL12 with an EC_{50} of 9.3 ± 2.5 nM (Fig. 4A, Table 3). CXCL11 acted as a full agonist towards CXCR3, with an EC_{50} of 4.3 ± 0.8 nM, whereas CXCL10 and CXCL9 were less efficient inducing, at the highest concentration tested (1 μM) about 90 and only 15 % of the maximum response observed with CXCL11. CXCL10 also showed lower potency as compared to CXCL11, with an EC_{50} of 25.5 ± 6.3 nM (Fig. 4B, Table 3).

CXCL12, CXCL11 and vCCL2 all induced arrestin recruitment to CXCR7 (EC_{50} of 2.3 ± 0.7 nM, 14.3 ± 1.6 nM and 27.8 ± 3.9 nM), with CXCL11 and vCCL2 showing reduced potency and slightly lower efficacy compared to CXCL12 (Fig. 4C, Table 3).

The kinetic analysis of arrestin recruitment revealed somewhat different profiles for CXCR4 and CXCR3 compared to CXCR7. Arrestin recruitment to CXCR3 and CXCR4 was more rapid and sustained, with the maximum response reached after 30 minutes of stimulation followed by a modest and delayed decrease, whereas CXCR7 activation led to a more progressive recruitment of arrestin, with the maximum reached only after 60 minutes, followed by a rapid decrease of about 20 % of the signal (Fig. 4D-F).

3.2 Binding and activity of chemokine N-terminal peptides towards CXCR4, CXCR3 and CXCR7

Next, the ability of synthetic peptides derived from CXCL12, CXCL11, CXCL10, CXCL9 and vCCL2 to interact with CXCR4, CXCR3 and CXCR7 was evaluated in the same binding and functional assays and compared to that of parental chemokines.

First, peptides comprising the flexible N terminus, the cysteine motif and the N-loop of the chemokines ($CXCL12_{1-17}$, $CXCL11_{1-17}$, $CXCL10_{1-17}$, $CXCL9_{1-17}$ and $vCCL2_{1-21}$) were tested (Fig. 1B).

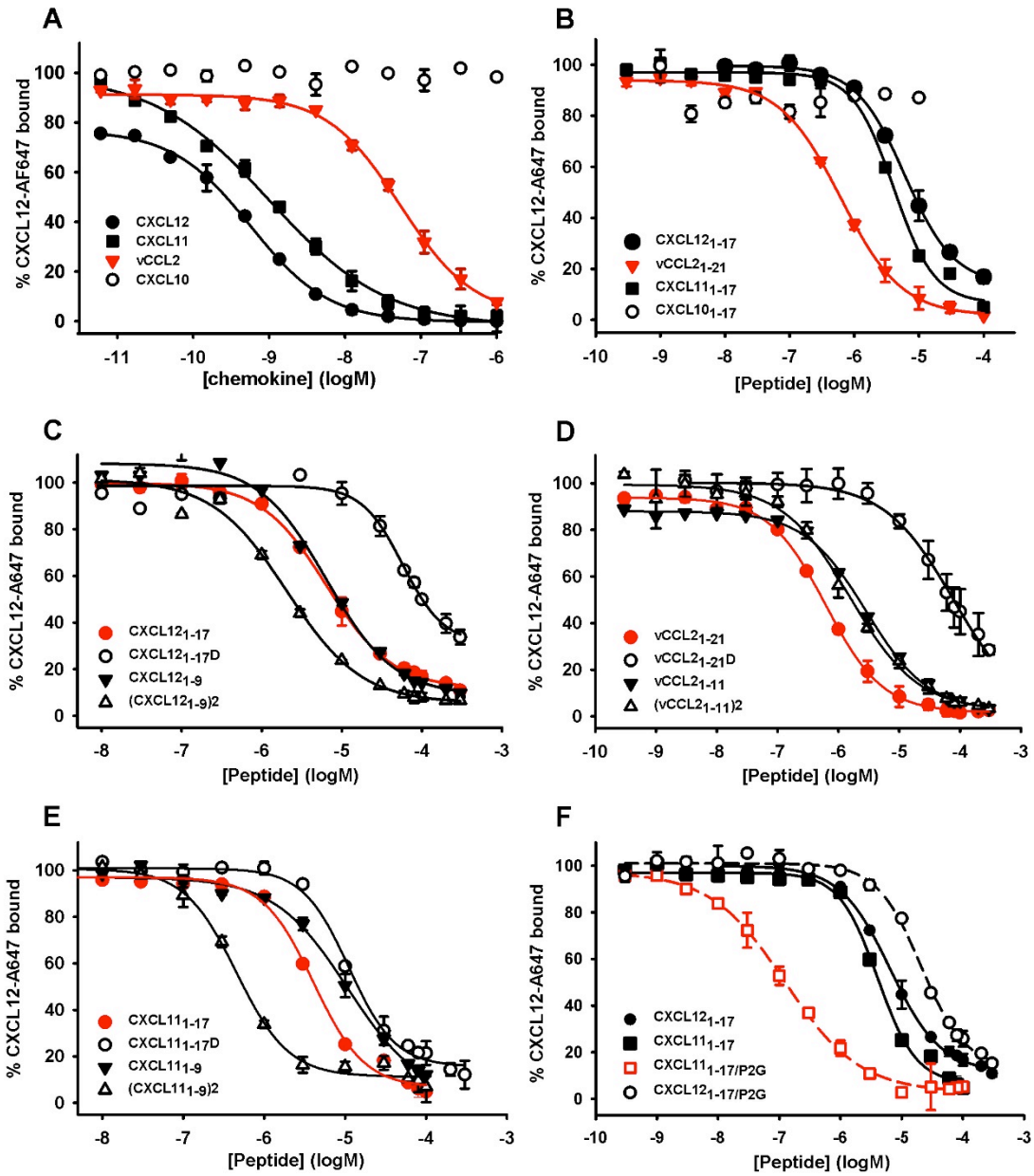

Fig. 3. Binding of full-length chemokines and chemokine N terminus-derived peptides to CXCR7. (A and B) Binding of full-length CXCL12, CXCL11, vCCL2, CXCL10 and peptides derived from their N terminus to CXCR7 assessed by binding competition assay with fluorescently labelled CXCL12. **(C and D**) Binding of CXCL12 and vCCL2 N terminus-derived peptides to CXCR7. **(E and F)** Binding of CXCL11 N terminus derived peptides to CXCR7 and comparison of the impact of P2G mutation in CXCL12 and CXCL11 derived peptides on binding to CXCR7. U87 cells stably expressing CXCR7 were incubated with increasing concentrations of peptides or non-labelled chemokines and Alexa Fluor 647-coupled CXCL12. The binding of CXCL12-AF647 was analysed by flow cytometry. Each experiment was performed in duplicates and the values correspond to average ± standard deviation. The sigmoidal curves were fitted to the data points using the four-parameter Hill equation.

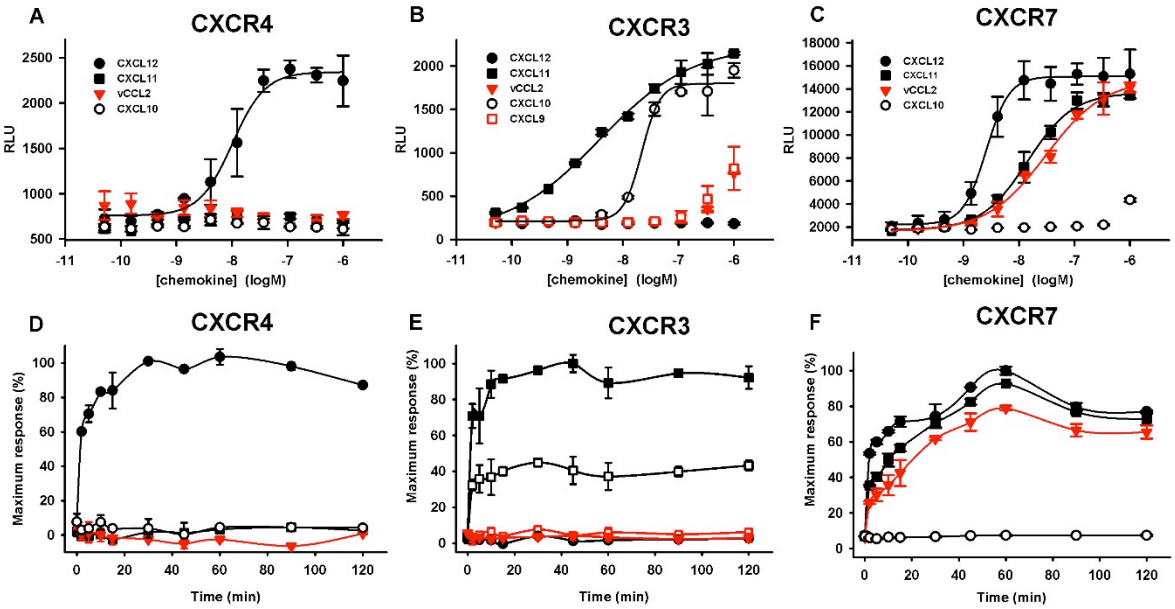

Fig. 4. β-arrestin-2 recruitment to CXCR4, CXCR3 and CXCR7 induced by full-length chemokines. (A to C) Comparison of β-arrestin-2 recruitment induced by different concentrations of full length CXCL12, CXCL11, CXCL10, CXCL9 and vCCL2 to **(A)** CXCR4, **(B)** CXCR3 and **(C)** CXCR7. **(D to F)** Comparison of kinetic profile of β-arrestin-2 recruitment to **(D)** CXCR4, **(E)** CXCR3 and **(F)** CXCR7, induced by the different chemokines (100 nM) at room temperature and monitored at different time points by firefly luciferase complementation assay. Each experiment was performed in duplicates and the values correspond to average ± standard deviation.

In CXCR4 binding assay, peptides $CXCL12_{1-17}$ and $vCCL2_{1-21}$ showed about 3000- and 100-fold decrease in potency compared with the full-length chemokines. Peptide $vCCL2_{1-21}$ was about 10 times more potent (IC_{50} = 7.4 ± 2.7 µM) in binding to CXCR4 than peptide $CXCL12_{1-17}$ (IC_{50} of 59.7 ± 6.4 µM), which reverses the relative order of potencies observed with their parental chemokines (Fig. 2A and B, Table 1). In functional assays, CXCL12- and vCCL2-derived peptides retained the activity of the parental chemokines, with only $CXCL12_{1-17}$ inducing a decrease in cAMP levels (EC_{50} = 21.1 ± 4.3 µM) and intracellular calcium release (EC_{50} = 16.4 ± 4.7 µM) and $vCCL2_{1-21}$ acting as an antagonist of CXCL12-induced calcium release. Surprisingly, although its capacity to trigger G protein signalling was maintained, the ability of $CXCL12_{1-17}$ to induce arrestin recruitment to CXCR4 was greatly reduced and, at the highest concentration tested (100 µM), reached only 20 % of the maximum signal observed with full-length CXCL12 (Fig. 5A, Tables 2 and 3).

Similar behaviours were observed with peptides derived from CXCL11, CXCL10 and CXCL9 towards CXCR3. Among the three peptides, only $CXCL11_{1-17}$ retained the ability to induce G protein signalling (EC_{50} = 18.2 ± 1.2 µM), albeit with a 700-fold reduction of

potency compared with the full-length CXCL11 (EC_{50} = 27.4 ± 1.6 nM). Unfortunately, the characterisation of the activity of $CXCL11_{1-17}$ at high concentrations (above 30 μM) was impossible due to peptide cytotoxicity. Peptides derived from CXCL10 and CXCL9 were not able to induce G protein signalling, which most probably originates from the already low affinities and potencies of their parental chemokines compared to CXCL11. At a concentration of 30 μM, $CXCL11_{1-17}$ induced only 20 % of the maximum signal induced by the parental chemokine (Fig. 5C, Tables 2 and 3).

In CXCR7 binding assay, the order of potencies of the three peptides derived from CXCL12, CXCL11 and vCCL2 was different compared to what we observed with their parental chemokines. $CXCL12_{1-17}$ and $CXCL11_{1-17}$ showed equivalent IC_{50} values of 6.4 ± 0.5 μM and 4 ± 1 μM corresponding to a 5000- and a 1000-fold loss of potency compared to their parental chemokines. This reduced potency was similar to what we observed for these peptides on CXCR4 and CXCR3, respectively. Peptide $vCCL2_{1-21}$ was considerably more potent in binding CXCR7 (IC_{50} = 0.6 ± 0.1 μM) than peptides derived from the endogenous chemokines (IC_{50} = 32.5 ± 1.0 nM), with only a 20-fold reduction compared to the full-length vCCL2. In contrast to what we observed with CXCR4 and CXCR3, peptides $CXCL12_{1-17,}$ $CXCL11_{1-17}$ and $vCCL2_{1-21}$ were all highly potent in inducing arrestin recruitment to CXCR7, with EC_{50} values of 0.9 ± 0.1 μM, 0.9 ± 2 μM and 0.8 ± 0.1 μM and maximum efficacies comparable to their parental chemokines (Fig. 4B and 5E, Table 3).

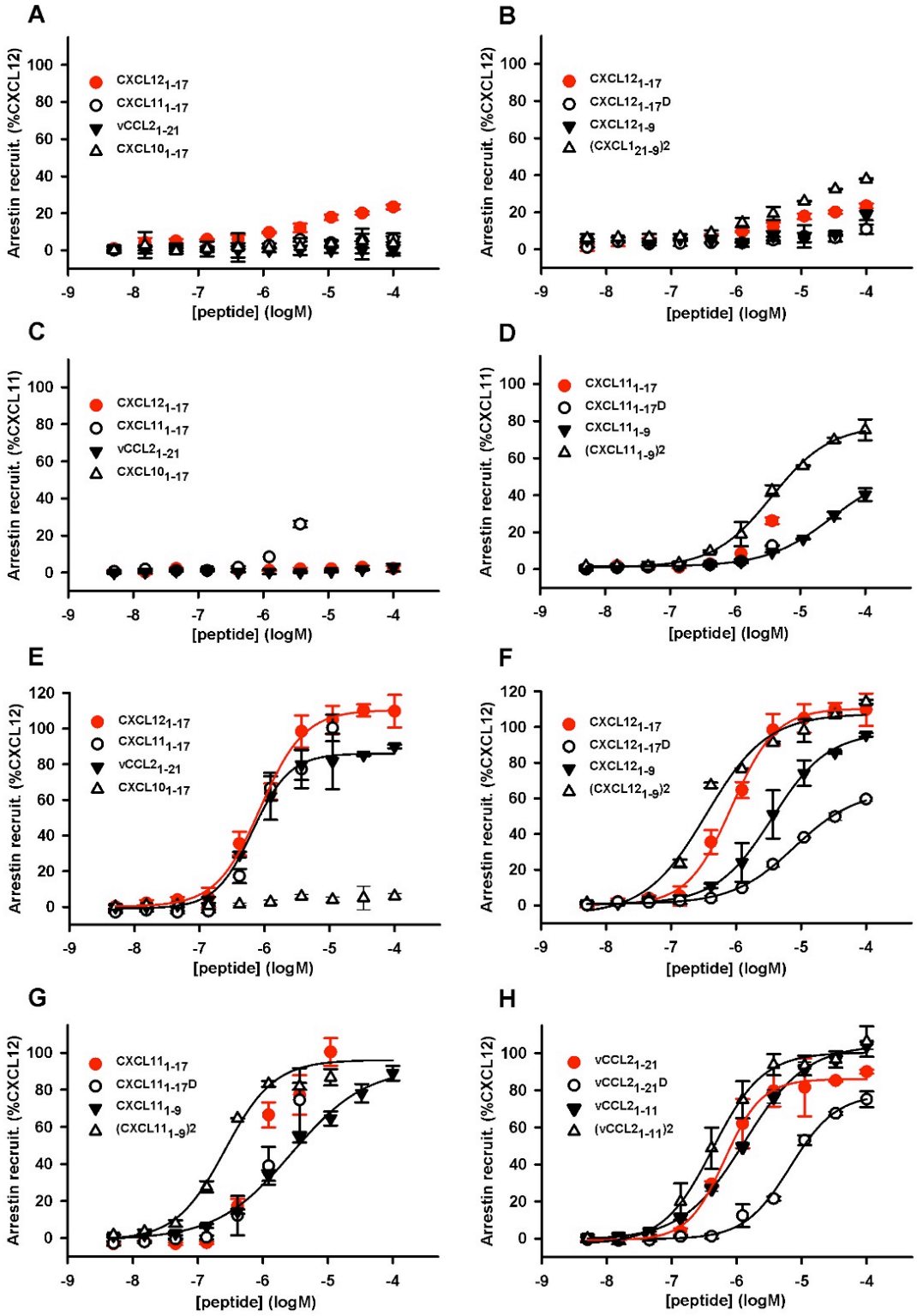

Fig. 5. β-arrestin-2 recruitment to CXCR4, CXCR3 and CXCR7 induced by chemokine N-terminal peptides. (A to B) Comparison of β-arrestin-2 recruitment to CXCR4 induced by CXCL12-, vCCL2-, CXCL11- and CXCL10-derived peptides. **(C to D)** Comparison of β-arrestin-2 recruitment to CXCR3 induced by CXCL12-, vCCL2-, CXCL11- and CXCL10-derived peptides. **(E to H)** Comparison of β-arrestin-2 recruitment to CXCR7 induced by CXCL12-, vCCL2-, CXCL11- and CXCL10-derived peptides. Each experiment was performed in duplicates and the values correspond to average ± standard deviation.

3.3 Impact of N-loop and cysteine motif truncation

Next, the binding and activity of truncated peptides devoid of the N-loop and the cysteine motif and comprising only the flexible N terminus of chemokines CXCL12, CXCL11 and vCCL2 were investigated ($CXCL12_{1-9}$, $CXCL11_{1-9}$, $vCCL2_{1-11}$) (Fig. 1B).

Truncation of $CXCL12_{1-17}$ after the first cysteine ($CXCL12_{1-9}$) resulted in a drastic loss of binding to CXCR4 (IC_{50} >100 μM) and calcium mobilisation but put the peptide ability to modulate cAMP production was conserved (EC_{50} = 23.4 ± 2.3 μM). Similar truncation of $vCCL2_{1-21}$ to peptide $vCCL2_{1-11}$ resulted in an 8-fold reduction in CXCR4 binding (EC_{50} = 61.3 ± 0.7 μM) but the peptide retained its ability to compete with labelled chemokine, which is consistent with the higher affinity of peptide $vCCL2_{1-21}$ compared to $CXCL12_{1-17}$ (Fig. 2C and D, Tables 1 and 2).

$CXCL11_{1-9}$ showed no toxicity even at concentration as high as 100 μM and maintained its ability to induce calcium mobilisation through CXCR3 (EC_{50} = 17.2 ± 7.5 μM) with potency equivalent to that of peptide $CXCL11_{1-17}$ (EC_{50} = 18.2 ± 1.2 μM) (Table 2).

$CXCL12_{1-9}$ and $CXCL11_{1-9}$ showed similar binding potencies to CXCR7 (IC_{50} = 10.8 ± 1.9 μM and 10.7 ± 1.7 μM) as $CXCL12_{1-17}$ and $CXCL11_{1-17}$, suggesting that the first nine residues support a large part of the binding of CXCL12 and CXCL11 N termini to the receptor. In line with this assumption, a peptide in which the residues following the first cysteine where permutated randomly ($CXCL12_{1-9}scrbld_{10-17}$) showed an IC_{50} of 7 ± 1 μM (data not shown), comparable with that of $CXCL12_{1-17}$ and $CXCL12_{1-9}$. For vCCL2, truncation to peptide $vCCL2_{1-11}$ resulted in a 5-fold decrease in affinity for CXCR7 (IC_{50} = 4.2 ± 0.4 μM) compared with $vCCL2_{1-21}$ (IC_{50} = 0.6 ± 0.1 μM) indicating a higher contribution of the N-loop and cysteine motif in vCCL2 binding to CXCR7 than in CXCL12 and CXCL11 (Fig. 3C-E, Table 1). Truncation resulted in a 4-fold decrease of potency in arrestin recruitment of $CXCL12_{1-9}$ and $CXCL11_{1-9}$ but only a 2-fold decrease in potency of peptide $vCCL2_{1-11}$ (Fig. 5F-H, Table 3).

3.4 Impact of chemokine N terminus dimerisation

Dimerisation of the C-truncated peptides ($(CXCL12_{1-9})_2$, $(CXCL11_{1-9})_2$ and $(vCCL2_{1-11})_2$) via a disulphide bridge between the terminal cysteine residues had a varying impact on the different peptide-receptor pairs (Fig. 1B).

For CXCR4, dimerisation of $CXCL12_{1-9}$ and $vCCL2_{1-11}$ substantially enhanced their binding and signalling potencies, as shown by the average 10-fold improvement observed in binding competition and G protein signalling assays. Similar results were observed for CXCR3 and $(CXCL11_{1-9})_2$ with a 3 and 10 times higher potencies in calcium release and cAMP modulation assay, respectively (Fig. 2C and D, Tables 1 and 2).

For CXCR7, binding and arrestin recruitment activities were different for CXCL12 and CXCL11 than for vCCL2 dimeric peptides. $(CXCL12_{1-9})_2$ and $(CXCL11_{1-9})_2$ both showed a 10-fold improvement of their binding and arrestin recruitment properties compared with their monomeric counterparts, whereas for vCCL2-derived peptide dimerisation led to only a 2-fold improvement, suggesting that the binding mode of the N-terminal region is slightly different in vCCL2 than in CXCL12 and CXCL11 (Fig. 3C-E and 5F-H, Tables 1 and 3).

3.5 Impact of D-stereoisomer replacement

The binding and activity of the N-terminal peptides in which each amino acid was replaced by the corresponding D-stereoisomer ($CXCL12_{1-17}$D, $CXCL11_{1-17}$D and $vCCL2_{1-21}$D) (Fig. 1B) towards the three receptors were also tested in order to evaluate the plasticity of the ligand-binding pocket and their tolerance to such modifications on the functional level.

D-amino acid replacement had no effect on the binding of vCCL2- and CXCL12-derived peptide to CXCR4. D-isomer replacement nevertheless turned $CXCL12_{1-17}$ into a CXCR4 antagonist, while the initial antagonist activity of $vCCL2_{1-21}$ was conserved (Fig. 2C and D, Tables 1 and 2). Similar effect was observed for CXCR3, as $CXCL11_{1-17}$D conserved the receptor binding properties but acted as an antagonist (Tables 1 and 2).

In contrast, D-isomer replacement significantly reduced the ability of peptides derived from vCCL2 and CXCL12 to bind CXCR7, with the most marked, over 100-fold, increase of IC_{50} for $vCCL2_{1-21}$D compared with the L-stereoisomer and a 10-fold increase for peptide $CXCL12_{1-17}$D. For CXCL11-derived peptide the decrease of potency was less marked with only a 3-fold reduction (Fig. 3C-E, Table 1). Remarkably, all three D-stereoisomer peptides conserved the parental agonist activity towards CXCR7, inducing arrestin recruitment, which contrasts with their inability to activate CXCR4 and CXCR3 (Fig. 5F-H, Table 3).

3.6 Impact of amino-terminal truncation and P2G mutation

The amino-terminal lysine and the proline at position 2 in CXCL12 have been shown to be critical for CXCR4 activation [31]. As this proline is conserved in CXCL11, N-terminal truncation and P2G mutation were introduced in peptides derived from both CXCL12 and CXCL11 (Fig. 1B) and their binding and activity towards the three receptors were evaluated and compared.

For CXCR4, the amino-terminal truncation and P2G mutation in peptide $CXCL12_{1\text{-}17}$ turned it into an antagonist but with no or little effect on the binding potency. Similar conversion from agonist to CXCR3 antagonist was observed with $CXCL11_{2\text{-}17}$ lacking the amino-terminal phenylalanine and $CXCL11_{1\text{-}17/P2G}$ bearing the P2G mutation (Tables 1 and 2).

The binding of $CXCL12_{2\text{-}17}$ and $CXCL12_{1\text{-}17/P2G}$ to CXCR7 was affected with a 3-fold increase in potency for peptide $CXCL12_{2\text{-}17}$ and 3-fold decrease for peptide $CXCL12_{1\text{-}17/P2G}$. Interestingly, truncation of the first residue of CXCL12 and CXCL11 peptides (Lys1 and Phe1, respectively) had opposite effects on their binding to CXCR7, with a 2-fold decrease of potency for $CXCL11_{2\text{-}17}$. The effect of P2G mutation on CXCL12- and CXCL11-derived peptides was reversed compared to the truncation of the first residue. While $CXCL12_{1\text{-}17/P2G}$ was 3 times less potent (IC_{50} = 20.8 ± 2.0 μM), the same substitution in CXCL11 peptide resulted in a surprising 30-fold increase of its binding potency and an IC_{50} in the nanomolar range (IC_{50} = 150 ± 5.0 nM). More remarkably, in contrast to what we observed for CXCR4 and CXCR3, all four modified peptides retained their agonist activity, with potencies reflecting their binding to CXCR7 (Fig. 3F, Tables 1 and 3).

Finally, the possibility to turn vCCL2-derived peptide from CXCR4 antagonist to agonist was investigated by exchanging its amino terminal residues with those of CXCL12 ($vCCL2_{1\text{-}21/G2P}$ and $vCCL2_{1\text{-}21/L1K\text{-}G2P}$). Both peptides showed slightly reduced CXCR4-binding potency with G2P mutation having the most negative effect. However, none of these peptides was able to induce CXCR4 G protein signalling, suggesting that either CXCL12 and vCCL2 occupy the ligand-binding pocket of CXCR4 in different ways or that other residues and interactions are required [26] (Fig. Tables 1 and 2).

4. Discussion

Chemokine receptors represent a fascinating subfamily of G protein-coupled receptors. Their chemokine ligands are large peptides that show little sequence identity but high degree of structural homology. Chemokines and their receptors form an intricate interaction network characterised by high promiscuity and multiple apparent redundancies [46]. The exact structural and functional bases of chemokine receptor selectivity and activation remain elusive. Chemokines play crucial roles in various vital processes and their activity needs therefore to be finely regulated at the molecular and functional levels to avoid exacerbated or inappropriate receptor activation. Chemokine availability for their cognate receptors is regulated by different means, including glycosaminoglycan (GAG) binding, oligomerisation and proteolysis but also through their binding to atypical chemokine receptors (ACKRs) that can act as scavenger receptors by internalising the chemokines [8, 22, 23, 34, 35, 47].

At the receptor level, the selectivity towards chemokines is dictated by the presence of multiple motifs and determinants located at their extracellular surface and within their transmembrane segments, which create a network of specific interactions ensuring the structural complementarity to chemokines, and molecular switches for activation [26, 48, 49]. Recent structural and mechanistic breakthroughs have demonstrated that chemokines make extensive contacts with their receptors in a 1:1 stoichiometry through at least three major recognition sites [12, 26-28, 48, 49]. However, in the absence of comparative structural data, the roles of the conserved features present in chemokines and especially in their N terminus remain obscure. Although a growing body of evidences indicates that the generally accepted two-site/two-step binding mode proposed for chemokine and their receptors is probably oversimplified, chemokine N termini still appear to be critical interacting elements, as their truncation or modification drastically impact the chemokine affinity and activity [26, 31]. However, most of the information available originates from studies conducted almost exclusively on the interactions between CXCR4 and CXCL12 or vCCL2, limiting our overall understanding of these processes for other chemokine-receptor pairs.

In this study, we examined and compared the importance of different features present in the N terminus of CXCL12, CXCL11 and vCCL2 and their impact on the binding and activation of a trio of receptors, including two conventional receptors, CXCR4 and

CXCR3, and the atypical chemokine receptor CXCR7. Using this subset of interconnected receptors, we also investigated how widespread the binding capacity of D-stereoisomers and the improved binding of dimeric ligands was, these properties being previously commonly recognised for CXCR4 [38, 39].

The binding and activity of peptides derived from CXCL12 and vCCL2 re-examined in this study were consistent with the previous reports [30, 31, 36, 37, 39-41] . The analysis of a larger set of modifications of chemokine-derived peptides, not only on CXCR4 but also on CXCR3 and CXCR7, provided new insights into the importance of different chemokine N-terminal features for receptor binding and activation. Noteworthy, this study showed that the use of peptides derived from chemokine N terminus to probe the ligand-binding pocket and to evaluate the importance of specific modifications on binding and signalling is not restricted to CXCR4 and can be extended to other receptors of the CXC and ACKRs families.

The impact of the modifications introduced in chemokine N terminus-derived peptides appears to be conserved between CXCR4 and CXCR3. Notably, truncations of the N-terminal residue and of the N-loop, P2G mutation, D-stereoisomer replacement as well as peptide dimerisation had similar effects on these two receptors, suggesting that the binding mode and activation mechanisms by their respective chemokine are comparable. CXCR3 was also permissive to D-amino replacement and dimeric peptides derived from CXCL11 were also more potent than the monomeric counterparts. The latter indicates that CXCR3 could form homodimers, possibly through TM5 and TM6 dimer interface, as described for CXCR4, bringing the two chemokine N terminus-binding pockets (CRS2) at close proximity [9, 10]. Furthermore, the loss of activity of peptides bearing simple modifications such as amino-terminal residue deletion, P2G mutation or D-amino acid replacement at both CXCR3 and CXCR4 confirmed the crucial role of the proximal N-terminal region of chemokines and the stringency of the contacts at CRS2 required for CXC receptors activation.

For CXCR7, which is not coupled to G proteins but instead exclusively recruits arrestin for its functions, the impact of peptide modifications was surprisingly different. Although some modifications introduced in peptides derived from the three chemokines affected the binding, none of them resulted in a loss of CXCR7 activation. Indeed, amino-terminal truncation, P2G mutation and even a complete D-amino acid

replacement in CXCL12- and CXCL11-derived peptides, which were all shown to convert the peptides from CXCR4 or CXCR3 agonists to antagonists, did not change their activity towards CXCR7. This suggests that the molecular interaction network within the binding pocket required for CXCR7 activation is different and less stringent than for CXCR4 and CXCR3 or that the continuum of active conformations is larger in CXCR7 than in CXCR4 and CXCR3 [50]. Such assumption is also reinforced by previous observations showing that CXCR4 antagonists such as AMD3100, TC14012 and vCCL2 all act as agonists towards CXCR7 [51, 52]. Recent screening of CXCL12 variants with randomised N terminus by phage display also led to the selection of CXCL12 variants all displaying CXCR7 agonist properties [53].

The higher permissivity of CXCR7 to ligand modifications, which at first sight may appear surprising, may nevertheless be required for its scavenging function. Indeed, in addition to scavenging native chemokines to limit their agonist activity on CXCR4 and CXCR3, our results suggest that CXCR7 may also bind and scavenge N terminus-modified chemokine species, such as those resulting form the action of proteases, including dipeptidyl peptidase IV (DPPIV or CD26) [54]. The higher propensity of CXCR7 to activation may be linked to its arrestin-biased activity and continuous cycling, possibly relying on a different activation mechanism than required for receptors coupled to G protein [50]. Recent structural studies uncovered the important role of interactions with specific structural determinants, the so-called micro-switches located in the major and minor subpockets of receptors [48, 55, 56] but also distinct helical movements of TM5 and TM6 or TM7 for G protein coupling or arrestin signalling, respectively [48, 57, 58]. Therefore, different subpocket occupancy or structural changes may be required for CXCR7 activation compared to CXCR4 and CXCR3, with the proximal region of chemokines playing a less important role. Alternatively, CXCR7 activation may depend on a less sophisticated mechanism, involving a simple, highly accessible molecular switch, leading to the observed “agonism bias”. These potential mechanisms of CXCR7 activation remain, nevertheless, purely speculative and structure-guided comparative mutational studies on CXCR4 and CXCR7 would now be necessary to gain more insight into the determinants involved in receptor interactions with its ligands.

Similarly to CXCR4 and CXCR3, dimeric peptides derived from the flexible N terminus of CXCL12 and CXCL11 were shown to bind with more affinity to CXCR7 in our system and induce arrestin recruitment to the receptor with an increased potency, suggesting that CXCR7 may also form homodimers. This observation is consistent with previous BRET analysis data [25, 51]. However, the lower potency of vCCL2-derived dimeric peptides in CXCR7 binding, which contrasts with what we observed for CXCR4, suggests that vCCL2 N terminus may occupy the subpockets differently or bind deeper in the TM region of CXCR7 than the N termini of CXCL12 and CXCL11.

Although our results indicate that the CXCR7 binding modes are similar for CXCL12 and CXCL11, some modifications in their N-terminal peptides had different effect on receptor interactions. Such differences may partly be explained by the poor sequence identity between CXCL12 and CXCL11 N termini, with only four identical residues including the proline at positon 2 and the two cysteine of the cysteine motif, leading to distinct contacts within the CXCR7 ligand-binding pocket. Interestingly, although CXCL12 and CXCL11 display a higher affinity for CXCR7 than vCCL2, the peptide derived from the N terminus of vCCL2 retained a higher proportion of the parental chemokine binding capacity than the endogenous chemokines. Similar observation was made for CXCR4 and may be related to the ability of vCCL2 to bind a broad spectrum of receptors of all the four classes (XC, CC, CXC and CX3C), implying that its core may have evolved less tight, and promiscuous binding capacity while the N terminus plays a more important role in specific binding and activity modulation [15, 41].

These data demonstrate that besides distinct functional roles, CXCR7 also presents clearly different activation mechanism than CXCR4 and CXCR3 with which it shares chemokines, giving ground for its recent classification as an ACKR [8]. However, whether the observation reported in this study is a hallmark of all ACKRs remains to be investigated. Furthermore, besides pure mechanistic considerations, the bias of CXCR7 towards agonism has also important consequences for the quest for efficient and specific CXCR7 antagonists. So far, no small molecule antagonist targeting the CXCR7 orthosteric site without inducing arrestin recruitment has been described, illustrating the importance to understand the interactions and mechanism underlying its activation.

Table 1. Binding properties of full-length chemokines and peptides derived from their N terminus towards CXCR4 and CXCR7.

Name	Sequence	Binding competition	
		CXCR4 IC_{50} (μM)	CXCR7 IC_{50} (μM)
CXCL12		**20.2 ± 4.6 nM**	**1.3 ± 0.4 nM**
$CXCL12_{1-17}$	KPVSLSYR**C**P**C**RFFESH	59.7 ± 6.4	6.4 ± 0.5
$CXCL12_{1-9}$	KPVSLSYR**S**	> 100	10.8 ± 1.9
$(CXCL12_{1-9})2$	$(KPVSLSYR\mathbf{C})_2$	26.2 ± 3.8	1.3 ± 0.1
$CXCL12_{1-17}$D	*KPVSLSYR**C**P**C**RFFESH*	54 ± 12	57.6 ± 15.2
$CXCL12_{2-17}$	**-**PVSLSYR**C**P**C**RFFESH	59.7.2 ± 2.4	1.9 ± 0.3
$CXCL12_{1-17/P2G}$	K**G**VSLSYR**C**P**C**RFFESH	41.5 ± 13.18	20.8 ± 2.0
vCCL2		**61.7 ± 9.7 nM**	**32.5 ± 1.0 nM**
$vCCL2_{1-21}$	LGASWHRPDK**CC**LGYQKRPLP	7.4 ± 2.7	0.62 ± 0.02
$vCCL2_{1-11}$	LGASWHRPDK**S**	61.3 ± 0.7	4.2 ± 0.4
$(vCCL2_{1-11})2$	$(LGASWHRPDK\mathbf{C})_2$	10.8 ± 1.7	2.3 ± 0.1
$vCCL2_{1-21\ D}$	*LGASWHRPDK**CC**LGYQKRPLP*	4.2 ± 1.2	77.6 ± 4.8
$vCCL2_{1-21/G2P}$	L**P**ASWHRPDK**CC**LGYQKRPLP	28.5 ± 5.9	24.2 ± 2.8
$vCCL2_{1-21/L1K-G2}$	**KP**ASWHRPDK**CC**LGYQKRPLP	15.0 ± 3.3	20.6 ± 6.2
CXCL11		**> 1000 nM**	**4.6 ± 0.4 nM**
$CXCL11_{1-17}$	FPMFKRGR**C**L**C**IGPGVK	> 30	4.1 ± 0.4*
$CXCL11_{1-9}$	FPMFKRGR**S**	-	10.7 ± 1.7
$(CXCL11_{1-9})2$	$(FPMFKRGR\mathbf{C})_2$	-	0.46 ± 0.04
$CXCL11_{1-17}$D	*FPMFKRGR**C**L**C**IGPGVK*	-	11.1 ± 0.9*
$CXCL11_{2-17}$	**-**PMFKRGR**C**L**C**IGPGVK	-	8.2 ± 1.8*
$CXCL11_{1-17/P2G}$	F**G**MFKRGR**C**L**C**IGPGVK	-	0.15 ± 0.05*
CXCL10		**> 1000 nM**	**> 1000 nM**
$CXCL10_{1-17}$	VPLSRTVR**C**T**C**ISISNQ	> 100	> 100

* Highest concentration tested 30 uM

Table 2. G protein signalling properties of full-length chemokines and peptides derived from their N terminus towards CXCR4 and CXCR3.

Name	Sequence	cAMP		Calcium	
		CXCR4 $EC_{50}/(IC_{50})$ (μM)	CXCR3 $EC_{50}/(IC_{50})$ (μM)	CXCR4 $EC_{50}/(IC_{50})$ (μM)	CXCR3 $EC_{50}/(IC_{50})$ (μM)
CXCL12		**0.2 ± 0.1 nM**	**>1000 nM**	**36.6 ± 3.8**	**>1000 nM**
$CXCL12_{1-17}$	KPVSLSYR**C**P**C**RFFESH	21.1 ± 4.3	>100	16.4 ± 4.7	>100
$CXCL12_{1-9}$	KPVSLSYR***S***	23.4 ± 2.3	ND	>100	ND
$(CXCL12_{1-9})2$	$(KPVSLSYR\mathbf{C})_2$	1.8 ± 0.1	ND	ND	ND
$CXCL12_{1-17}D$	*KPVSLSYR**C**P**C**RFFESH*	(TBD)	ND	(>100)	ND
$CXCL12_{2-17}$	**-**PVSLSYR**C**P**C**RFFESH	(TBD)	ND	(>100)	ND
$CXCL12_{1-17/P2G}$	K**G**VSLSYR**C**P**C**RFFESH	(TBD)	ND	(>100)	ND
vCCL2		**(TBD)**	**>1000 nM**	**(390 ± 70 nM)**	**>1000 nM**
$vCCL2_{1-21}$	LGASWHRPDKCCLGYQKRPLP	(TBD)	>100	(6.1 ± 0.7)	>100
$vCCL2_{1-11}$	LGASWHRPDK***S***	(TBD)	ND	>100	ND
$(vCCL2_{1-11})2$	$(LGASWHRPDK\mathbf{C})_2$	(TBD)	ND	ND	ND
$vCCL2_{1-21}D$	*LGASWHRPDK**CC**LGYQKRPLP*	(TBD)	ND	(6.3 ± 0.0)	ND
$vCCL2_{1-21/G2P}$	L**P**ASWHRPDK**CC**LGYQKRPLP	(TBD)	ND	(>100)	ND
$vCCL2_{1-21/L1K-G2P}$	**KP**ASWHRPDK**CC**LGYQKRPLP	(TBD)	ND	(>100)	ND
CXCL11		**>1000 nM**	**1.7 ± 0.3 nM**	**>1000 nM**	**27.4 ± 1.6 nM**
$CXCL11_{1-17}$	FPMFKRGR**C**L**C**IGPGVK	>30*	(TBD)	>30*	18.2 ± 1.2*
$CXCL11_{1-9}$	FPMFKRGR***S***	ND	78 ± 14.5	ND	17.2 ± 7.5
$(CXCL11_{1-9})2$	$(FPMFKRGR\mathbf{C})_2$	ND	7.8 ± 2.1	ND	5.0 ± 2.4
$CXCL11_{1-17}D$	*FPMFKRGR**C**L**C**IGPGVK*	ND	(TBD)	ND	(29.6 ± 5.0)*
$CXCL11_{2-17}$	**-**PMFKRGR**C**L**C**IGPGVK	ND	(TBD)	ND	(63.0 ± 0.0)*
$CXCL11_{1-17/P2G}$	F**G**MFKRGR**C**L**C**IGPGVK	ND	(TBD)	ND	(28.5 ± 10.7)*
CXCL10		**>1000 nM**	**8.2 ± 2.2 nM**	**>1000 nM**	**198.5 ± 1.8 nM**
$CXCL10_{1-17}$	VPLSRTVR**C**T**C**ISISNQ	>100	>100	>100	> 100
CXCL9		**>1000 nM**	**128.5 ± 31.2 nM**	**>1000 nM**	**760.5 ± 1.3 nM**
$CXCL9_{1-17}$	TPVVRKGRCSCISTNQG	>100	>100	>100	> 100

* Highest concentration tested 30 μM
ND: Not determined
TBD: To be determined (only data at a single concentration of 100 μM are available)

Table 3. β-arrestin-2 recruitment induced by full-length chemokines and peptides derived from their N terminus to CXCR4, CXCR3 and CXCR7.

Name	Sequence	Arrestin recruitment					
		CXCR4		CXCR3		CXCR7	
		EC_{50} (μM)	% max	EC_{50} (μM)	% max	EC_{50} (μM)	% max
CXCL12		**9.3 ± 2.5 nM**	**100**	**> 1000 nM**	**0**	**2.3 ± 0.7 nM**	**100**
$CXCL12_{1-17}$	KPVSLSYR**C**P**C**RFFESH	> 100	23 ± 1	> 100	2 ± 1	0.9 ± 0.1	109 ± 9
$CXCL12_{1-9}$	KPVSLSYR**S**	> 100	18 ± 3	ND	ND	3.6 ± 0.5	95 ± 1
$(CXCL12_{1-9})2$	$(KPVSLSYR\mathbf{C})_2$	> 100	37 ± 0	ND	ND	0.3 ± 0.1	114 ± 1
$CXCL12_{1-17}D$	*KPVSLSYR**C**P**C**RFFESH*	> 100	10 ± 2	ND	ND	7.3 ± 0.5	60 ± 1
$CXCL12_{2-17}$	-PVSLSYR**C**P**C**RFFESH	> 100	3 ± 3	ND	ND	2.1 ± 0.3	88 ± 4
$CXCL12_{1-17/P2}$	K**G**VSLSYR**C**P**C**RFFESH	> 100	7 ± 10	ND	ND	7.7 ± 1.3	79 ±1
vCCL2		**> 1000 nM**	**0**	**> 1000 nM**	**9**	**27.8 ± 3.9 nM**	**86**
$vCCL2_{1-21}$	LGASWHRPDKCCLGYQKRPLP	> 100	0 ± 3	> 100	2 ± 2	0.8 ± 0.1	90 ± 1
$vCCL2_{1-11}$	LGASWHRPDK**S**	> 100	0 ± 2	ND	ND	1.3 ± 0.1	96 ± 2
$(vCCL2_{1-11})2$	$(LGASWHRPDK\mathbf{C})_2$	> 100	0 ± 2	ND	ND	0.4 ± 0.1	106 ± 8
$vCCL2_{1-21}D$	*LGASWHRPDK**CC**LGYQKRPLP*	> 100	0 ± 6	ND	ND	7.5 ± 0.9	75 ± 4
$vCCL2_{1-21/G2P}$	L**P**ASWHRPDK**CC**LGYQKRPLP	> 100	0 ± 4	ND	ND	3.4 ± 0.4	92 ± 0
$vCCL2_{1-21/L1K-G2P}$	**KP**ASWHRPDK**CC**LGYQKRPLP	> 100	0 ± 4	ND	ND	7.0 ± 1.1	78 ± 12
CXCL11		**> 1000 nM**	**5**	**4.3 ± 0.8 nM**	**100**	**14.3 ± 1.6 nM**	**87**
$CXCL11_{1-17}$	FPMFKRGR**C**L**C**IGPGVK	> 30*	0 ± 2	> 30*	26 ± 2*	0.9 ± 0.2*	89 ± 19*
$CXCL11_{1-9}$	FPMFKRGR**S**	ND	ND	> 100	40 ± 4	3.4 ± 0.3	88 ± 4
$(CXCL11_{1-9})2$	$(FPMFKRGR\mathbf{C})_2$	ND	ND	8 ± 3	75 ± 6	0.3 ± 0.1	107 ± 3
$CXCL11_{1-17D}$	*FPMFKRGR**C**L**C**IGPGVK*	ND	ND	> 30	13 ± 0*	3.0 ± 1.5*	74 ± 17*
$CXCL11_{2-17}$	-PMFKRGR**C**L**C**IGPGVK	ND	ND	> 30	4 ± 1*	3.0 ± 1.2*	41 ± 5*
$CXCL11_{1-17/P2}$	F**G**MFKRGR**C**L**C**IGPGVK	ND	ND	> 30	4 ± 4*	0.3 ± 0.1*	103 ± 1*
CXCL10		**> 1000 nM**	**0**	**25.5 ± 6.3 nM**	**88**	**> 1000 nM**	**3**
$CXCL10_{1-17}$	VPLSRTVR**C**T**C**ISISNQ	ND	ND	> 100	2 ± 1	> 100	6 ± 1
CXCL9		**ND**	**ND**	**> 1000 nM**	**15**	**ND**	**ND**
$CXCL9_{1-17}$	TPVVRKGRCSCISTNQG	ND	ND	> 100	2 ± 0	> 100	2 ± 2

* Highest concentration tested 30 μM
ND: Not determined

Highlights

- Peptides derived from the N terminus of chemokines represent useful probes to investigate the binding and activation of chemokine receptors.
- CXCR4 and CXCR3 seem to use similar chemokine-binding modes and activation mechanisms with a crucial role of the chemokine N terminus for receptor activation.
- CXCR7 is more permissive to peptide modifications than CXCR4 and CXCR3, suggesting a different activation mechanism than CXCR4 and CXCR3 and that CXCR7 could also scavenge modified chemokines.

This chapter presented our very recent results on the chemokine N-terminal features, which are part of a manuscript currently in preparation. The experiments were partly performed in collaboration with the laboratories of Prof. Dominique Schols and Dr. Julien Hanson. Additional experiments are still foreseen to better evaluate the antagonist properties of the peptides and to further investigate the effect of their dimerisation. Complementary data obtained by other collaborating groups (Prof. Brian Volkman and Prof. Nikolaus Heveker) with full-length chemokines bearing N terminus modifications similar to the ones presented above, will also be included in the manuscript.

The next chapter will describe a study on the determinants involved in chemokine N terminus recognition within the receptor CXCR4.

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Chapter 7

Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 and vCCL2 binding to CXCR4 and CXCR7

Adapted from Chevigné A, Fievez V, Szpakowska M, Fischer A, Counson M, Plesséria J-M, Schmit J-C and Deroo S, Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1, Biochim Biophys Acta 2014 May;1843(5):1031-41.

The previous chapter discussed the molecular determinants of receptor-activating interaction at site 2 (or CRS2) from the perspective of chemokines, considering various features in their N terminus. Here, the CRS2 interaction will be approached from the receptor's side.

The study described in this chapter focused on CXCR4 and was an attempt to characterise the receptor residues delimiting CRS2, the binding site of chemokine N terminus, using various mutated and truncated peptides derived from the receptor extracellular loops. Moreover, as CXCR4 and CXCR7 together with their shared ligand, CXCL12, represent valuable drug targets, being involved in various pathologic processes such as inflammation and many cancers, we also investigated the possibility of simultaneously blocking their interactions using these same receptor extracellular loop-derived peptides.

1. Introduction

The chemokine receptor CXCR4 is a class A G protein-coupled receptor (GPCR) expressed at the surface of various cells including T lymphocytes, monocytes, neutrophils, dendritic and endothelial cells [1-3]. The interaction of CXCR4 with its unique endogenous ligand, the chemokine CXCL12 plays a crucial role in processes such as hematopoietic stem cell [4, 5] and leukocyte trafficking [6, 7], vascular and neuronal development as well as inflammation and immune-modulation [5, 7, 8]. In addition to its physiological role, CXCR4 is involved in several pathologies including inflammatory diseases, WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelokathexis) syndrome, cancer and HIV-1 infection [9-13]. In addition, CXCR4 together with CCR5, acts as co-receptors for the entry of HIV-1 into host cells by interacting with the viral envelope protein gp120 after its engagement with CD4 [11, 14-21].

CXCR4 is also expressed on a large variety of cancer cells and its interaction with CXCL12 has been demonstrated to favour tumour cell survival, proliferation and mobility, leading to metastasis development [13, 22-24]. Besides CXCL12, the broad-spectrum human herpesvirus 8-encoded chemokine vCCL2, interacts with CXCR4 but acts as an antagonist [25]. In 2005, CXCR7 was identified as the second CXCL12-binding chemokine receptor [26, 27]. Similarly to CXCR4, CXCR7 promotes cancer metastasis and its over-expression is often associated with more aggressive tumour phenotypes and bad prognosis [28-30]. Importantly, the biology and regulation of the activity of CXCR4, CXCR7 and their common ligand CXCL12 was suggested to be interdependent [31].

CXCR4 was shown to adopt a typical GPCR fold, consisting of a seven-transmembrane helix bundle. However, the location and shape of its ligand-binding pocket differs from that of other protein-binding GPCRs and is situated closer to the receptor surface [32] suggesting a greater implication of the N terminus and the three extracellular loops (ECL1, ECL2 and ECL3) in ligand binding and receptor activation [33-35].

The three-dimensional structure of chemokines consists of (1) an elongated and flexible N terminus, (2) a cysteine motif, (3) a loop of approximately ten residues, referred to as the N-loop, (4) a single-turn of 3_{10} helix, (5) three anti-parallel β-strands and (6) a C-terminal α-

helix. These secondary structures are connected by turns known as the 30s, 40s, and 50s loops, which reflects the numbering of residues in the mature protein [36, 37]. The chemokine structure is further stabilised by two disulphide bridges connecting the cysteine residues of the N terminus with those located in the 30s and 50s loops [36].

Based on the large amount of information available for CXCL12 and CXCR4, a general two-step mechanism was proposed to describe the interaction of chemokines with their cognate receptors [38, 39]. The initial step of this model corresponds to the anchoring of the chemokine to the receptor's N terminus (Chemokine Recognition Site 1, CRS1) and is followed by the binding of the flexible N terminus of the chemokine to a second site (CRS2) located in the vicinity of the transmembrane segments (TMs) and the extracellular loops of the receptor. In line with this model, studies using sulfated peptides derived from the N-terminal domain of CXCR4 demonstrated that peptide corresponding to CRS1 binds the surface of CXCL12 in an extended conformation close to the chemokine N-loop [40, 41]. Furthermore, these studies highlighted the importance of sulfotyrosines present on CRS1 and sulfotyrosine-binding pockets present on the chemokine. Binding of the chemokine N terminus to CRS2 was suggested to induce conformational changes in the receptor and in its subsequent activation. In agreement with this model, short peptides derived from the flexible N terminus of CXCL12 were sufficient to specifically bind CXCR4, and displayed agonist activity [42-45]. Further analyses conducted with affinity-purified CXCR4 identified several amino acids located on the CXCL12 β-sheet and 50s loop as additional receptor interacting residues [46]. Although all these results corroborate the two-step binding model, the exact stoichiometry of the CXCR4-CXCL12 interaction as well as the receptor determinants forming the CRS2 remain to be clarified [32, 40].

The critical role of CXCR4 in cancer biology and HIV-1 infection makes this receptor and its ligands valuable targets for drug development. To date, several small molecule CXCR4 antagonists, including AMD3100, T140 or CTCE-9908 have been described [47-51]. Although these molecules are potent in blocking HIV-1 infection and metastasis development, they are often associated with important side effects and/or inverse action on other chemokine receptors [47, 52, 53]. Therefore, other inhibition strategies need to be explored. Over the last few years, ligand neutralisation by small molecules, peptides and

antibody fragments has emerged as an interesting alternative to the classical receptor inhibition [54-61]. However, peptide derivatives of receptor extracellular loops have never been reported as potential chemokine neutralisers. In the context of CXCR4 and CXCR7, targeting their common chemokine, CXCL12, would allow to simultaneously interfere with its binding to both receptors [62].

In the present study, we investigated the neutralising properties of individual peptides corresponding to the first, second and third extracellular loop (ECL1, ECL2, ECL3) of CXCR4 towards CXCL12 binding to CXCR4 and CXCR7. Analyses with mutated and truncated peptides provided new insights into the molecular basis of receptor-ligand recognition, opening new perspectives for the development of CXCR4 ligand neutralisers.

2. Materials and methods

2.1 Peptides, proteins and cell lines

Peptides corresponding to the extracellular loops of CXCR4 (ECL1-X4, ECL2-X4 and ECL3-X4) were designed based on the receptor topology predicted prior to its X-ray structure resolution [33].

Table 1. Sequence and length of peptides derived from CXCR4 extracellular loops.

Name	Length	Position	Sequence
ECL1-X4	14	97-110	DAV**ANWYF**GNFLCK
ECL2-X4	27	176-202	**NVSEADDRYICDRFYPN**DLWVVVFQFQ
ECL3-X4	21	262-282	DSFILL**EIIKQG**CEFENTVHK

Residues in bold are solvent-exposed in the X-ray structure of CXCR4.

All peptides including the scrambled control peptides ECL1-X4$_{scrbl}$ (FNYSGAKFVNDLWA) and ECL2-X4$_{scrbl}$ (DVQDPRVLDWRNDVYSFYAFQFVCINE) were purchased from JPT and contain an amide group at the C terminus to avoid additional negative charges. Peptide ECL2-X4 was also purchased biotinylated at its N terminus, with a Ttds linker ([N1-(9-Fluorenylmethoxycarbonyl)-1,13-diamino-4,7,10-trioxatridecan-succinamic acid) separating the biotin moiety from the peptide. The CXCL12 N-terminal peptide comprises the first 17 residues of the chemokine (KPVSLSYRCPCRFFESH). Control peptide (SPAPERRGYSGYDVPDY) (Ctrl) corresponds to a HCDR3 sequence binding to an

antibody directed against human influenza haemagglutinin [63]. Chemokines CXCL12 (SDF1α), vCCL2 (vMIP-II), CCL5 (RANTES), CCL3 (MIP-Iα) and CCL4 (MIP-Iβ) were purchased from Peprotech. Alexa Fluor 647-labelled CXCL12 was purchased from Almac. MT-4, Cf2Th-CXCR4, CEM.NK^R, CEM.NK^R-CCR5 and U87.CD4.CXCR4 cell lines were obtained through the NIH AIDS program from Dr. D. Richman, Dr. J. Sodroski and Dr. A. Trkola [64-66]. Cells stably expressing CXCR7 were obtained by transfecting U87.CD4 cells with pBABE-CXCR7 vector.

2.2 Binding of fluorescently labelled CXCL12 to CXCR4

Alexa Fluor 647-labelled CXCL12 (100 ng/ml) was incubated 30 minutes at room temperature with ECL-X4 peptides (50 μM). Cf2Th-CXCR4 cells were incubated with the mix 90 minutes at 4°C. Cells were then washed, incubated 30 minutes at 4°C with an amine-reactive cell viability dye (LIVE/DEAD® Fixable Dead Cell Stain, Lifetechnologies) and analysed on a BD FACS Canto cytometer (BD Biosciences) using BD FACS Diva software. Unlabelled CXCL12 chemokine (100-fold excess) was used as positive control for Alexa Fluor 647-labelled chemokine displacement and the viability dye to determine peptide cytotoxicity.

2.3 Surface plasmon resonance measurements

Biotinylated ECL2-X4 peptide (1 μM) was immobilised on a streptavidin chip (GE Healthcare) by injection at a flow rate of 5 μl/min for 20 minutes in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20 (HBS-EP) on a BIAcore 3000. Typically, a signal ranging from 1000 to 1500 RU was obtained. For all sensorgrams, signal obtained with an irrelevant peptide (Ctrl) was subtracted from signal obtained with ECL2-X4. Binding analyses were performed by injecting 200 nM of CXCL12, vCCL2, CCL5, CCL3, CCL4 for 3 minutes at a flow rate of 30 μl/min. All binding measurements were performed in triplicate and were presented as average ± standard deviation. Kinetic analyses were performed by injecting various concentrations of CXCL12 and vCCL2 (7 to 500 nM) in HBS-EP at a flow rate of 30 μl/min. Association and dissociation was recorded during 2 minutes and 20 minutes, respectively. Measurements were carried out in duplicate. Surface

regeneration was performed by a single injection of 10 µl of 10 mM glycine buffer pH 1.5. The presence of mass transfer phenomena and linked reactions was excluded by performing the control assays as recommended by the manufacturer. Kinetic data analysis was performed using the BIAevaluation 4.1 software. The overall dissociation constant (K_D) values and on (ka) and off (kd) rates for the complexes were obtained after global fits of the experimental data using a simple model for 1:1 (Langmuir) binding.

2.4 cAMP modulation

Cyclic AMP (cAMP) modulation upon CXCL12 binding in the presence or absence of ECL-X4 peptides was evaluated on MT-4 cells using the TR-FRET LANCE cAMP assay (Perkin Elmer) adapted for a 96-well plate format. MT-4 cells (2.10^4 cells/well) were diluted in HBSS stimulation buffer (5 mM HEPES, 0.1% BSA, 0.5 mM IBMX pH 7.4) containing Alexa Fluor 647-labelled anti-cAMP antibody. Cells were incubated with forskoline (FSK) and CXCL12 pre-incubated 30 minutes at room temperature with ECL-X4 or control peptides. cAMP modulation was measured by adding Europium-labelled streptavidin and biotin-cAMP for 1 hour at room temperature. The signal was measured at 665 nm in a TECAN Genios Pro fluorimeter and compared with cAMP standard curves (10^{-6} to 10^{-11} M).

2.5 Intracellular calcium release

Intracellular calcium release induced by CXCL12 was measured using indo-1-acetoxymethyl ester (Interchim) as calcium-responsive fluorescent probe. Calcium release was monitored in MT-4 cells in 20 mM HEPES buffer containing 2.5 mM probenecid and 0.1 % BSA. All measurements were performed at 37°C in a 1 ml stirred cell using wavelengths of 355 nm for excitation and 475 and 405 nm for emission in a PTI QM-4 QuantaMaster fluorimeter. For inhibition experiments, CXCL12 (7.5 nM) was incubated 10 minutes in the presence of CXCR4-derived peptides (100 µM).

2.6 Internalisation of CXCR4, CXCR7 and CCR5

Internalisation of CXCR4, CXCR7 and CCR5 receptors from the cell surface was monitored by flow cytometry. Phycoerythrin-conjugated monoclonal antibodies (mAb) 12G5 (BD Pharmingen) and 4G10 (Santa Cruz Biotechnology) were used to follow CXCR4 internalisation from the surface of MT-4 cells. Phycoerythrin-conjugated monoclonal antibodies T21/8 (EBiosciences) and 11G8 (R&D Systems) were used to monitor CCR5 and CXCR7 internalisation from CEM.NK^R-CCR5 and U87-CXCR7 cells, respectively.

Cells were incubated for 30 minutes in the presence of CXCL12 (50 nM), CCL5 (20 nM) or vCCL2 (400 nM). For neutralisation experiments, chemokines were pre-incubated 30 minutes at 37°C with ECL-X4 and control peptides. Internalisation was stopped after 30 minutes by addition of NaN_3 (0.1 %) and placing cells on ice. Cells were then stained with the adequate antibody 30 minutes at 4°C. Cell viability and the potential cytotoxic effect of peptides was monitored using the LIVE/DEAD® Fixable Dead Cell Stain. Samples were analysed on a BD FACS Canto cytometer (BD Biosciences) using BD FACS Diva software.

2.7 Chemotaxis

Chemotaxis assays were performed in ChemoTx 96-well cell migration systems (Neuro Probe) equipped with a 5-µm pore polycarbonate membrane filter. Migration buffer (RPMI 1640) containing CXCL12 (12 nM) and two-fold serial dilutions of ECL-X4 and control peptides (3 µM to 100 µM) were loaded in the lower chamber. Calcein-AM-loaded Jurkat cells (2.5 10^5 cells) were added to the upper chamber. Migration was allowed for 2 hours and 15 minutes at 37°C. Cells in upper and lower chambers were quantified by measuring fluorescence (E_{ex} 485 nm, E_{em} 525 nm) using a Tecan Genios Pro fluorimeter.

3. Results

3.1 Chemokine binding and neutralisation by ECL-X4 peptides

ECL-X4 peptides were analysed for their ability to inhibit the binding of fluorescently labelled CXCL12 to CXCR4. This labelled chemokine specifically bound to Cf2Th cells overexpressing CXCR4 and not to the parental Cf2Th cells (data not shown). Only ECL2-X4 inhibited the binding of labelled CXCL12 to CXCR4 in a concentration-dependent manner (IC_{50}= 2 ± 1 µM) (Fig. 1). No inhibition was observed with ECL1-X4, ECL3-X4, scrambled ECL2-X4 (ECL2-$X4_{scrbl}$) and the control peptide.

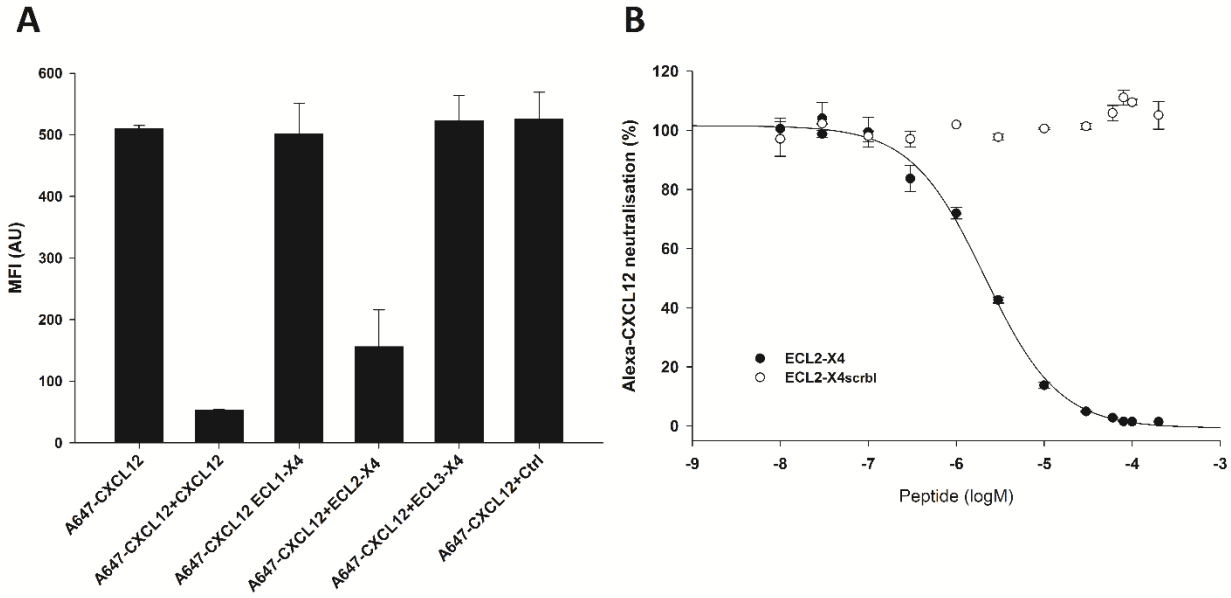

Fig. 1. Inhibition of CXCL12 binding to CXCR4 by ECL-X4 peptides. (A) Inhibition of CXCL12 binding to Cf2Th cells expressing CXCR4 by ECL1-X4, ECL2-X4, ECL3-X4 and control (Ctrl) peptides (50 µM). **(B)** Comparison of inhibitory properties of ECL2-X4 and ECL-$X4_{scrbl}$ (200 µM to 10 nM) towards CXCL12. Alexa Fluor 647-labelled CXCL12 (AF647-CXCL12) (100 ng/ml) was pre-incubated 30 minutes at RT with CXCR4 or control peptides before addition on Cf2Th-CXCR4 cells for 90 minutes at 4°C. All experiments were performed in duplicate and are presented as average ± standard deviation.

Surface plasmon resonance experiments demonstrated a strong and specific binding of ECL2-X4 to CXCR4-related chemokines in solution (CXCL12 K_D = 22 ± 0.5 nM, and vCCL2 K_D = 27 ± 0.5 nM). This data indicated that the reduced binding of fluorescent CXCL12 in the presence of ECL2-X4 was related to chemokine neutralisation and not receptor-peptide interactions (Fig. 2).

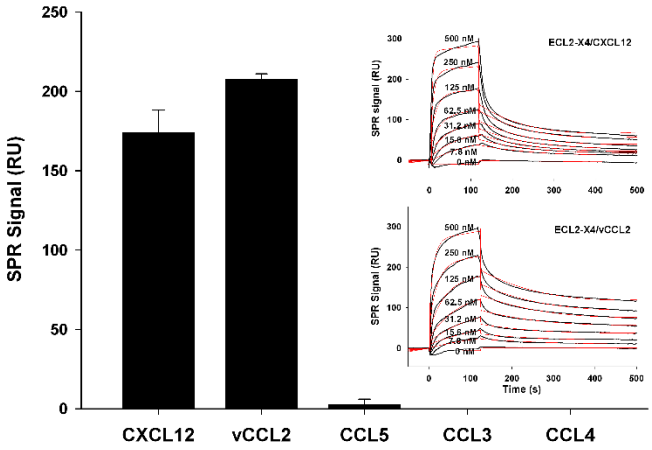

Fig. 2. Binding specificity of peptide ECL2-X4. Binding was evaluated by surface plasmon resonance (SPR) using a biotinylated ECL2-X4 peptide immobilised on a SA-chip. CXCL12, vCCL2, CCL5, CCL3, CCL4 chemokines were injected at 200 nM. Binding intensities corresponding to SPR signals recorded at the end of the association phase are presented as average values ± standard deviation of triplicate experiments. Insets: kinetic analysis of the binding of peptide ECL2-X4 to chemokines CXCL12 (upper panel) and vCCL2 (lower panel). Measurements were performed with two-fold dilutions of chemokine starting at 500 nM. Kinetic rate constants (ka and kd) for both complexes were fitted globally according to a Langmuir 1:1 model using biaevaluation 4.1 software. The fitting results were ka= 4.01 ± 0.05x10^5 $M^{-1}s^{-1}$, kd= 8.84 ± 0.07x10^3 s^{-1}, K_D= 22.1 ± 0.5 nM for ECL2-X4/CXCL12 and ka= 1.86 ± 0.02 x 10^5 $M^{-1}s^{-1}$, kd= 5.05 ± 0.04 x 10^3 s^{-1}, K_D= 27.1 ± 0.5 nM for ECL2-X4/vCCL2, respectively.

3.2 Inhibition of CXCL12-induced CXCR4 and CXCR7 activation by ECL-X4 peptides

The effect of ECL-X4 peptides on the G protein signalling through CXCR4 was then investigated. In agreement with the binding data, in cAMP modulation assay, only peptide ECL2-X4 inhibited CXCL12-induced CXCR4 activation (IC_{50}= 35 ± 0.5 µM), whereas ECL1-X4, ECL3-X4 and the control peptide had no effect (Fig. 3A). In the absence of CXCL12, none of the ECL-X4 peptides acted as a CXCR4 agonist (data not shown). Similar results were obtained with CXCL12-induced calcium release measurements. ECL2-X4 peptide abolished calcium response induced by CXCL12 with an IC_{50} of 5.7 µM (Fig. 3B inset), whereas ECL1-X4, ECL3-X4 and the control peptide did not exert any effect (Figure 3B). The inhibitory properties of ECL2-X4 were confirmed in a receptor internalisation assay, using the 4G10 mAb directed against the CXCR4 N terminus and not interacting with any of CXCR4 ECLs. Only ECL2-X4 inhibited CXCL12-induced CXCR4 internalisation (IC_{50}= 19 ± 2 µM) (Fig. 3C and 4). The ability of ECL-X4 peptides to inhibit CXCL12-induced

migration of leukaemia-derived T cells was also monitored using a Transwell system. ECL2-X4 abolished chemotaxis of Jurkat cells at concentrations higher than 50 μM, while only partial or no inhibition was observed with peptides ECL3-X4 and ECL1-X4, respectively (Fig. 3D).

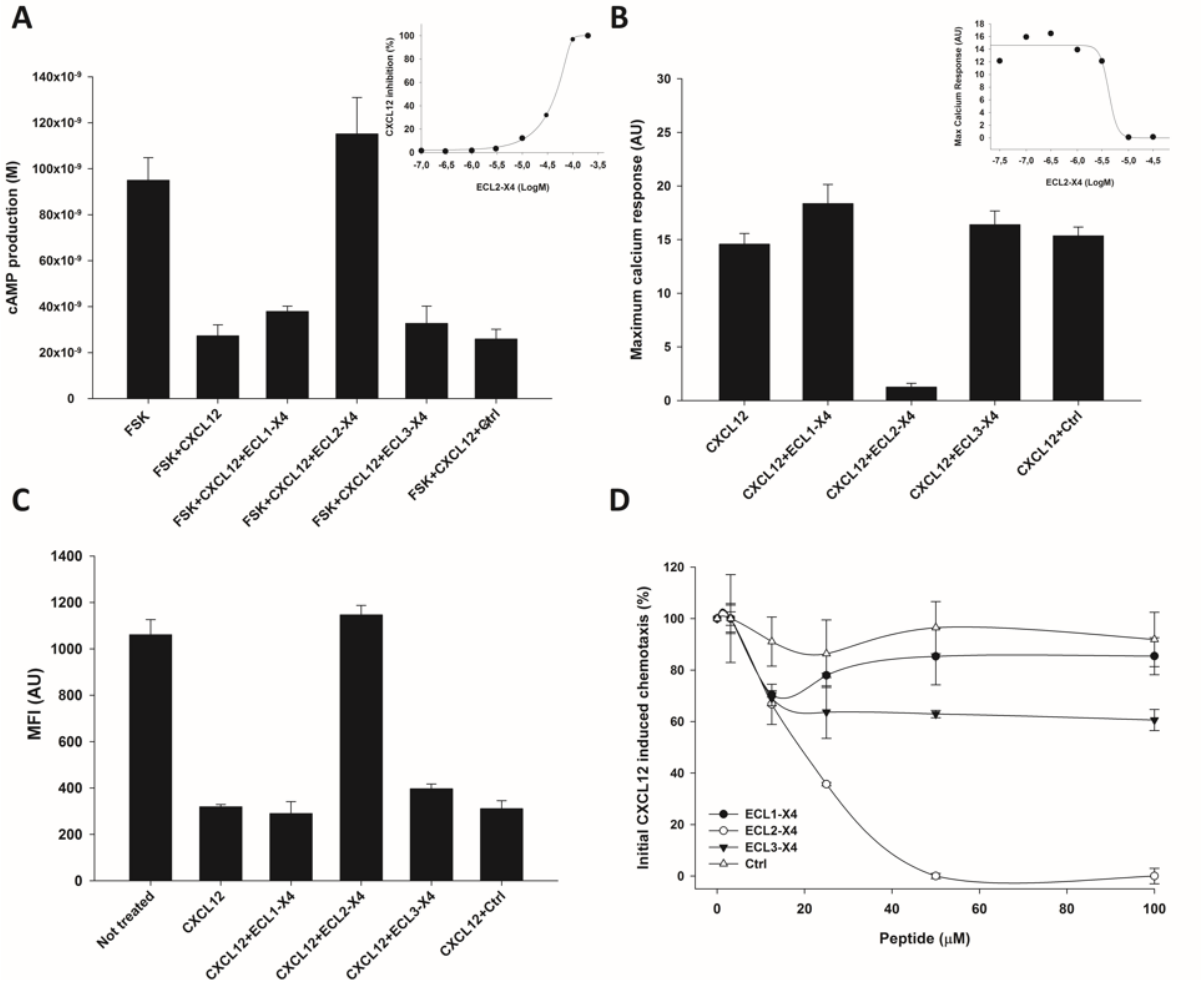

Fig. 3. Inhibition of CXCL12-induced CXCR4 G protein signalling by ECL-X4 peptides. (A) cAMP modulation by ECL1-X4, ECL2-X4, ECL3-X4 and the control peptide. Modulation of forskolin-induced cAMP production was monitored using TR-FRET-based assay. CXCL12 (30 nM) was pre-incubated 30 minutes at 37°C with peptides (100 μM) before addition on MT-4 cells. **Inset**: Concentration-dependent inhibition of CXCL12 by peptide ECL2-X4 (10 nM to 200 μM). **(B)** Inhibition of CXCL12-induced calcium release by ECL-X4 peptides. Antagonist properties were monitored in the presence of CXCL12 (7.5 nM) by measuring calcium response using Indo-1 fluorescence. **Inset:** Concentration-dependent inhibition of CXCL12-induced calcium release by ECL2-X4 (100 μM to 10 nM). **(C)** Inhibition of CXCL12-induced CXCR4 internalisation in MT-4 cells. CXCL12 (50 nM) was pre-incubated 30 minutes at 37°C with ECL-X4 peptides (100 μM) before addition on MT-4 cells for 30 minutes at 37°C. CXCR4 surface expression was monitored by flow cytometry using mAb 4G10. **(D)** Inhibition of CXCL12-induced migration of leukaemia-derived Jurkat T-cells by ECL-X4 peptides. All experiments were performed in duplicate and are presented as average ± standard deviation.

Finally, the inhibitory properties of ECL2-X4 towards CXCL12-induced CXCR7 internalisation were evaluated (Fig. 4). ECL2-X4 inhibited CXCR7 internalisation albeit with less potency than was observed for CXCR4 (IC_{50}= 100 ± 24 μM vs. IC_{50}= 19 ± 2 μM).

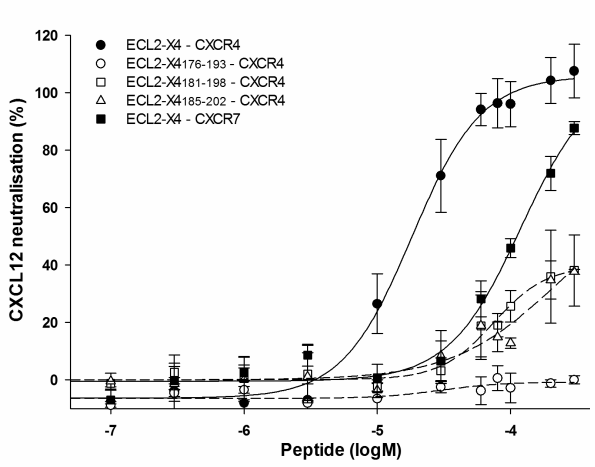

Fig. 4. Inhibition of CXCL12 interaction with CXCR4 and CXCR7 by ECL2-X4-derived peptides. Neutralisation properties towards CXCL12 were evaluated in receptor internalisation assays. CXCL12 (50 nM) was pre-incubated with different concentrations (10 nM to 300 μM) of ECL2-X4 and truncated analogues for 30 minutes at 37°C prior addition on cells for 30 minutes at 37°C. Full-length ECL2-X4 peptide covered positions 176 to 202. Truncated ECL2-X4 peptides (ECL2-$X4_{176-193}$, ECL2-$X4_{181-198}$ and ECL2-$X4_{185-202}$) are partially overlapping 18-mers covering the entire ECL2 sequence (Fig 7C). Data are presented mean ± standard deviation.

3.3 vCCL2 neutralisation by peptide ECL2-X4.

The ability of ECL2-X4 to neutralise vCCL2 was also evaluated (Fig. 5). This chemokine binds to CXCR4 as well as to other CXC and CC chemokine receptors, including CCR5. As vCCL2 displays an inherent antagonist activity, its binding to chemokine receptors was monitored in competition experiments. To avoid interference between ECL-X4 peptides and the competing mAbs, the binding of vCCL2 was monitored on CCR5-expressing cells using the anti-CCR5 T21/8 mAb. At concentration of 400 nM vCCL2 abolished the binding of the anti-CCR5 antibody to the receptor, while in the presence of peptide ECL2-X4, this binding was fully restored, demonstrating that ECL2-X4 also neutralised vCCL2. This interaction was concentration-dependent with a potency equivalent to that observed for CXCL12 (IC_{50}= 29 ± 6 μM vs. IC_{50}= 19 ± 2 μM). Peptide ECL2-X4 had no effect on CCL5 binding to CCR5 confirming its specificity.

3.4 Inhibition of CXCL12 and its N terminus-derived peptide by truncated and mutated ECL2-X4 analogues

To unravel the structural basis of CXCL12 and vCCL2 neutralisation by ECL2-X4, partially overlapping truncated 18-mer peptides covering the full-length sequence of ECL2-X4 (176-193, 181-198 and 185-202) were analysed in a CXCR4 internalisation assay (Fig. 4, 5 and 6).

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ECL2-X4176-202   NVSEADDRYICDRFYPNDLWVVVFQFQ
ECL2-X4176-193   NVSEADDRYICDRFYPND---------
ECL2-X4181-198   -----DDRYICDRFYPNDLWVVV----
ECL2-X4185-202   ---------ICDRFYPNDLWVVVFQFQ
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Fig. 6. Full-length and truncated ECL2-X4 peptides. Residues within ECL2-X4 critical for CXCL12 neutralisation (IC_{50} values of alanine mutant twice as high as the wild-type peptide, see fig. 7A) are coloured in blue. Sequences of the truncated overlapping 18-mer ECL2-X4 peptides are shown. Underlined asparagines (N) correspond to putative N-glycosylation sites (N_XS/T).

Truncation of the C-terminal residues (ECL2-$X4_{176-193}$) abrogated the inhibitory properties of the peptide, while peptides ECL2-$X4_{181-198}$ and ECL2-$X4_{185-202}$, at the highest concentration tested (300 µM), displayed only 30 % of the full-length ECL2-X4 activity (Fig. 4). Similar profiles were observed for vCCL2, although the analogue ECL2-$X4_{181-198}$ displayed stronger inhibition potency (Fig. 5).

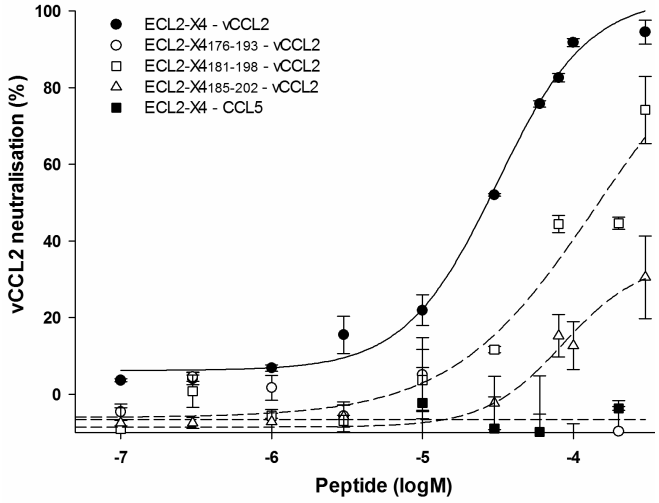

Fig. 5. Inhibition of vCCL2 interaction with CCR5 by ECL2-X4-derived peptides. Neutralisation properties towards vCCL2 were evaluated in internalisation assay (-vCCL2). vCCL2 (400 nM) was pre-incubated 30 minutes at 37°C with ECL2-X4 peptides (10 nM to 300 µM) before 30-minute incubation at 37°C with CCR5-expressing cells. Control experiment was performed using CCL5 (20 nM) (-CCL5). CCR5 surface expression was detected with the T21/8 antibody. Full-length ECL2-X4 peptide covers positions 176 to 202. Truncated ECL2-X4 peptides (ECL2-$X4_{176-193}$, ECL2-$X4_{181-198}$ and ECL2-$X4_{185-202}$) are partially overlapping 18-mers covering the entire ECL2 sequence (Fig 7C). Data are presented as mean ± standard deviation.

To assess the importance of individual amino acids for CXCL12-neutralising properties of ECL2-X4, the IC_{50} values of alanine mutants were determined (Fig. 7A). Only the P191A mutant almost completely lost its capacity of inhibiting CXCL12-induced receptor internalisation (IC_{50} > 300 μM), while replacement of residues Asp181 (D181A, IC_{50}= 38 ± 3 μM), Asp182 (D182A, IC_{50}= 63 ± 7 μM), Asp187 (D187A, IC_{50}= 46 ± 5 μM), Asp193 (D193A, IC_{50}= 75 ± 9 μM) and Cys186 (C186A, IC_{50}= 91 ± 8 μM) as well as the hydrophobic residues Leu194 (L194A, IC_{50}= 38 ± 8 μM), Trp195 (W195A, IC_{50}= 50 ± 5 μM), Val196 (V196A, IC_{50}= 44 ± 11 μM), Val197 (V197A, IC_{50}= 47 ± 6 μM) and Phe201 (F201A, IC_{50}= 50 ± 15 μM) located at the C terminus of peptide ECL2-X4 resulted in more than two-fold reduction in CXCL12-neutralising property. In contrast, the removal of positively charged residues in mutants R183A (IC_{50}= 15 ± 3 μM) and R188A (IC_{50}= 14 ± 3 μM) slightly favoured chemokine binding.

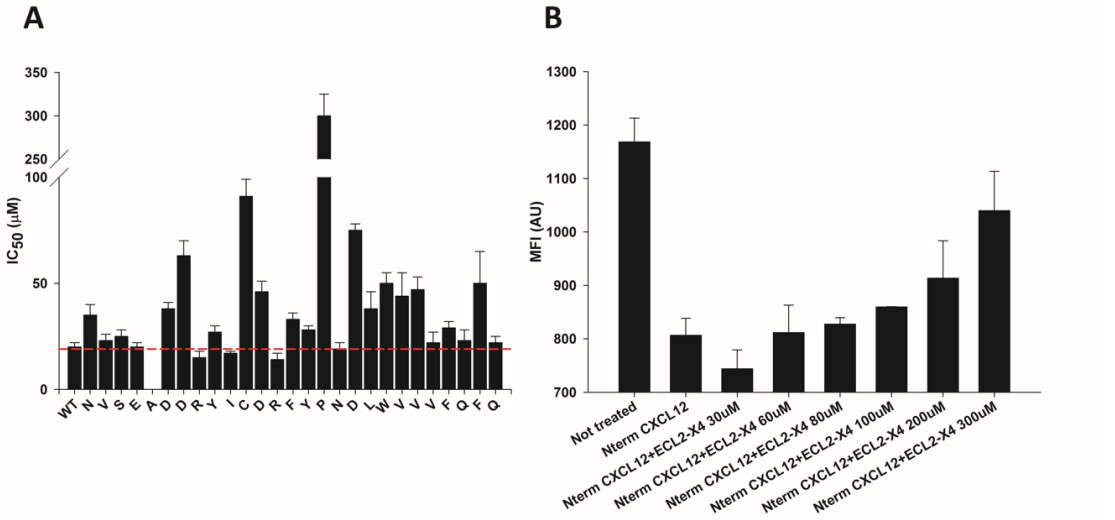

Fig. 7. Mutational scanning of peptide ECL2-X4 and its inhibitory properties towards CXCL12 and the agonist peptide derived from CXCL12 N terminus. (A) Inhibition of CXCL12-induced CXCR4 internalisation by ECL2-X4 mutants monitored by flow cytometry. IC_{50} values are compared to that recorded with wild-type (WT) peptide ECL2-X4 (IC_{50}= 19 ± 2 μM) (red dashed line). **(B)** Neutralisation of the agonist peptide derived from CXCL12 N terminus (residues 1-17) by ECL2-X4. CXCL12 N terminus peptide (50 μM) was pre-incubated with ECL2-X4 (10 μM to 300 μM) and neutralisation was monitored as the decrease of CXCL12 N terminus-induced CXCR4 internalisation. Experiments were performed in duplicate and are presented as average ± standard deviation.

To pinpoint the region of CXCL12 involved in the interaction with ECL2-X4, neutralisation of the short peptide derived from the N terminus of CXCL12 (residues 1-17) was evaluated in internalisation assay, as this peptide alone was shown to induce receptor internalisation with an EC_{50} of 50 µM (Fig. 7B). As observed for full-length chemokine, ECL2-X4 inhibited over 70 % of CXCR4 internalisation induced by this agonist peptide indicating that ECL2-X4 neutralises CXCL12 predominantly by binding to its flexible N-terminal extremity.

4. Discussion

4.1 ECL2-X4 neutralises CXCL12 by binding to its flexible N terminus - molecular basis for CRS2 interaction

Based on our data, ECL2 is most likely to be the major determinant of CXCR4 CRS2. Indeed, only peptides derived from this loop were able to specifically bind CXCL12 and inhibit its interactions with CXCR4 thereby preventing receptor activation. The binding of CXCL12 to CXCR4 is generally described as a two-step mechanism [38]. While the receptor N terminus is commonly accepted as the major determinant of the initial chemokine recognition (CRS1), the residues constituting CRS2 involved in the subsequent receptor-activating interaction with the chemokine N terminus are not precisely defined. *In silico* predictions pointed out residues located in ECL2, ECL3 as well as the TM5 and TM6 regions [35, 38, 67]. In our study, while full-length ECL2-X4 was needed for efficient chemokine neutralisation, mutational analysis highlighted the crucial role of Pro191. Furthermore, Cys186, the four aspartate residues scattered along the peptide (Asp181, Asp182, Asp187 and Asp193) as well as the LWVV cluster and Phe200 located at the C-terminal part of ECL2 were important for neutralisation (Fig. 6, 7A and 8B). In the CXCR4 crystal structure, all four aspartates are solvent-exposed and ideally positioned on the loop to interact with ligands (Fig. 8B). Pro191 was crucial for CXCL12 neutralisation most probably by reducing the flexibility of this region and introducing a kink at the C terminus of ECL2 upstream of the LWVVVFQFQ sequence (annotated as the top of TM5 in the X-ray structure). This kink may be necessary for correct positioning of the N- and C-terminal parts of the peptide for optimal ligand binding. Based on this structural arrangement, a plausible mechanism for the initial interactions of CXCL12 at CRS2 would rely on

stabilising contacts of the four aspartates with the core and the N terminus (Arg8 and Arg12) of the chemokine, ensuring the correct orientation of its flexible N terminus for receptor-activating insertion in the transmembrane cavity, close to the top of TM5 (Fig. 2SD). This insertion would result in conformational changes in the TM5 and TM6 region, allowing the formation of new interactions between the N-terminal lysine of CXCL12 and Asp262 or Glu288 located at the inner segment of TM6 and TM7, respectively [38].

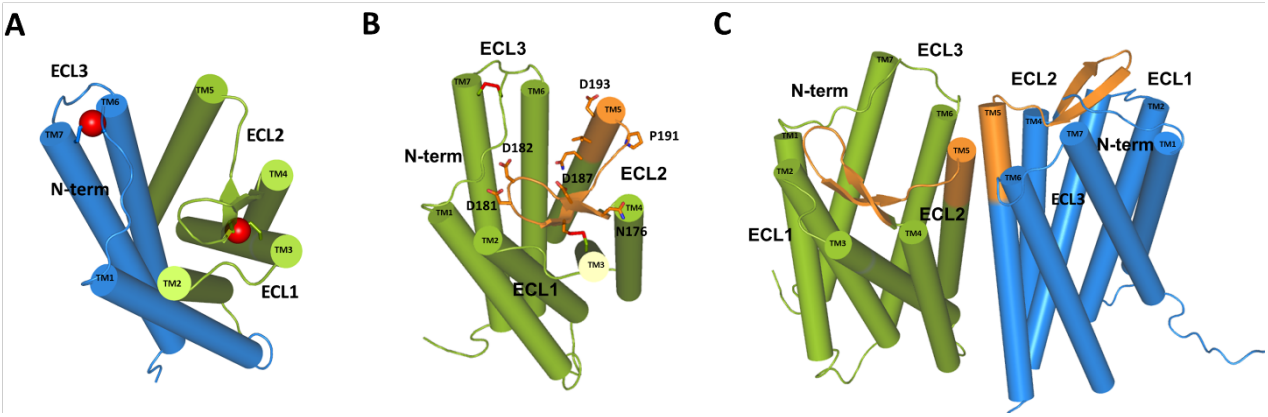

Fig. 8. Spatial arrangement of CXCR4 extracellular domains and positioning of ECL2 residues involved in interactions with CXCL12 and its N terminus. (A) Top-down view of CXCR4 extracellular surface (PDB 3ODU). Disulphide bridges (red dots) of the extracellular parts of CXCR4 divide the receptor into two distinct domains (Nterm-ECL3 in blue and ECL1-ECL2 in green) potentially involved in different steps of ligand binding. **(B)** Overall CXCR4 receptor and localisation of ECL2. Residues located in ECL2/top of TM5, which when mutated to alanine resulted in at least two-fold higher IC_{50} values than the wild-type peptide ECL2-X4 are represented as sticks. Side chains of residues D181, D182, D187 and D193 point towards the inner face of the receptor. **(C)** Structure of CXCR4 dimer and location of CXCL12 N terminus-binding sites (ECL2 and the top of TM5 coloured orange). CXCR4 monomers are coloured green and blue. Receptor dimerisation mainly involves the extracellular surface of TM5 and TM6 and brings two CXCL12 N terminus-binding sites in close vicinity.

In accordance with the proposed mechanism, the agonist activity of the peptide derived from CXCL12 N terminus was inhibited by ECL2-X4. Interestingly, dimeric peptides derived from CXCL12 and vCCL2 N termini showed ten times stronger activity in inhibiting HIV-1 infection or CXCL12 binding than their monomeric counterparts [42-45]. The molecular basis for this increase in potency is not entirely understood. Nevertheless, it is noteworthy that in the crystal structure, CXCR4 is present in a dimeric form (Fig. 8C). The dimerisation, which takes place at the extracellular side of the TM5-TM6 region, brings

the two CXCL12 N terminus-binding sites closer together in a symmetric manner, which could therefore account for more favourable bivalent interactions of the dimeric peptides with a dimeric form of the receptor.

To date, the exact stoichiometry of CXCL12-CXCR4 interactions remains unclear [32]. As observed for CXCR4, CXCL12 can also form dimers and monomeric or dimeric forms of the chemokine were shown to elicit different signalling and cellular responses [40, 68]. Our data indicate that peptide ECL2-X4 binds to CXCL12 by forming multiple contacts with an important contribution of the hydrophobic and negatively charged residues, reminiscent of the interactions described for CXCR4 N terminus peptides (CRS1) and CXCL12 [40]. Indeed, peptides corresponding to CRS1 have been shown to bind the chemokine in an extended conformation and to occupy a cleft delimited by the N-loop and the β-sheet, leaving the flexible N terminus of the chemokine free for an interaction with ECL2 (CRS2). It is, therefore, conceivable that the interaction at CRS1 positions the chemokine, induces conformation changes or creates larger interaction interface facilitating the subsequent binding of the chemokine at CRS2. However, the binding of CRS1-derived peptides to CXCL12 has also been demonstrated to induce its dimerisation and the formation of a symmetric 2/2 complex, in which the N terminus-binding sites of the two receptors are located at opposing faces of the dimer [40]. Therefore, it is also plausible that the CRS1- and CRS2-derived peptides (N-term and ECL2) recognise equivalent sites on each monomer providing structural basis for the binding of dimeric CXCL12.

4.2 Therapeutic potential of ECL-X4-derived peptides

Considering the increasing number of studies reporting the implication of CXCR4 and CXCR7 in the spread and survival of tumour cells, neutralising their common ligand may be a highly relevant therapeutic strategy [29, 69-71]. In this study, the peptide derived from ECL2 of CXCR4 strongly and specifically interacted with CXCL12, blocking its interactions with these two receptors. ECL2-X4 was however less potent in inhibiting the binding of CXCL12 to CXCR7 than to CXCR4, which may be in part explained by the ten times higher affinity of CXCL12 towards CXCR7 (Fig. 5) [26, 27]. In contrast to peptides derived from the N terminus of chemokine receptors, peptides derived from the extracellular loops do not

require tyrosine sulfation to be fully active [61]. The N terminus of CXCR4 bears three sulfotyrosines at position 7, 12 and 21, which were previously shown to be critical for CXCL12 binding [40, 72]. We have previously observed that an unsulfated peptide derived from the N terminus of CXCR4 (residues 1-40) displayed no CXCL12 inhibition properties in the different assays presented above. This post-translational modification is difficult to introduce at multiple sites of long synthetic peptides due to the lability of sulfate group and usually results in a heterogeneous mixture of sulfated peptide species. This observation emphasises the therapeutic potential of peptides derived from the receptor extracellular loops. However, their potency, affinity (low micromolar range) and stability as well as the pharmacokinetic properties remain to be largely improved through stabilisation in a protein scaffold or by incorporating non-natural residues such as D-amino acids or chemical derivatives. A study on CCL5-derived peptides has previously demonstrated that rational design modification at hot spots allowed improving the overall potency of these peptides by over 100 times to reach the nanomolar range [73, 74]. Therefore, the mutational analysis conducted in this study provides valuable positional information for such further improvements.

In the near future, additional work will be needed to better understand the structural determinants of CRS1 and CRS2 in CXCR4 and CXCR7, as well as to elucidate the ligand-receptor stoichiometry and to determine if the chemokine neutralising properties observed with ECL2-derived peptides can be extended to other CXC receptors.

Highlights

- Peptides corresponding to ECL2 of CXCR4 neutralise CXCL12 interaction with CXCR4 and CXCR7.
- These peptides have also similar properties towards vCCL2, blocking its interactions with the receptor.
- ECL2 of CXCR4 forms multiple contacts with the N terminus of CXCL12 and therefore represents an important structural determinant in CXCR4 activation.
- The binding of CXCL12 at CRS2 is mainly driven by four aspartate residues of ECL2 and the top of TM5.

This study was chronologically the first one that was published in the course of my project. Although it is mainly focused on the CRS2 determinants of CXCR4 and little elements seem directly related to CXCR7, it was in fact during this study that I acquired the skills, experience and developed first tools, which were later to be applied in CXCR7 characterisation. Since the publication of this study, we have extended the range of available methods and some questions, which could not be tackled back then, could easily be addressed now. For instance, by using the functional assays that we established to study the interaction between vCCL2 and CXCR7, described in chapter 4, it would be possible to evaluate whether ECL2-X4 can also block the binding of vCCL2 to CXCR7 and whether it uses similar determinants as it does to bind CXCR4. It would now also be interesting to investigate the vCCL2-binding determinants within the extracellular domains of CXCR7, using a similar peptide neutralisation strategy as described in the above study.

The last chapter will provide a wider view on how the until recently widely accepted models and paradigms for chemokine-receptor interactions have lately been gradually given a lot more nuances.

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Chapter 8

New paradigms in chemokine receptor signal transduction: moving beyond the two-site model

Adapted from Kleist AB, Getschman AE, Ziarek JJ, Nevins AM, Gauthier P-A, Chevigné A, Szpakowska M, and Volkman BF, New paradigms in chemokine receptor signal transduction: moving beyond the two-site model, submitted to Biochemical Pharmacology, March 2016.

Chemokine-receptor interactions are traditionally described by a two-step/two-site mechanism, in which the receptor N terminus *recognises* the chemokine globular core (site 1 interactions), followed by *activation* when the unstructured chemokine N terminus is inserted into the receptor TM bundle (site 2 interactions). Several recent studies challenge the structural independence of sites 1 and 2 by demonstrating physical and allosteric links between these supposedly separate sites. These developments emerge within a rapidly changing landscape in which chemokine receptor function is influenced by receptor post-translational modifications, chemokine and receptor dimerisation, and endogenous non-chemokine ligands. Moreover, advances in the structural and functional characterisation of biased 7TM receptor signalling have altered how we understand promiscuous chemokine-receptor interactions.

In this chapter, new paradigms in chemokine receptor signal transduction will be explored by considering studies that depict more intricate mechanisms governing the chemokine-receptor interactions.

1. Introduction

Chemokine receptors are cell-surface seven transmembrane domain receptors (7TMRs) that mediate a diverse repertoire of functions, such as immune surveillance and embryonic development, by directly regulating cellular migration, adhesion, growth, and survival. They are also implicated in many pathological processes such as atherosclerosis, HIV infection, tumour metastasis, and autoimmune disorders [1]. Due to their prominent roles in so many disease processes, chemokine receptors have been the target of considerable drug development efforts since the discovery of the chemokine-receptor system in the late 1980s [2, 3].

Chemokines and their receptors demonstrate widespread promiscuity, wherein chemokines may bind multiple receptors and *vice versa*. Of the nearly 50 chemokines and 20 chemokine receptors identified in humans, most bind multiple counterparts, with a minority involved in monogamous interactions. Promiscuous interactions among chemokines and their receptors are increasingly recognised as a mechanism to generate diverse signalling and functional outcomes using a discrete set of chemokines and receptors [4, 5]. This characteristic promiscuity may be explained, in part, by their conserved tertiary structure, composed of an unstructured N terminus, conserved mono- or di-cysteine motif (C, CC, CXC, CX_3C, where X represents a non-cysteine residue), extended loop, three anti-parallel β-strands, and C-terminal α-helix (Fig. 1.A) [1]. One or two conserved disulphide bridges constrain the chemokine fold by linking the cysteine motif with the β1-β2 turn (a.k.a. the 30s loop) and the β3-strand.

Chemokine receptor binding and activation is described as proceeding via a two-step/two-site mechanism, a model which dates back to the mid-1990s. This model is alternatively framed by segregating chemokine-receptor interactions *functionally* (two-step) and *spatially* (two-site). In the functional formulation, site 1 provides affinity and specificity, followed by site 2, which elicits receptor activation. In the spatial formulation, site 1 refers to interactions between the receptor N terminus (chemokine recognition site 1, CRS1) and the chemokine globular core, and site 2 refers to contacts between residues in the receptor transmembrane (TM) domain (CRS2) and the unstructured chemokine N terminus [3].

Notably, interactions between chemokines and the receptor extracellular loops (ECLs) are variously ascribed to site 1, site 2, or not included in these models at all [1, 6-9].

Isolation of the receptor N-terminal domain has enabled structure determination of several site 1 complexes but numerous difficulties hindered the characterisation of full-length receptors. Since 2007, technological innovations have made possible the purification and crystallisation of over 100 family A 7TMRs, including chemokine receptors. Until recently, only apo structures or those bound to small molecule antagonists were available [10-12]. In 2015, the first structures of chemokine-receptor complexes were solved, detailing chemokine interactions in the TM domain (site 2) [8, 9]. Combination of these site 1 and site 2 structures recently enabled construction of the most detailed chemokine-receptor model to date [13]; and together, these data highlight numerous contacts that fall outside of the conventional spatial and functional definitions of sites 1 and 2. This, coupled with an increased awareness of biased agonism (preferential activation of G protein or β-arrestin pathways), non-chemokine ligands (*e.g.* ubiquitin, β-defensins), and the expanding roles of post-translational modifications (PTMs; *e.g.* sulfation, polysialylation), underscores how the two-site model may overlook the complexity and diversity of chemokine receptor signalling that we now appreciate two decades after it was proposed [4, 5, 14-17].

The chapter will highlight instances in which the two-site model inadequately addresses more complex features of chemokine-receptor interactions. Although the two-site model has served as a useful framework to understand chemokine receptor activation, and in some cases may sufficiently describe binding and activation, it is advantageous to look beyond the functional and structural roles segregated into site 1 or site 2, to delineate new capacities for interactions that have not been well described by either site, and to include new features that have been discovered since the original conception of the two-site model.

2. Beyond Site 1

2.1 Origins of the two-site model and early studies of the site 1 interface

The two-step/two-site model was realised almost 20 years ago through the work of three contemporaneous studies [18-20]. First, Monteclaro *et al.* used a chimera of the CCR2 N-

terminal domain and the CCR1 transmembrane region to show that the receptor N terminus was sufficient to recognise CCL2 with high affinity and recapitulate the native interaction [18]. Interestingly, the complementary CCR1-CCR2 chimera exhibited a 30-fold decrease in G protein signalling, demonstrating that the CCR2 N terminus is essential for chemokine recognition but not signalling. In a follow-up study it was shown that high-affinity CCL2 binding was dependent upon the presence of the CCR2 N terminus and could be fully recapitulated using only a membrane tethered N-terminal peptide [19]. Crump *et al.* also hypothesised a two-site mechanism through studies of the chemokine rather than the receptor. They showed that mutation of the CXCL12 N terminus attenuated signalling activity without significant loss of affinity [20]. Taken together, these studies suggested that the site 1 and site 2 interactions were spatially and functionally independent, with site 1 conferring receptor specificity and affinity, and site 2 mediating receptor activation. Over time, other functional studies led to the consensus that this model was broadly applicable to the chemokine-receptor system [21].

At the same time other studies began to probe the site 1 interface in greater detail. Alanine scanning of CXCL8 first identified site 1-interacting residues within its N-loop [22]. Unlike CXCL8, CXCL1 is a high-affinity CXCR2 ligand with weak affinity for CXCR1. Exchange of seven CXCL1 N-loop residues with those of CXCL8, a high-affinity CXCR1 ligand, resulted in a molecule capable of recognising both receptors [23, 24]. Using a similar chimera approach, Crump *et al.* showed that insertion of the CXCL12 N-loop into unrelated CXC-family chemokines (CXCL1 and CXCL10) rendered them capable of binding CXCR4 [20]. Subsequent studies expanded the importance of this region for site 1 interactions to other CC and CXC chemokines, establishing the N-loop as a critical motif for receptor recognition [19, 20, 25, 26].

While these and related mutational studies have helped define roles for the N-loop, NMR titration experiments have historically been used to define structural interactions contributing to site 1 recognition. One of the most common NMR-based approaches has been to titrate unlabelled, receptor N-terminal peptides into purified, [*U*-^{15}N]-labelled chemokines to identify chemokine residues that participate in direct site 1 interactions. This and related approaches have been used to map the site 1 for CCL11:CCR3 [27],

CCL21:CCR7 [28], CCL24:CCR3 [29], CXCL8:CXCR1 [30, 31], CXCL10:CXCR3 [25], CXCL12:CXCR4 [13, 32-36], and CX3CL1:CX3CR1 [37]. Collectively, these studies demonstrate the essential role of the chemokine N-loop in directly binding chemokine receptor N termini, and have now been validated by soluble chemokine-receptor structures (discussed in section 2.4).

2.2 Site 1 interactions: chemokine allostery and conformational dynamics

While early studies of chemokine-receptor interactions suggested that site 1 interactions play only a chemokine-anchoring role and are unrelated to functional outcomes, more recent studies argue the opposite. In a study of CXCL8 activation of CXCR2 and CXCR1, Rajarathnam *et al.* identified an important GP sequence in the 30s-loop that, when mutated, had large conformational effects on CXCL8, causing it to activate CXCR1 and CXCR2 in unique ways [38]. While some GP mutants activated both receptors with similar potencies, two mutants (G31A and P32G) displayed a modest reduction in affinity at CXCR2 but completely lost the ability to elicit CXCR2-mediated calcium release. These same mutants lost their capacity of binding and signalling at CXCR1. The study demonstrated that the change in signalling was due to intramolecular interactions between the GP motif of the 30s-loop and the conserved "ELR" motif of the CXCL8 N terminus, which is known to be important for CXCR2 activation. The authors employed molecular dynamics (MD) simulations to show conformational switching for some CXCL8 mutants by converting the 30s-loop between a type-I and type-II β-turn and thereby altering the conformation and orientation of the chemokine N-loop and N terminus allosterically. They suggested that chemokines exist in conformational ensembles, and receptor binding and activation involves conformational selection both on the part of the ligand and the receptor. In this way, different receptor N termini may selectively bind specific orientations of the chemokine ensemble, thereby eliciting unique functional outcomes at various receptors (see section 5.1 on biased signalling).

In another study, the same group examined the role of the residue sandwiched between the conserved cysteines of CXCL8. Conversion of the CXC motif to a CC motif greatly reduced the binding affinity for both CXCR1 and CXCR2 and rendered it incapable of activating

CXCR2 [39]. The mutation did not affect the chemokine fold, dimerisation, or glycosaminoglycan (GAG) binding, suggesting that the attenuated binding and signalling properties were a consequence of altered intramolecular dynamics. Supported again by MD simulations, the authors suggested that allosteric site 1 interactions may in effect 'steer' the orientation of the chemokine N terminus within the receptor orthosteric pocket. Similar studies of vCCL2 and CX3CL1 cysteine motifs suggest that conformational switching may be a more general phenomenon among the chemokine family [40]. These studies demonstrate that subtle structural changes in one chemokine domain can significantly alter receptor activation by eliciting conformational changes in another domain. In effect, these studies challenge the structural and functional independence of site 1 and site 2 interactions.

2.3 Complex roles for receptor post-translational modifications

Farzan *et al.* expanded the scope of interactions underlying site 1 recognition by showing that sulfation of tyrosines in the CCR5 N terminus enhanced affinity for CCL3 and CCL4 [41]. This and later studies broadened the repertoire of post-translational modifications (PTMs) to include glycosylation, demonstrating that in addition to enhancing chemokine affinity, PTMs can regulate functional outcomes of site 1 interactions [17, 21].

Chemokine receptors undergo enzymatic, O-sulfate modification by tyrosylprotein sulfotransferase (TPST) during processing in the *trans*-Golgi network. The presence of these sulfotyrosines (sTyr or sY) at receptor N termini enhances binding of chemokine ligands and affects receptor activation in many chemokine-receptor systems [16, 41-48]. The structural contributions of sTyr modifications to site 1 interactions were defined by the NMR solution structure of a covalently linked CXCL12 dimer bound to the first 38 amino acids of CXCR4 that was enzymatically sulfated at three tyrosine positions. The observation that tyrosine sulfation enhanced CXCR4 peptide binding, which in turn promotes CXCL12 dimerisation, defined an allosteric model in which binding of sTyr peptides at the conserved sTyr pocket causes CXCL12 self-association. For instance, Ziarek *et al.* found that a sulfated heptapeptide corresponding to the Tyr21 region of CXCR4 specifically and preferentially binds to the CXCL12 dimer while promoting dimerisation of WT-CXCL12.

These studies represent the first evidence that binding at a pocket, disconnected from the CXC dimer interface, could allosterically regulate chemokine self-association [49]. Specifically, dynamic NMR studies of CXCL12 revealed that to accommodate dimer formation, the α-helix of CXCL12 rearranges to an almost 90° angle, perpendicular to the β-strands [50]. This large motion is triggered by two residues adjacent to the sulfotyrosine binding pocket that serve as a link to the C-terminal α-helix of CXCL12 [51]. Importantly, self-association of CXCL12 has significant functional effects, as the locked, dimeric version of CXCL12 elicits a unique signalling profile compared to WT-CXCL12 [35].

Glycosylation of receptor extracellular domains occurs during processing in the endoplasmic reticulum (N-linkage of asparagine residues) or Golgi (O-linkage of serine/threonine residues). Recent studies described a novel functional role for chemokine receptor glycosylation in which polysialic acid (polySia) addition to receptor glycans allows them to discriminate between chemokine binding partners [17]. CCR7 is polysialyated by the enzymes ST8Sia II and ST8Sia IV on the surface of patrolling dendritic cells. This rare PTM is used by CCL21, which has an unusual extended C-terminal tail not present in the other CCR7 ligand, CCL19. When CCR7 is polysialylated, CCL21 binds CCR7 with high affinity due to an interaction between the polySia of CCR7 and the C terminus of CCL21. This interaction is thought to release CCL21's tail from an autoinhibitory interaction with its chemokine core, freeing its N terminus to bind and activate CCR7. In the examples described for both tyrosine sulfation and polysialylation, interactions between these receptor PTMs and chemokine site 1 domains dictate unique functional outcomes.

2.4 Complex interactions between receptor N termini and the chemokine core

An early model for the interaction of CXCL8 with the CXCR1 N terminus set the structural precedent for site 1 formation, corroborating the direct involvement of the N-loop and expanding the interface to include the *chemokine cleft*, formed by the N-loop and β2/β3 turn [31]. To date, six site 1 complexes (four NMR [31, 34, 52, 53] and two crystallographic [8, 9]) have been determined in which the receptor peptide adopts three different orientations. Indeed, in all structures except the CCL11:CCR3 complex, the receptor lies nearly perpendicular to the β-sheet axis primarily contacting the N-loop, chemokine cleft and β-

strands, validating the site 1 interface defined by the CXCL8:CXCR1 structure. All six receptors form apolar and electrostatic contacts, often through a highly conserved tyrosine with the chemokine cleft. However, despite this common interface, the site 1 complexes demonstrate considerable architectural diversity. For instance, the direction of the N and C termini of the CXCR4 peptide is inverted when bound to CXCL12 compared to other site 1 complex structures. While a recent review has suggested that this orientation is incompatible with chemokine N-terminal insertion into the TM domain [54], flexible docking of the monomeric CXCL12:$CXCR4_{1-38}$ structure into CXCR4 demonstrates that this distinct directionality may facilitate rotation of CXCL12 relative to the CXCR4 orthosteric pocket so that it is positioned to form extensive site 2 and intermediate-site interactions [13]. Specifically, this model predicts that CXCR4 assumes a bent conformation adjacent to the conserved N-terminal Pro-Cys motif (Fig. 1B, discussed in section 4.1) [13].

Dimeric CXCL12 was recently identified as a biased agonist that induces G protein signalling but is incapable of promoting β-arrestin recruitment or cellular migration [53, 55, 56]. Regardless of quaternary structure, CXCR4 makes specific contacts with the N-loop and chemokine cleft, contributing 50% of the total site 1 binding energy [57], but residues of the CXCR4 N-terminal domain adopt two distinct conformations when bound to CXCL12 monomer and dimer (Fig. 1C-E). For example, CXCR4 residues 7-9 form an intermolecular β-strand with monomeric CXCL12 and residues 4-6 tuck into a hydrophobic pocket bordering the chemokine C-terminal helix (Fig. 1C, D) [53, 58, 59]. Self-association with a second CXCL12 molecule competes for the monomer-specific β1 and helix contacts and displaces those residues of the receptor, which instead form less stable contacts with the opposite protomer (Fig. 1E) [34].

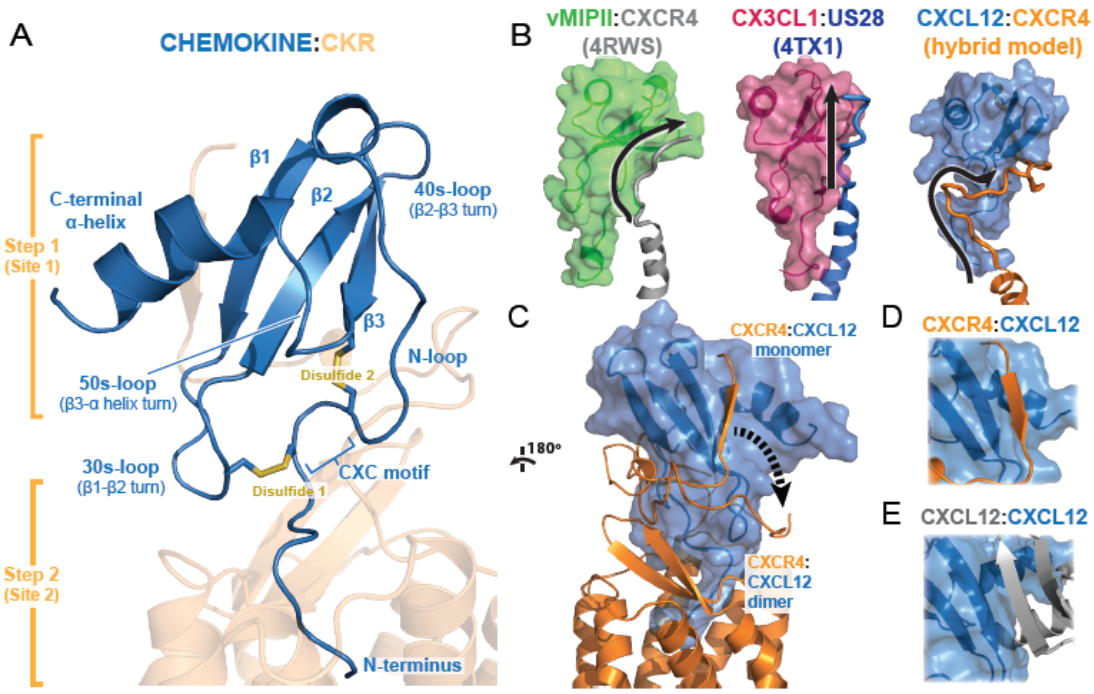

Fig. 1. Chemokine tertiary structure and implications of site 1 binding modes in chemokine-RECEPTOR interactions. (A) The structure of monomeric CXCL12:CXCR4$_{1\text{-}38}$ (PDB ID 2N55) modelled into full-length CXCR4 (PDB ID 3ODU) demonstrates conserved features of chemokine tertiary structure [13]. The two steps/sites of the two-step/two-site model are also depicted. (B) Binding sites of the receptor N-termini are shown on chemokines from the two recent chemokine-receptor co-crystal structures with the chemokine represented in the same view (vCCL2:CXCR4: PDB ID 4RWS; CX3CL1:US28: PDB ID 4TX1) [8, 9]. Only TM1 from each receptor is depicted for clarity. The CXCL12:CXCR4$_{1\text{-}38}$ structure-based model from Ziarek, *et al.* is shown for comparison [13]. The orientation of the receptor N termini varies among the three complexes, suggesting that site 1 contacts may alter subsequent chemokine-receptor interactions. (C) The CXCL12:CXCR4 model based on the CXCL12-monomer:CXCR4$_{1\text{-}38}$ structure is shown ("CXCR4:CXCL12 monomer") along with the CXCR4 N terminus from the CXCL12-dimer:CXCR4$_{1\text{-}38}$ structure ("CXCR4:CXCL12 dimer"; PDB ID 2K01) [34]. CXCL12 dimerisation alters the binding orientation of the CXCR4 N terminus, which may cause unique binding modes at CXCR4 and ultimately lead to different functional outcomes. (D) Close-up view of CXCL12:CXCR4 contacts from (C). (E) The CXCL12 dimer is shown with one subunit in orange (aligned to the CXCL12 monomer from C) and the second subunit in grey. CXCL12 dimerisation occludes the binding site of CXCR4 N terminus residues 1-9.

It is reasonable to assert that the degree to which the receptor N terminus wraps around the globular core modulates the chemokine's orientation and interactions with the receptor ECL and TM regions. In the context of biased agonism, the receptor N terminus may mask or expose epitopes to the receptor ECLs. Taken together, the site 1 interactions may generate functional complexity via unique interactions rather than simply tethering the

chemokine and contributing binding energy. Classic definitions of site 1 fail to recognise the diversity of binding modes and unique domains that can interface with receptor N termini, suggesting a greater spatial and functional complexity than the traditional model would suggest.

3. Beyond Site 2

3.1 A more complicated site 2: the major and minor binding pockets

The recent crystal and NMR structures of chemokine-receptor complexes provide clues that, far from following a two-site convention, interactions are diverse and highly specific for each individual chemokine-receptor pair at the extracellular surface [8, 9, 13]. Similarly, over the past decade, crystal structures of other 7TM receptors have helped to understand how the conserved 7TM architecture recognises diverse ligand types and triggers unique signalling outcomes [60, 61]. In particular, the early 7TM receptor crystal structures divided the orthosteric-binding site into two subpockets [60, 62-64]: the *major subpocket* consists of the cavity defined by TMs 4, 5, and 6, and the *minor subpocket* by TMs 1 and 2. TMs 3 and 7 occupy the interface between the two subpockets and stabilise ligand-receptor interactions in either subpocket [3, 60, 65, 66].

An analysis of over 40 7TM receptor structures revealed that the majority of co-crystallised family A ligands contacted the major subpocket (especially TMs 3, 5, and 6) with few ligands forming contacts in the minor subpocket [67]. Peptide-binding receptors (including chemokine receptors) represented a small minority of the crystallised receptors in this study. Nevertheless, reviews of chemokine receptor binding determinants show a more equitable distribution of contact points among major and minor subpockets, with many chemokine receptor agonists and antagonists preferentially binding the minor subpocket alone [3, 7-10, 66, 68]. This trend is confirmed among the five receptor-ligand co-complexes, with three of the five co-crystallised ligands primarily occupying the minor subpocket (IT1t:CXCR4, vCCL2:CXCR4, and CX3CL1:US28), one ligand primarily occupying the major subpocket (CVX15:CXCR4), and one ligand straddling the two subpockets (maraviroc:CCR5) [8-10, 68].

Chemokine receptors possess a number of unique features that may explain why ligands more readily sample the minor binding-pocket relative to other family A receptors. Firstly, the extracellular portion of TM1 in all chemokine receptor structures is inwardly oriented towards the centre of the TM bundle, with CXCR4-IT1t displaced 9 Å relative to a prototypical family A member, β_2-adrenergic receptor (β_2AR) [10]. TM1 is positioned closer to the adjacent TM7 and creates a more contiguous helix-helix interface [8-10, 68]. Secondly, compared to the β_2AR, the extracellular side of TM1 is 1-2 turns longer when the receptor (CXCR4 or US28) is bound to a chemokine ligand (vCCL2 or CX3CL1) and TM7 is 1-2 turns longer regardless of the associate ligand (discussed in section 4.1). The overall effect of the elongated TM1 and TM7 helices, and the inward orientation of TM1, is to create a larger minor pocket. Another feature that may enrich minor subpocket contacts is that chemokines almost universally bind receptor N termini [7], which themselves are linked to TM7 via a disulphide bond, chemokines are positioned directly above the minor subpocket (Fig. 2A, discussed in section 5.1).

3.2 Role of subpocket specificity in receptor activation

The diversity of chemokine receptor ligand-binding sites emphasises the additional level of regulation built into receptor activation compared to major subpocket-biased 7TM receptors. The major and minor subpockets contain unique sets of residues that comprise *molecular switches* [4, 66, 70]. *Molecular switch*es are conserved receptor "hotspots" that undergo conformational rearrangements following agonist binding, helping to drive global conformational rearrangements required for receptor activation [70]. Interestingly, antagonists frequently engage the same subsets of receptor contacts as agonists [71]. Consequently, site 2 binding might itself be broken down into a series of "choices" dictated by the ligand: 1) selection of the binding-pocket (major subpocket, minor subpocket or a combination of both), and 2) stabilisation of subpocket residues in active (or inactive conformations), both of which will have the effect of engaging (or preventing engagement of) a particular subset of molecular-switch residues required to elicit the ligand-associated functional response (Fig. 2B).

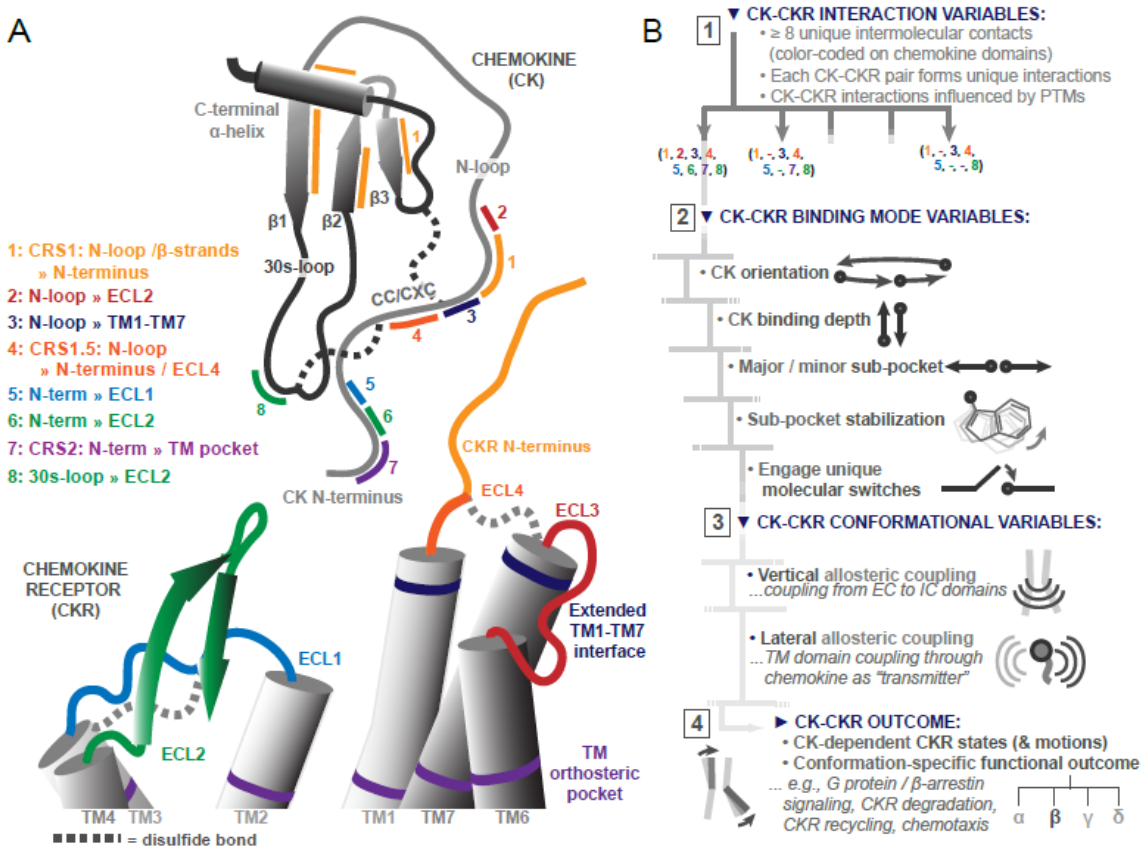

Fig. 2. (A) Chemokine (CK) -chemokine receptor (CKR) interactions encompass many other interactions than those between the chemokine core and the receptor N terminus (site 1) and those between the chemokine N terminus and the receptor TM domains (site 2). **(B)** A "multi-site"/multiple-variable model of chemokine receptor activation [69]. Functionally distinct outcomes following chemokine-receptor interactions may be generated by a unique subset of interactions, binding modes, and conformations. These variables are listed numerically to demonstrate how pairs of chemokine-receptor interactions generate complex outcomes.

Consequently, the diverse binding modes in the major and minor subpockets place each ligand in the proximity of a unique subset of molecular switches. For instance, below the minor pocket is the TxPxW motif (Thr$^{2.56}$-x-Pro$^{2.58}$-x-Trp$^{2.60}$) conserved among most chemokine receptors, although its specific role in receptor activation is not well understood [66, 72]. Pro$^{2.58}$ is important for receptor activation in multiple receptors, including CCR5 [66, 73]. Trp$^{2.60}$ has been consistently identified as a principal binding contact for small molecule antagonists of chemokine receptors, and when mutated, disrupts their inhibitory effects [3, 74]. Interactions between the TxPxW motif and residues in TMs 3 and 7 were also proposed to initiate a concerted rearrangement of the hydrophobic TM region, resulting in receptor activation [13].

Given the unique distribution of both ligand contacts and residues involved in conserved motifs among different TM domains, it is becoming clear that ligand-specific receptor outcomes are a consequence of the stabilisation of specific rotameric states in the receptor-binding pocket followed by engagement of unique subsets of molecular switches.

A chemokine's subpocket "preference" may also depend upon its N-terminal cysteine motif. Qin *et al.* aligned multiple chemokine structures belonging to the CC and CXC subgroups, and noted that CXC chemokines display a characteristic bend immediately preceding the CXC motif, causing their N terminus to run parallel to the N-loop [8]. In models of CXCL12 bound to CXCR4, they predicted that the bend directs the N terminus toward the major pocket, whereas vCCL2 (a viral CC chemokine) directs its N terminus towards the minor subpocket. Since most chemokines are thought to form interactions with receptor N termini, CC chemokines might be predicted to preferentially utilise the minor pocket, whereas CXC chemokines would be able to take advantage of the major binding pocket by redirecting their N termini via the CXC bend. However, despite possessing a distinct "bulge" at its CX3C motif, the N terminus of CX3CL1 inserts into US28's minor subpocket [9, 37]. This suggests that subpocket preference may be more complicated than can be predicted by the CC/CXC/CX3C motif [8, 13]. More structures of chemokine-receptor complexes will be needed to see to what extent the cysteine-motif influences subpocket binding preferences.

3.3 Role of binding depth and chemokine N-terminal length in receptor activation

In addition to the ligand's "choices" to 1) specify a binding pocket, and 2) stabilise subpocket residues, a ligand may also "choose" to bind at a particular depth within that pocket. While four of five chemokine receptor-ligand complexes bind high in the orthosteric binding-pocket relative to other 7TM receptors, maraviroc binds CCR5 at a depth resembling that of many aminergic ligands [8-10, 68]. A review of mutagenic and functional studies suggests diversity in the depths at which different chemokines contact their respective receptors, with some N-termini potentially achieving depths comparable to those of deep-binding aminergic ligands [7]. Additional complex structures will be needed to validate that chemokines may contact receptors at different depths within the TM

domain, however current data suggest that depth variation presents yet another level of complexity within site 2 manipulated by chemokines to achieve specific signalling outcomes.

Chemokine N-terminal length does not necessarily correlate with the chemokine's binding depth, or its functional properties. Early chemokine structure-function studies showed that truncation of the chemokine N terminus transforms chemokine agonists into antagonists [20, 75, 76]. Recent studies of the CCL5 N terminus demonstrate that extension of the chemokine N terminus produces variant-specific functional outcomes, such as receptor internalisation, degradation, recycling, or biased signalling [54, 76]. Similar approaches have since been applied to other chemokines [76]. A recent study by Hanes *et al.* utilised phage display and modelling to suggest how N-terminal length influences receptor function [77]. The authors screened two phage display libraries of CXCL12 for CXCR4 antagonists: a "N-addition" library with a single amino acid addition to CXCL12 and the first four residues of the lengthened chemokine randomised, and a "N-truncation library," where the first four residues were deleted and residues 5-8 randomised. Two results were conclusively found: 1) the N-addition library produced more antagonists, whereas the N-truncation library produced none, and 2) of the N-addition antagonists found, many bound with greater affinity than WT-CXCL12. Interestingly, the screen selected for a subset of variants possessing neutral polar and aliphatic residues, independent of amino acid sequence. The authors propose that despite the "scrambled sequence," similar intermolecular contacts form due to the conformational dynamics of the chemokine N terminus and receptor pocket. These results are consistent with recent NMR studies of the MOR peptide agonist dynorphin, which was highly dynamic even in a receptor-bound state [77, 78]. In sum, these studies suggest that it may be difficult to make generalisations with respect to chemokine N-terminal length as it relates to receptor activation, as examples of elongated and shortened chemokine variants demonstrate diverse outcomes. Moreover, the dynamic nature of the chemokine N terminus suggests that elongated peptides may adopt a more folded structure in the orthosteric pocket, as opposed to "diving" more deeply into the TM bundle [77]. Indeed, comparison of the vCCL2:CXCR4 structure and the CXCL12:CXCR4 model suggests that despite two additional N-terminal residues in vCCL2,

both chemokines reach the same depth by virtue of vCCL2 forming a short N-terminal helix [13].

4. Site 1.5

4.1 Defining unique, non-site 1, non-site 2 interactions at the receptor surface

The first chemokine-receptor crystal structure showed that in contrast to recognising spatially distinct receptor domains, the chemokine formed interactions spanning from the receptor N terminus (site 1) to the receptor TM domain (site 2) [8]. Noting a region that lacked precedence as either site 1 or site 2, Qin *et al.* named an interaction between the chemokine's CC motif and the receptor N-terminal base *chemokine recognition site 1.5* (CRS1.5) (Fig. 2.A) [8]. Similar interactions were observed in the CX3CL1:US28 structure, confirming previous predictions that the N-terminal stalk region serves a direct and essential role in chemokine recognition [9, 79]. An analogous site (CRS1.5-*like*) was identified in the CXCL12:CXCR4 model and its role in binding and activation was validated experimentally [13]. The existence of multiple intermediate interfaces calls into question the assumed *spatial* and *functional* separation between sites 1 and 2, suggesting that other interactions may be overlooked by the two-site model. This section will highlight intermediate chemokine-receptor interactions that do not fall into traditional spatial designations of sites 1 or 2, and will speculate on the functional implications of these interactions.

Diverse chemokine orientations: The most striking difference between the vCCL2:CXCR4 and CX3CL1:US28 structures is the substantial deviation in chemokine orientation relative to the orthosteric pocket of the two receptors [8, 9]. Specifically, vCCL2 and CX3CL1 are rotated ~35^{o} about the C-terminal ends of their C-terminal α-helices (Fig. 3A,B). The CXCL12:CXCR4 hybrid model diverges even more drastically, with CXCL12 rotated ~80^{o} relative to vCCL2 [13]. Importantly, variation in chemokine orientation allows these ligands to form unique but overlapping subsets of interactions with receptor ECLs and TM domains.

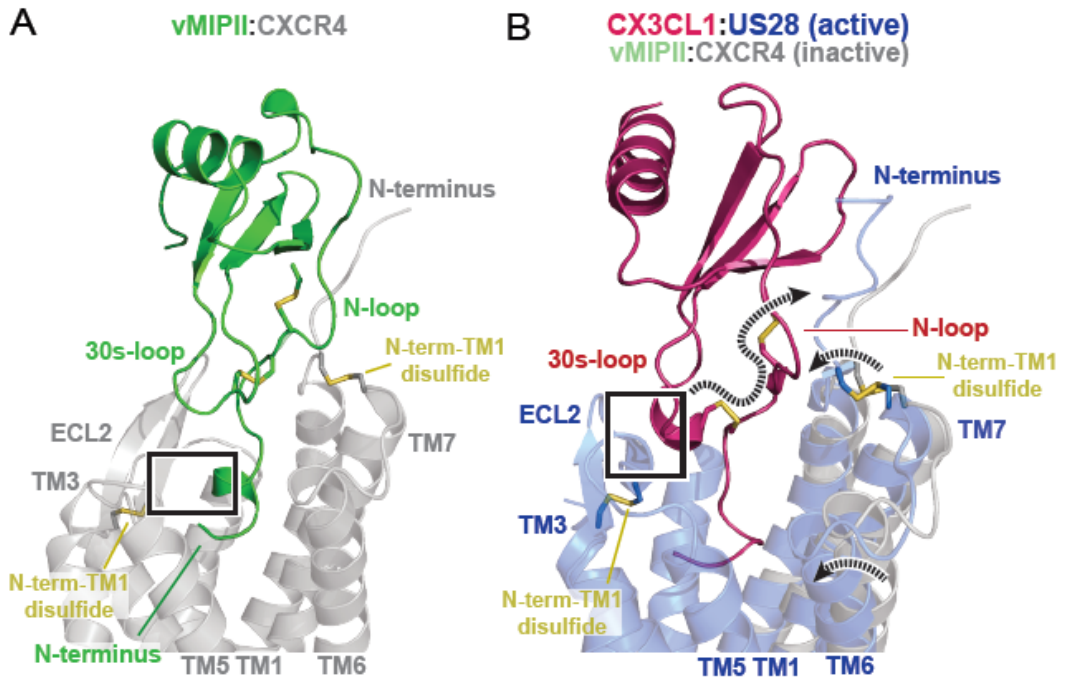

Fig. 3. Unique intermediate interactions specify chemokine orientation in two chemokine-receptor complexes. (A) vCCL2 binds CXCR4 such that its 30s-loop is sequestered from forming extensive interactions with extracellular or TM domains of CXCR4 (PDB ID 4RWS). ECL2 of CXCR4 interacts with the vCCL2 N terminus, boxed. (B) Unlike the vCCL2, CX3CL1 forms extensive intermediate interactions with US28 (PDB1D 4TX1) using its 30s-loop, such that CX3CL1 is stabilised extensively at two separate receptor sites: the N terminus (site 1) and ECL2. These extensive interactions may help stabilise US28 in an active state by simultaneously drawing together extracellular domains of US28, aided by a disulphide network comprised of receptor (N terminus-TM7 or 'ECL4' and TM3-ECL2) and chemokine (CX3C-30s-loop and CX3C-N-loop) disulphides. The active-state US28 structure is overlaid with an inactive-state CXCR4 structure (PDB ID 4RWS) to demonstrate how this disulphide network could facilitate receptor activation (arrows). Unlike the vCCL2:CXCR4 structure, ECL2 of US28 primarily stabilises interactions with the 30s-loop of CX3CL1, boxed.

ECL2 links a 'disulphide network': The orientations of CX3CL1 and vCCL2 relative to their receptor binding pockets demonstrates that CX3CL1, but not vCCL2, is ideally positioned to interact with ECL2 (Fig. 3A,B). CX3CL1 forms multiple interactions between its 30s-loop and ECL2 of US28, which, intriguingly, completes a *disulphide network* spanning from TM3 to TMs 1 and 7. In addition to a family A-conserved disulphide bond between ECL2 and TM3, chemokine receptor possess a disulphide bond that connects the N terminus with TM7 to form an additional ECL, termed "ECL4" [79]. Owing to these two disulphide bridges, rigid body motions of receptor TM domains elicited by a chemokine at one site (*e.g.* N-loop interactions with TM7) could be efficiently communicated to a distant receptor domain (*e.g.* TM3) via a third interface (ECL2-30s-loop interactions), as suggested by

Rajagopalan *et al.* [6]. In short, this structure may provide a glimpse of how multiple-site coupling at extracellular domains might influence chemokine receptor conformation via *lateral (through-chemokine) allostery*.

Chemokine loop engagement: The two chemokine-receptor co-crystal structures exhibit an extended TM interface (relative to other non-chemokine family A 7TM receptors), formed by 1-2 additional α-helical loops at the extracellular portions of TMs 1 and 7, and the disulphide bond linking TM7 to the receptor N terminus (Fig. 2.A) [8, 9]. This extended TM1-TM7 interface may also be important for chemokine binding and orientation, analogous to the 30s-loop-ECL2 interactions of CX3CL1 and US28. For instance, Leu13 in the vCCL2 N-loop forms contacts, albeit weakly, with the extended TM1-TM7 interface (residues Cys274$^{7.25}$ and Glu277$^{7.28}$), as well as the closely positioned Gly273^{ECL3}. These interactions in turn may contribute to the rotation of vCCL2 relative to CX3CL1, causing the vCCL2 30s-loop to be spatially sequestered, preventing the formation of multiple interactions with CXCR4. In effect, these chemokine-receptor co-crystal structures suggest that chemokines utilise different loops to stabilise intermediate (non site 1/site 2) interactions with the receptor, thereby guiding chemokine orientation and likely influencing the signalling behaviour of unique chemokine-receptor pairs.

Diverse functional uses of ECL2: Some descriptions of the two-site model categorise chemokine interactions with ECLs as site 1 interactions due to their *functional* contribution toward specificity and affinity, as well as their *spatial* interactions with the chemokine body [3, 9]. Nevertheless, ECLs also interact with chemokine N termini and strongly influence receptor activation (for instance the ECL2 of CXCR4 interaction described in chapter 6) [6, 7, 80, 81]. The recent chemokine-receptor structures also support the resistance of ECL interactions to site 1 or site 2 classification: CXCR4 preferentially uses ECL2 to stabilise the vCCL2 N terminus, whereas US28 preferentially uses ECL2 to stabilise the CX3CL1 30s loop, making few N-terminal contacts (Fig. 3A, B) [8, 9]. Evidently, each receptor utilises ECL2 for different purposes, likely contributing to the unique binding modes of the respective chemokines.

These structural examples illustrate that in some instances, *spatial* delineation of site 1 and site 2 may be artificial. Moreover, each chemokine-receptor complex uses distinct

combinations of structural domains to specify unique functional outcomes. Having now seen the first comprehensive structural evidence of multiple intermediate, non-site 1/2 interactions, the next section will consider how receptor ECLs take on *functional* characteristics of sites 1 and 2 alike.

4.2 A "multi-site model" accounts for diverse, interdependent chemokine-receptor interactions

While we are only now beginning to appreciate the extent and diversity of chemokine-receptor interactions following high-resolution structural data, pharmacological evidence predating the two-site model supported a more complex "multi-site model" of receptor activation [82, 83]. Studies of CXCR1 and CXCR2 in the 1990s established a number of important principles concerning chemokine receptor recognition and activation as they relate to the receptor extracellular surface, including: 1) different chemokines utilise unique combinations of extracellular domains for the binding and activation of a single receptor [84, 85], 2) a single chemokine may utilise unique combinations of extracellular domains when binding different receptors [84], and more generally 3) chemokine receptor binding and activation is a consequence of multiple, interdependent variables, particularly the identity of the chemokine and the simultaneous interactions it makes with all adjacent extracellular domains (N terminus, ECLs 1-3) (Fig. 2A, B) [82-85]. Similar pharmacological and structural studies expanded these principles to other chemokine-receptor pairs, including CCR1 [80, 86, 87], CCR2 [18, 88, 89], CCR3 [90, 91], CCR5 [18, 87, 92-95], CXCR1 [96-98], CXCR2 [96, 97], CXCR3 [99], CXCR4 [81, 100], CX3CR1 [101].

CXCR3 provides an illustrative example of the interdependence of chemokine "multi-site" binding, chemokine preference for unique receptor conformations, and associated functional outcomes. Xanthou *et al.* disputed the universality of the two-site model for chemokine ligands, proposing instead a "multi-site model in which several distinct extracellular domains are required for efficient ligand binding and receptor activation" [69]. The authors created "gain-of-function" chimeras by individually replacing the N terminus, ECL1, ECL2, and ECL3 of CXCR3 with the equivalent regions of CXCR1 (which does not share ligands with CXCR3) and *vice versa* to create "loss-of-function" chimeras. They found

that while absence of ECL2 did not abolish chemokine binding, it completely attenuated CXCR3-mediated signalling, supporting a role beyond chemokine recognition. In addition, they showed that each chemokine required a unique subset of interactions to activate CXCR3: CXCL9 required ECL2 and ECL3; CXCL10 required all extracellular domains; and CXCL11 required the N terminus, ECL1, and ECL2.

From these experiments it is clear that in addition to possessing *spatial* characteristics of sites 1 and 2, ECL2 may in some instances possess *functional* characteristics of sites 1 and 2. Moreover, this study suggests a "multi-site" mechanism by which different chemokines could, in principle, elicit functionally distinct downstream outcomes. Two lines of evidence support such a mechanism. A recent study demonstrated that the three CXCR3 ligands elicit unique patterns of $G\alpha_i$ actvation, β-arrestin recruitment, and internalisation following CXCR3 stimulation [5]. Secondly, CXCL10 preferentially binds inactive conformations of CXCR3, whereas CXCL11 binds both inactive and active conformations [102]. In all, these data show that chemokines can preferentially bind unique subsets of extracellular domains and/or available receptor conformations to elicit specific functional outcomes. By illustrating the interdependence of multiple extracellular domains on chemkokine receptor signal transduction, these studies undermine the functional separation of sites 1 and 2 into recognition and activation, and imply that cooperative interactions influence chemokine recognition at extracellular chemokine receptor domains and subsequent functional outcomes.

4.3 Molecular switches at the extracellular surface: allosteric coupling to the TM region

As suggested in the previous section, the role of ECLs is more nuanced than static stabilisation of chemokine ligands. Dynamic interactions between ECL and TM residues may act as molecular switches regulating receptor activation, especially in the case of ECL2. While ECL2 displays high variability in sequence, length, and structure even among related receptor subtypes, an impressive number of family A receptors seem to utilise ECL2 in this capacity, including rhodopsin [103-106], the serotonin 5-HT4 receptor [107], the V(1a) vasopressin receptor (V1aR) [108], the cannabinoid receptor 1 (CB1) [109-111], the β_2AR [112], the angiotensin II type 1a receptor (ATII1aR) [113, 114], the D2 dopamine receptor

(D2R) [115], the C5a complement receptor (C5aR) [116], and protease activated receptor 1 (PAR-1) [117], among others [118, 119]. Considering CXCR4, it has recently been suggested that manipulation of ECL2 by CXCL12 draws ECL2 toward the orthosteric pocket, thereby moving TMs 2 and 3 closer to one another [13]. These TM movements may then help initiate receptor activation, in an analogous mechanism to that predicted for CCR5 [120].

In addition to ECL2, ECLs 3 and 4 have been suggested to act as a tandem molecular switch required for CXCR4 activation [79, 100]. Using mutagenesis and a yeast-based $G\alpha_i$ protein activation screen, Rana *et al.* showed that interaction between TM7 and the CXCR4 N terminus is essential for receptor activation, and that replacement of the disulphide-bonded cysteines with an electrostatic pair (Arg-Glu) conserves CXCR4 signalling. The authors suggest a model in which the N-terminal-TM7 disulphide undergoes a conformational change during receptor activation that is transmitted to ECL3 and TM6. Comparison of the active state CX3CL1:US28 and inactive state vCCL2:CXCR4 structures supports this model (Fig. 3.B) [8, 9]. Compared to CXCR4, US28 demonstrates an inward motion of ECL4, seemingly driving an inward motion of ECL3 and the extracellular portion of TM6. In a well characterised mechanism, inward motion of the top of TM6 causes it to rotate about a conserved proline "kink," resulting in substantial outward movement at the intracellular face to accommodate G protein binding [121]. Similar ECL4 motions may contribute to CXCR4 activation [13].

These examples suggest that chemokine binding to extracellular domains "primes" the receptor for activation by stabilising an intermediate conformation, followed by chemokine N-terminal insertion into the receptor TM core and intracellular coupling of signalling effectors (G protein or β-arrestin).

5. Beyond canonical chemokine receptor signalling

5.1 Beyond 1:1 interactions: stoichiometry of chemokine-receptor interactions

5.1.1 Chemokine dimerisation

Initially thought to be a crystallisation artefact, chemokine dimerisation has been re-examined over the past years in numerous structural and biochemical studies. It appears now that the vast majority of chemokines are able to form dimeric species, with the monomer-dimer equilibrium being regulated by factors such as pH, anions and interactions with glycosaminoglycans [122, 123].

Depending on the family, chemokines adopt two main oligomeric states with unique structural arrangements and interaction interfaces. CC chemokines form flexible and extended dimers mainly through residues surrounding the cysteine motif [124, 125], whereas CXC chemokines self-associate in more compact dimers via interactions involving the first β-strand [123, 126, 127]. In both types of interactions the N termini of the two monomers are pointing in opposite directions. Chemokines of the XC family, XCL1 and XCL2, have recently been shown to exist in a monomer-dimer equilibrium, unusually requiring complete protein unfolding. XCL dimers adopt a novel dimer conformation that also creates a six-stranded β-sheet [128, 129]. CX3CL1, the only member of the CX3C family, dimerises in a similar manner to that of CC chemokines [130]. Additionally, some chemokines have been observed to form tetramers (CCL2, CCL27, CXCL4, and CX3CL1) or higher-order oligomers [130-133]. Heterodimers of two different CC or CXC chemokines as well as cross-family CC/CXC heterodimers have also been reported [134-136]. Furthermore, HMGB1 (high mobility group protein B1) protein was reported to form complexes with CXCL12, promoting different conformational rearrangements of CXCR4 from that of CXCL12 alone [137]. These findings further challenge the two-step binding model for chemokine-receptor interactions and complicate the question of which stoichiometries are capable of generating functional responses.

Immobilisation of chemokines on glycosaminoglycans (GAGs) is an important step for chemokine function as it creates a gradient to direct cell migration and regulates the local chemokine concentration and availability for their receptors. Likewise, GAG binding can

favour dimer formation as demonstrated for CCR2-binding chemokines [135], CXCL8 [138, 139], CXCL12 [140-143], XCL1 and XCL2 [128, 129]. Oligomerisation has also been shown to increase GAG affinity by creating a more extensive surface for interactions [123].

The biological relevance of chemokine dimerisation is still a matter of debate and its exact impact on receptor binding, stoichiometry and biased signalling remains to be unravelled [123, 127, 144, 145]. As an illustration it has been demonstrated that monomeric and dimeric CXCL12 induce different intracellular signalling responses and opposite effects on cell migration, but other recent studies suggested that this receptor interacts with CXCL12 in a 1:1 stoichiometry [35, 146].

5.1.2 Receptor dimerisation

Throughout the past two decades, it has been assumed that chemokine receptors exist as monomers, which behave as fully competent signalling units. This assumption, in part, forms the basis of the classical two-site binding model. However, a number of studies demonstrated that chemokine receptors can form homodimers and/or heterodimers (Fig. 4) [150]. Chemokine receptor dimerisation has been investigated by various biochemical approaches such as co-immunoprecipitation (co-IP) [151-154], protein fragment complementation (PFC) [155, 156], Förster/bioluminescence resonance energy transfer (FRET/BRET) [157-159] and GPCR heteromer identification technique (GPCR HIT) [160, 161]. The first structural evidence of chemokine receptor dimerisation however was provided by the first inactive-state crystal structures of CXCR4 in which the receptor was present as a dimer with the interface between the subunits located at the top of TM5 and TM6 and stabilised by hydrogen bonds [10]. Chemokine receptors from all four subfamilies (C, CC, CXC, CX3C) have now been described to form homo- or heterodimers *in vitro* [152, 162-164] and some of them, including CXCR4 and CCR5, were shown to interact with other families of GPCRs such as the α1A/B-adrenergic receptors [165], opioid receptors [166] or non-GPCR membrane proteins that modulate the activity of the receptor or act as co-receptor for certain non-conventional ligands (Fig. 4) [167]. Receptor dimerisation has been shown to modify ligand binding properties [153, 168] and receptor signalling [151, 165, 169, 170] as well as intracellular trafficking [156]. However, so far there is no *in vivo* data

reporting the existence of chemokine receptor dimers and therefore their biological relevance remains controversial [150, 171].

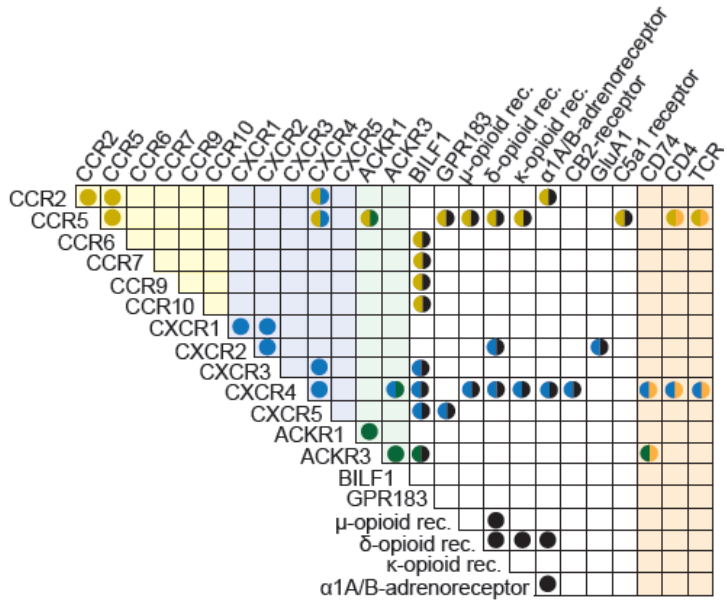

Fig. 4 Chemokine receptor homo- and heterodimerisation. Interactions between receptors are represented by dots. CC, CXC and atypical chemokine receptor subfamilies are represented in yellow, blue and green, respectively. Non-chemokine receptor GPCRs and non-GPCRs are represented in black and orange, respectively. Homodimers are indicated with one-colour dots and heterodimers between receptors from different families by two-colour dots (monomer 1/ monomer 2).

5.1.3 Stoichiometry of chemokine-receptor complex

Another poorly understood and highly debated facet of chemokine-receptor interactions is their stoichiometry in functional signalling complexes. As both chemokines and receptors can homo- and heterodimerise, novel hypotheses around the stoichiometry of their interactions have emerged, leading to more complex models than the initially proposed two-step/two-site model. Among them, the 1:2 stoichiometry model where one chemokine binds two receptors simultaneously (Fig. 5A), the 2:1 stoichiometry model in which a chemokine dimer binds one receptor (Fig. 5B), and finally, the 2:2 stoichiometry model in which both the chemokine and the receptor interact as dimers (Fig. 5C) [8, 21, 146, 147]. Complementation studies carried out with CXCR4 mutants partially deficient in site 1 (CRS1) or site 2 (CRS2) were inconsistent with a 1:2 stoichiometry model and supported CXCR4 monomers as fully competent signalling units [146]. These results were later supported by the crystal structure resolution of the viral chemokine vCCL2 in complex with CXCR4 and CX3CL1 in complex with US28, both revealing a 1:1 stoichiometry interaction and an extensive contact surface between the two partners [8, 9]. However,

more recent investigation of the preferential binding of monomeric CXCL12 to either monomeric (1:1) or dimeric (1:2) CXCR4 by molecular dynamics simulations proposed that in the 1:2 stoichiometry model, the N terminus of the chemokine could make more tight contacts with the CRS2 of the second monomer to more efficiently favour signalling than in the 1:1 stoichiometry [147]. Finally, studies of CXCR4:CXCR7 heterodimers suggest that upon CXCL11 binding to CXCR7, conformational changes propagate through the dimer interface activating CXCR4 without the need of its own ligand (1:2*stoichiometry) (Fig. 5D) [148]. Gathering structural and mechanistic information on receptor dimerisation, chemokine/receptor stoichiometry and relating it to functional observations remains challenging and necessitates state-of-the-art techniques to strengthen or to invalidate the currently accepted but oversimplified two-step/two-site model [9, 21, 146].

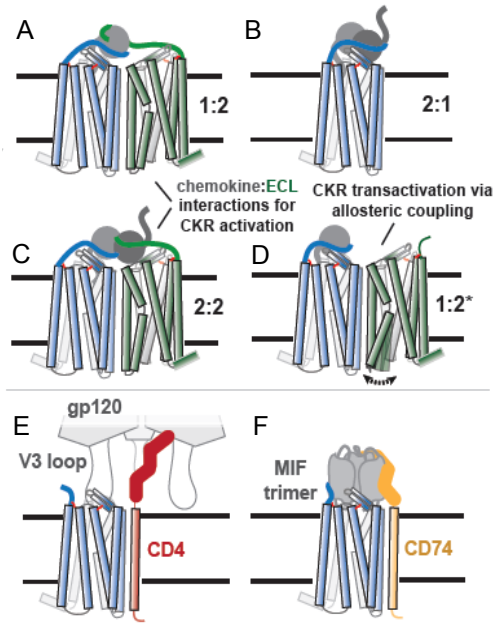

Fig. 5. Models of chemokine-receptor stoichiometry and non-canonical ligands. (A) 1:2 stoichiometry in which a chemokine monomer binds a receptor dimer. The disulphide bridges between N terminus/ECL3 and ECL2/TM3 are depicted as red lines. **(B)** 2:1 stoichiometry in which a chemokine dimer binds a receptor monomer. **(C)** 2:2 stoichiometry in which a receptor dimer binds a chemokines dimer. **(D)** 1:2* stoichiometry in which a receptor dimer interacts with a monomeric chemokine. Upon the binding of the chemokine to one monomer (receptor 1, blue), the conformational changes induced in receptor 1 are propagated to receptor 2 (green) through the dimer interface, activating receptor 2 without the need of chemokine binding. **(E)** Binding and activation of chemokine receptors CCR5 or CXCR4 by the HIV gp120 envelope protein require another membrane protein, CD4 (red), which acts as a primary receptor, inducing conformational rearrangements to expose the V3 loop. **(F)** Binding and activation of CXCR2, CXCR4 and CXCR7 by the pseudo-chemokine MIF also requires the presence of a primary receptor, CD74 (yellow). Like for chemokine interactions, the stoichiometry of MIF:CD74:receptor is not well established.

5.2 Beyond G protein signalling: biased signalling at chemokine receptors

Once proposed to serve redundant signalling and functional roles, promiscuous chemokine-receptor interactions are now widely believed to confer signalling and functional complexity [1, 4-6]. Individual chemokines regulate multiple essential functions via independent chemokine receptor interactions. In turn, the consequences of each unique interaction depends upon concurrent spatial and temporal expression of both partners [149]. Further complicating matters, chemokine receptors were until recently accepted to signal exclusively through canonical G protein pathways and to couple exclusively to the $G\alpha_{i/o}$ G protein subtype. However, mounting evidence shows that some they may also signal through other G protein subtypes ($G\alpha_s$, $G\alpha_{q/11}$ or $G\alpha_{12/13}$) and activate G protein-independent signalling cascades (*e.g.* via β-arrestin) in ligand- and cell-specific contexts [150-152]. Analogous findings have been described for countless non-chemokine 7TM receptors for over a decade, signifying the new paradigm known as biased signalling or functional selectivity [153, 154]. Biased signalling appears. Biased signalling has been subdivided into three categories: ligand bias, receptor bias and tissue or cell bias [4, 5, 155, 156].

Chemokine ligand bias occurs when different chemokines bind the same receptor to elicit distinct cellular responses. Ligand bias has been well documented for both CC and CXC chemokines, including CCL19 and CCL21 at CCR7 [151, 157, 158], CCL27 and CCL28 at CCR10 [5], three chemokine ligands at CXCR3 [5, 158], CXCL7 and CXCL8 at CXCR2 [159], as well as for chemokine ligands at CCR1 [5, 160], CCR2 [161] and CCR4 [162, 163]. Intriguingly, biased responses may be elicited by chemokines bearing unique PTMs, including truncation, citrullination or dimerisation as reported for CCL14 [164] and CXCL12 [35]. The characteristic promiscuity of the chemokine-receptor network and poor sequence identity among chemokines, may partly explain the prevalence of bias [5]. Indeed, variation of the structural interactions discussed in previous sections (summarised in Fig. 2B) such as chemokine orientation, ECL contacts, and major/minor subpocket selection likely stabilises distinct active forms of the receptor, eliciting preferential coupling to different intracellular effectors [165]. While we are far from defining the precise structural mechanisms underpinning biased signalling, studies of other family A 7TMRs suggest a

role for helical movements of and direct physical interactions with TMs 5 and 6 for G protein and TM7 for β-arrestin coupling, respectively [4, 121, 165-168].

Furthermore, a chemokine agonist at one receptor can act as an antagonist towards another receptor. Chemokines activating CXCR3 can also bind CCR3, blocking CCL11-induced cell migration and G protein signalling [169]. Similarly, CXCL11 and CCL7, well-characterised agonists at CXCR3 and CCR1/CCR2, respectively, were found to be antagonists at CCR5 [170-172]. In another example of *dual activity* chemokines, vCCL2 binds human and viral chemokine receptors across all four families, acting as an antagonist or an agonist at different receptors [173]. Dual activity is often observed in cross-family interactions and could relate to differences in chemokine N-terminal orientation among CC and CXC chemokines, as discussed above (see section 3.2) [8]. Nevertheless, antagonism within the same family has also been reported, suggesting that other determinants may also play a role [172, 174].

Receptor bias occurs when a particular receptor preferentially or exclusively couples to a particular effector even in the context of multiple different ligands. Receptor bias has been well-characterised at atypical and viral chemokine receptors [175]. For instance, CXCL12 binding to CXCR4 elicits both G protein and β-arrestin signalling [150, 176], whereas binding to CXCR7 elicits G protein-independent β-arrestin signalling exclusively [177].

Cellular bias occurs when the same chemokine-receptor pair triggers distinct signalling pathways or cellular responses in different cellular contexts. For instance, CCL19 binding to CCR7 induces chemotaxis only in certain cell types [178, 179]. Such cellular bias is unsurprising considering the large variety of cells expressing chemokine receptors, each of which carry unique expression profiles of signalling effectors (*e.g.* G protein subtypes and β-arrestin isoforms), receptor modifying enzymes (*e.g.* GRKs, TPSTs), as well as of other chemokine receptors or receptor-modulating partners involved in dimeric receptor interactions.

5.3 Beyond chemokines: binding and signalling by non-chemokine ligands

The chemokine receptors CCR2, CCR3, CCR5, CXCR2, CXCR4 and CXCR7 bind endogenous or virus-encoded ligands other than chemokines. These unconventional ligands vary widely in size, ranging from large proteins (*e.g.* > 100 kDa) to peptides, and often have no sequence or structural similarities with chemokines [15, 180-182]. Despite their structural dissimilarities, non-chemokine ligands can trigger signalling pathways similar to those induced by endogenous chemokines, although in some cases they initiate unconventional signalling responses [15, 180-185]. For some, binding and signalling relies on the chemokine receptor alone [15], while for others, the chemokine receptor operates in tandem with another membrane protein that usually serves as primary receptor [183, 186].

One of the best-known examples of non-chemokine ligands for chemokine receptors is the HIV envelope protein gp120 (120 kDa), which uses CCR5 and CXCR4 as co-receptors for cell type-specific recognition and entry into host cells. To initiate viral membrane fusion with host cell membranes, gp120 first binds to CD4, a primary single TM segment receptor, initiating conformational changes (Fig. 5E). These changes then expose gp120's third variable loop (V3 loop), which in turn interacts with CCR5 or CXCR4 [186-188]. The second interaction was suggested to occur in a two-site binding mechanism similar to that initially proposed for chemokines, with common interacting determinants [184]. Importantly, not only is the interaction of gp120 with CXCR4 or CCR5 required for cell-specific HIV entry but it also leads to the activation of signalling pathways such as JNK and MAPKs, facilitating the early steps of viral replication [189, 190]. Tat, the HIV-trans-activating protein (14 kDa) released extracellularly by infected cells, triggers G protein-mediated signalling and chemotaxis through CCR2 and CCR3 [191, 192] and acts as an antagonist of CXCR4 [193]. Similarly, the HIV-1 matrix protein p17 binds CXCR1 and CXCR2, inducing chemokine-like activity on monocytes through Rho/ROCK activation [194, 195].

More recently, the pseudo-chemokine MIF (macrophage migration inhibitory factor), a pleiotropic and proinflammatory chemotactic cytokine of 12.3 kDa highly expressed by tumour cells, has been identified as a ligand for CXCR2 [181], CXCR4 [182] and CXCR7 [183], inducing ERK1/2 and ZAP-70 signalling and chemotaxis. As with gp120, the binding of MIF to chemokine receptors requires a primary receptor, CD74, a single segment

membrane-spanning protein also known as HLA class II histocompatibility antigen gamma chain (Fig 5F). Although MIF possesses some chemokine-like features, including a pseudo-ELR motif (D45-X-R129) and an N-loop-like region (amino acids 48-57), it lacks the canonical cysteine motif and is therefore classified among the chemokine-like function (CLF) chemokines.

In addition to CXCL12, CXCR4 also binds extracellular ubiquitin (eUb, 8.6 kDa). The eUb-CXCR4 interaction was proposed to follow a two-site binding mode, leading to G protein signalling similar to that induced by CXCL12 [15]. Other endogenous non-chemokine ligands such as human β3-defensin (HDB-3) (5.1 kDa) [196] and EPI-X4 (1.8 kDa) a 16-amino acid peptide derived from human albumin [197] also interact with CXCR4 but fail to induce intracellular signalling. Finally, human cytosolic proteins such as histidyl- and asparginyl-tRNA synthetases, released in some inflammatory pathologies, were shown to induce leukocyte migration through CCR3 and CCR5 [198]. Similar results were reported for parasitic asparginyl-tRNA synthetases which act as agonists for CXCR1 and CXCR2 [199].

The identification of non-cognate ligands for chemokine receptors, some exclusive to a single receptor, others interacting with several receptors across several subfamilies, further emphasises the complexity of the chemokine-receptor network, which seems now more promiscuous and predisposed to bias than initially thought. These new ligands will certainly help to uncover other important physiological and pathological functions for this family of receptors, explain past observations and provide new therapeutic opportunities to modulate chemokine or receptor activity.

Highlights

- Despite the seemingly redundant character of chemokine-receptor interactions, each chemokine-receptor pair uses a unique set of interacting residues, leading to stabilisation of a particular receptor conformation and to various functional outcomes, which, together with the cellular context, is at the basis of functional selectivity.
- Post-translational modifications of chemokine receptors control their ligand affinity and selectivity, and hence also the functional outcomes of ligand-receptor interactions.
- Both chemokine and their receptors can dimerise and the stoichiometry of their interactions to date is not well understood but the oligomeric state of the interacting partners may also influence the functional outcomes.
- Chemokines seem to preferentially use the minor subpocket within the TM region of chemokine receptors, which is larger relative to other receptors due to the elongated TM1 and TM7 helices and the inward orientation of TM1 and the positioning of chemokines directly above the minor subpocket following its interactions with the receptor N terminus.
- Site 1 and site 2 interactions are far from being structurally and functionally independent. Interactions at site 1 can for instance induce conformational changes within the chemokine and hence affect the interactions at site 2. The extracellular loops of chemokine receptors can also spatially and functionally contribute to both site 1 and site 2 interactions.
- The existence of endogenous but non-chemokine ligands able to bind and signal through chemokine receptors may be more widespread than initially thought.

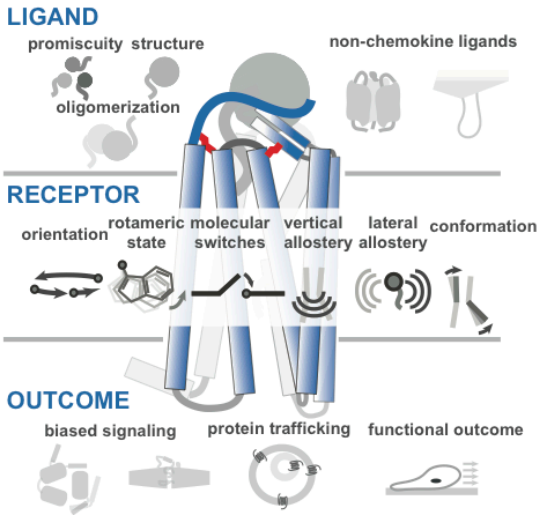

Fig. 6. Diversity of chemokine-receptor interactions. Multiplicity of ligands, structural consequences within the receptor and the functional outcomes.

This chapter is a fruit of highly stimulating discussions that we had during a recent chemokine-receptor congress with Brian Volkman and his collaborators. Their group is well known for their work on the structural aspects of chemokines and chemokine receptors and for proposing the first NMR data describing the interactions between CXCL12 and the N terminus of CXCR4. Together, we have decided that there were enough structural and functional data available to defend our shared view on how the generally accepted model of chemokine-receptor interactions should be revised because of its numerous limitations. The discussions resulted in a collaborative manuscript, which was recently submitted and on which this final chapter of the thesis was based.

It has to be metioned that, although we have actively participated in the revision and editing of the initial sections on site 1 and site 2 interactions, it was the collaborating group that took the lead in writing these parts. We were in turn in charge of tackling the questions on biased signalling, chemokine and chemokine receptor dimerisation as well as the emergence of alternative non-chemokine ligands for chemokine receptors.

The numerous examples given in this final chapter illustrate well how rapidly the understanding of chemokine-receptor interactions is evolving, challenging the past models and paradigms. Yet, the emergence of previously unsuspected mechanisms of biased agonism and alternative ligands for chemokine receptors testifies for how much in the field still remains to be elucidated.

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Conclusions and perspectives

During the last years, from the start of the project until today, the understanding of chemokines and their receptors has evolved considerably. Personally, it was an entirely novel experience to witness such changes within a field of research. Many of the paradigm-altering discoveries concerned CXCR7.

During the course of the project, we have obtained a series of interesting data about the molecular recognition and activation of CXCR7 by it ligands. This thesis presented our contribution to the understanding of this atypical chemokine receptor. In addition to the practical work done in the framework of my project, the thesis also included a substantial theoretical part. Indeed, before approaching each question experimentally, we needed guidance on its relevance and the direction to take. Our group being relatively new to the field of chemokine receptors, an in-depth study of the literature was often necessary. In addition, while reviewing the work of others, we were able to propose new research tracks and to identify new signatures within the N termini of chemokine receptors.

The field of interest of our group had been extended to CXCR7 when the first reports suggestive of its uncommon properties appeared in the literature. My project was the first one in the laboratory centred on CXCR7 and at beginning most of the tools and assays needed to be set up and validated, which was not always a straightforward task. Nevertheless, although it surely has taken me more time than it would have, had I worked within an already well-established group, it also taught me a lot and was an enriching experience.

The identification of vCCL2 as a new ligand for CXCR7 was one of the most important outcomes of the project and has uncovered the potential role of this interaction in HHV-8 infection. However, more efforts are now needed to understand the biological relevance of this finding and whether the ability of vCCL2 to bind CXCR7 gives an advantage to the virus or the host.

The identification of vCCL2-CXCR7 interaction also offers the possibility to better understand the receptor molecular biology through comparative studies with other CXCR7 endogenous ligands. Further extending the investigation to CXCR3, CXCR4 and the chemokine ligands shared with CXCR7 provides a simplified model for the complex interaction network of chemokine receptors allowing to discern differences in their ligand recognition and activation mechanisms.

We were able to show that various modifications in N termini of chemokines have less impact on their interactions with CXCR7 than with the classical receptors CXCR3 and CXCR4, suggesting that CXCR7 has different activation mechanism, which could be linked to its scavenging functions not only for native chemokines but also modified truncated chemokines.

The constantly growing number of reports stressing the important roles of CXCR7 in both physiologic and pathologic processes emphasises the need to better understand this receptor and at the same increases the competitively in the field, as more and more groups become interested in this receptor. Yet, there is still a lot of mystery behind CXCR7, as its functions or its interplay with chemokines and other receptors remain not well understood. One of the reasons for this may be the difficulty of choosing the appropriate cellular background to study CXCR7, independently of the ubiquitously expressed CXCR4, with which it shares a ligand.

Probably the liveliest debate around CXCR7 concerns its signalling properties. A consensus seems now to have been reached that CXCR7 cannot trigger G protein signalling but its ability to signal via arrestin has lately also been questioned. In our experience, we have never observed CXCR7 signalling in response to its chemokine ligands, leading us to believe that CXCR7 is a silent receptor, its predominant role being ligand scavenging. However, it cannot be excluded that depending on the cellular context, CXCR7 may indeed trigger signalling, be it the canonical Gi protein, arrestin-mediated signalling or even signalling through other G protein isoforms.

The results obtained in the course of my project seem to legitimise the recent classification of CXCR7 among the atypical chemokine receptors. Indeed, in addition to its controversial signalling properties, the ability to scavenge chemokines of both CC and CXC families and its general apparent “agonist bias”, CXCR7 also carries two additional cysteines within its N terminus. The analysis of the N terminal sequences of chemokine receptors, initially done for the two publications presented in the first chapters of this thesis, made us realise how unique this characteristic was. We were, therefore, very excited and had for long put most of our interest in the part of the project examining the two additional cysteines and the potential four-amino acid loop they may create through a disulphide bridge. However, to our disappointment, the preliminary results indicate that these cysteines seem to have little effect on chemokine binding or

CXCR7 activation. Nevertheless, in parallel to other work, we continue our investigation of the disulphide bond, to first confirm its existence by mass spectrometry, with the final goal to determine the role of the still hypothetical arch.

Interestingly enough, at a conference held last June, during which we proposed vCCL2 as a third ligand for CXCR7, a group from the University of North Carolina presented their data on the potentially crucial role of CXCR7 in the development of the cardio-vascular system through its interaction with a fourth ligand, a non-chemokine protein, adrenomedullin, belonging to the calcitonin gene-related peptide family (CGRPs) [1]. Interestingly, before its deorphanisation, CXCR7 had already been proposed, in an unnoticed study, as an adrenomedullin-binding receptor, triggering classical G protein signalling, which contrasts with its response to chemokines [2]. Nothing is yet known on the mechanism and the molecular determinants of the interaction between adrenomedullin and CXCR7. However adrenomedullin in known to bind to another GPCR (CGRPR), although a receptor activity-modifying protein (RAMP), which defines the specificity of CGRPR for either adrenomedullin or other CGRPs, is required for this interaction. Remarkably, adrenomedullin, like all the members of the CGRP family, bears a four-amino acid loop reminiscent of the hypothetical TPA in the N terminus of CXCR7 (Fig. 1).

Fig. 1. Structural similarities between the N terminus of CXCR7 and CGRPs and conservation of the internal ring/arch. (A) Sequence alignment of human adrenomedullin (ADM), calcitonin gene-related peptide (CGRP), amylin and adrenomedullin-2 (ADM-2) and the N terminus of CXCR7 (Nterm-X7). Conserved cysteine residues involved in the disulphide bridge forming the internal arch/ring are represented in blue. **(B)** Possible structural mimicry of adrenomedullin by the N terminus of CXCR7.

The internal loop of adrenomedullin and of other CGRPs is proposed to interact with RAMPs. The identification of adrenomedullin as a ligand for CXCR7 and the presentence of similar structural features in both interacting partners is intriguing and raises many questions about the potential interplay between CXCR7, RAMPs,

adrenomedullin and chemokines and the possible signalling outcomes of such a crosstalk (Fig. 2).

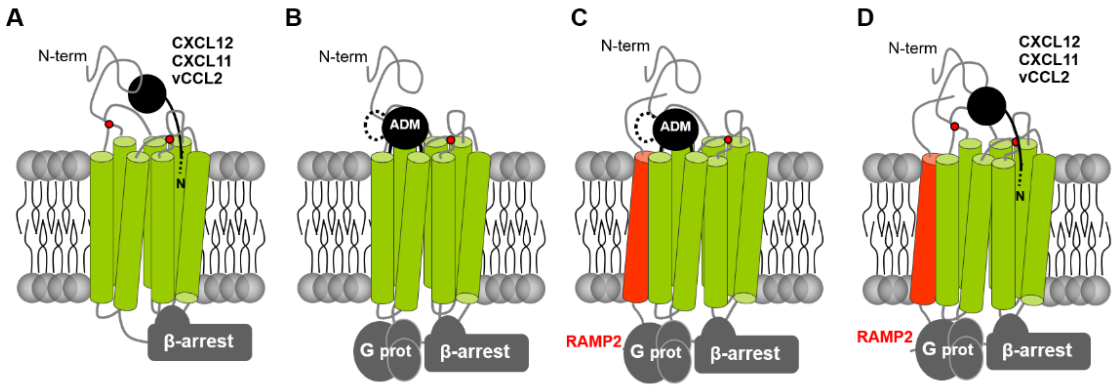

Fig. 2. Interaction and signalling network of CXCR7 with chemokines, adrenomedullin and interacting partners, RAMPs. (A) Interactions of CXCR7 with its endogenous chemokines (CXCL12 and CXCL11) and the viral chemokine vCCL2 only β-arresting recruitment. **(B)** Hypothetic binding of adrenomedullin to CXCR7 in the absence of RAMP2 is proposed to induce G protein signalling and possibly arrestin recruitment. **(C)** Binding of adrenomedullin to the CXCR7-RAMP2 oligomer may trigger G protein and arrestin recruitment **(D)** CXCR7-RAMP2 oligomers show different signalling properties in response to chemokines than CXCR7 alone.

A follow-up project aiming at investigating these questions has recently been submitted and will hopefully provide additional interesting data on CXCR7.

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Appendices

Biochemical Pharmacology 84 (2012) 1366–1380

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Review

Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors

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ARTICLE INFO

Article history:
Received 16 June 2012
Accepted 9 August 2012
Available online 21 August 2012

Keywords:
Chemokine receptor
Receptor N-terminus
Chemokine
CXCR4
Sulfotyrosine

ABSTRACT

Chemokines and their receptors play fundamental roles in many physiological and pathological processes such as leukocyte trafficking, inflammation, cancer and HIV-1 infection. Chemokine-receptor interactions are particularly intricate and therefore require precise orchestration. The flexible N-terminal domain of human chemokine receptors has regularly been demonstrated to hold a crucial role in the initial recognition and selective binding of the receptor ligands. The length and the amino acid sequences of the N-termini vary considerably among different receptors but they all show a high content of negatively charged residues and are subject to post-translational modifications such as O-sulfation and N- or O-glycosylation. In addition, a conserved cysteine that is most likely engaged in a receptor-stabilizing disulfide bond delimits two functionally distinct parts in the N-terminus, characterized by specific molecular signatures. Structural analyses have shown that the N-terminus of chemokine receptors recognizes a groove on the chemokine surface and that this interaction is stabilized by high-affinity binding to a conserved sulfotyrosine-binding pocket. Altogether, these data provide new insights on the chemokine-receptor molecular interplay and identify the receptor N-terminus-binding site as a new target for the development of therapeutic molecules. This review presents and discusses the diversity and function of human chemokine receptor N-terminal domains and provides a comprehensive annotated inventory of their sequences, laying special emphasis on the presence of post-translational modifications and functional features. Finally, it identifies new molecular signatures and proposes a computational model for the positioning and the conformation of the CXCR4 N-terminus grafted on the first chemokine receptor X-ray structure.

Contents

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URL: http://www.crp-sante.lu

0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2012.08.008

1. Introduction

Chemokine receptors are rhodopsin-like G protein-coupled receptors (GPCRs) displaying a structure typical of this family that consists of seven hydrophobic membrane-spanning α-helices separated by alternating hydrophilic extracellular (ECL) and intracellular (ICL) loops. The N-terminus of the receptor is situated on the outside of the cell and participates in ligand binding whereas the C-terminal tail is located on the intracellular side. Upon ligand binding, chemokine receptors activate intracellular heterotrimeric G proteins triggering downstream signaling pathways that result in a variety of cellular responses. Additionally, non-signaling receptors such as decoy receptors were shown to control the cellular response to chemokines by sequestration and modulation of their local concentration [1,2].

Chemokines and their receptors regulate vital cellular mechanisms including migration, adhesion as well as growth and survival [3,4]. Chemokines control processes such as embryonic development, angiogenesis and hematopoiesis but can be also released under stress. These inducible chemokines play crucial roles in adaptive and innate immune response, wound healing and organ repair by attracting effector cells to the site of infection or injury [5–7]. Many chemokines are also involved in pathological processes including inflammatory and autoimmune diseases as well as cancer [4,8–10]. In addition, some pathogens interfere with the host chemokine/chemokine receptor network to promote their own survival by either encoding chemokine receptors/chemokines or co-opting chemokine receptors for host cell entry.

Chemokines are generally believed to interact with their cognate receptors according to a two-step model [11,12]. The initial step corresponds to the anchoring of the chemokine to the N-terminus of the receptor and is followed by the binding of the flexible chemokine N-terminus to the extracellular loops and the transmembrane segments of the receptor. Numerous studies illustrate the great importance of the extracellular parts, and in particular the receptor N-terminus, in discriminating between the various chemokine ligands.

Despite their low level of sequence identity, all chemokines display a common monomeric structure consisting of a flexible N-terminus followed by an N-loop, three anti-parallel β-strands and a C-terminal α-helix [13,14]. The N-terminal domain contains one or two cysteines implicated in structure-stabilizing disulfide bonds. Based on the positioning of these cysteines, chemokines are divided into four groups: C, CC, CXC and CX_3C [15]. Accordingly, chemokine receptors are named CR, CCR, CXCR or CX_3CR. The chemokine-receptor network is very complex and a given chemokine may bind to several receptors, while a chemokine receptor usually has multiple ligands. To date, 50 chemokines and 20 receptors have been identified in humans.

The N-terminal domains vary considerably in length between different chemokine receptors, also within subfamilies. They do however display a number of common features, including high content of negatively charged residues, tyrosine sulfation motifs and N-glycosylation sites. In addition, a highly conserved disulfide bond links the N-terminus and the third extracellular loop. Some of these characteristics of the chemokine receptor N-terminal domain have been shown to strongly influence ligand binding as well as the cellular responses.

Given the implication of the chemokine network in many pathologies, a better understanding of the mechanisms driving ligand binding to chemokine receptors is essential for the development of highly specific therapeutic molecules targeting either the receptors or more recently the chemokines. To accurately comprehend these interactions, three-dimensional structures of chemokine receptors would be needed. Yet, their resolution has proven particularly arduous mainly due to the difficulties in purifying and crystallizing these proteins. To date, CXCR4 is the only chemokine receptor for which the three-dimensional structure has been resolved [16]. The spatial arrangement of its N-terminus, however, could not be determined. Nevertheless, multiple alternative approaches have been used to investigate the interactions of the N-terminus of chemokine receptors with their ligands. Chimeric, mutated or truncated receptors have long been widely exploited [17–23]. In parallel, soluble synthetic peptides derived from the N-termini of chemokine receptors have been used as models for the binding of ligands to full-length receptors [24,25]. In particular, the NMR studies of interactions between the receptor N-terminus-derived peptides and chemokines have provided substantial functional and structural information in this regard [26–33]. Additionally, grafting of the N-terminus together with another extracellular loop on the B1 domain of protein G soluble scaffolds allowed examining ligand interactions in contexts that are more reminiscent of native receptors [34,35]. Other approaches aimed to investigate the N-terminus of chemokine receptors in more membrane-like environments such as micelles or phospholipid bilayers as well as in fusion with membrane proteins [24,36,37]. Thanks to this constantly growing arsenal of methods and increasingly powerful tools, remarkable progress has been made towards the elucidation of ligand interactions with chemokine receptors.

The present review gives an outline of the information currently available on the diversity and function of human chemokine receptor N-terminal domains. Additionally, it provides a comprehensive annotated inventory of the chemokine receptor N-terminal sequences, laying special emphasis on the presence of post-translational modifications, sequence signatures and functional features. In this review, chemokines and chemokine receptors will be referred to by their systematic nomenclature.

2. Sequence diversity of chemokine receptor N-terminal domains

2.1. Length and molecular signatures

Chemokine receptors present relatively short N-terminal domains ranging from 26 (CX3CR1) to 65 (DARC) amino acids compared to the N-terminal domains of up to 600 amino acids in other GPCRs. Notably, in all chemokine receptors except for CXCR6, the N-terminal domains bear a conserved cysteine residue in their second moiety. This cysteine is likely to be engaged in a disulfide bridge with the third extracellular loop of the receptor (ECL3) and delimits two functional parts characterized by different sequence features: the M–C part including residues from the N-terminal methionine (M) to the cysteine (C) and the C–TM part including the residues from the cysteine to the first transmembrane segment (TM). While the M–C parts are in general described as very flexible, the C–TM parts link the TM1 and TM7 through a disulfide bridge forming a pseudo-loop at the surface of the receptor (Fig. 1A and B) (see Section 2.2.1).

In all chemokine receptor families, the M–C parts show variable length, low sequence identity, overall negative charges and contain multiple tyrosine and asparagine residues that are post-translationally modified. The size of the M–C parts varies from 21 to 51 amino acids and is not fixed within a family. Moreover, there seems to be no correlation between their length and the selectivity of the receptor. Low identity observed in the M–C parts supports their implication in ligand selectivity. Except for their overall negative charges, the presence of sulfotyrosines (see Section 2.2.2) and of potential N-glycosylation sites (see Section 2.2.3), no specific signatures seem to be present and conserved in the M–C parts.

The C–TM parts are shorter (5–20 residues), display variable net charges within the CC and decoy receptor families and are neutral

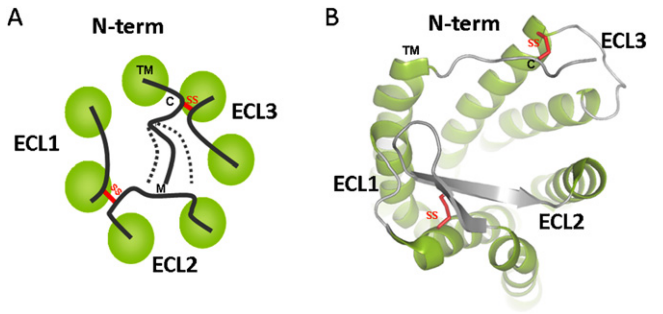

Fig. 1. Top-down representation of chemokine receptor surface (A) Schematic representation. The seven transmembrane (TM) segments are represented as green circles. The two disulfide bridges connecting the N-terminus to ECL3 and ECL1 to ECL2 are colored in red and indicated by SS. The N-terminal part of the N-terminus (M–C part) is flexible and unstructured in the absence of chemokine. The conserved cysteine (C) forms a disulfide bridge (red) with ECL3 linking TM1 and TM7. (B) X-ray structure (PDB ID: 3OEO), clear density was only observed for C–TM part, starting at residue P28.

or negative in CXC receptors, contain no sulfated tyrosines or glycosylation sites. The only exception is CXCR7 which bears a putative N-glycosylation site three residues before the predicted TM1. Despite the apparent low identity and size variation, we identified new signatures conserved within the C–TM parts of different chemokine receptor families. Receptors CCR1, CCR2, CCR3, CCR4, CCR5 and CCR9 present longer C–TM parts characterized by a length of 18 residues and the conservation of a scattered motif K-X_3-K/R-X_7-PPLYS/W separated from the cysteine by one residue. In contrast, CCR6, CCR7, CCR8, CCR10, all CXC receptors, D6 and CCX-CKR display shorter C–TM parts (10 or 11 residues) characterized by the conservation of a negative charge ($E/D_{+3/+4}$) 3 or 4 residues after the cysteine and a positive charge ($K/R_{+9/+10}$) preceding the TM1. In other receptors such as XCR1, CX3CR1 and DARC, no particularities or features allowing their classification in one of these two families were found.

2.2. Post-translational modifications

2.2.1. Disulfide bridge

Chemokine receptors typically bear one cysteine residue in each extracellular domain. While the two cysteines present in ECL1 and ECL2 are a characteristic of nearly all rhodopsin-like GPCRs and form a structurally and functionally critical disulfide bridge [38], the other two cysteines situated in the N-terminus and ECL3 are a particularity of chemokine receptors and their role is not as well-established. Indeed, although the conservation of these residues as a pair in all chemokine receptors except CXCR6 indicates their importance for receptor biology, most likely through disulfide bridge formation, somewhat diverging results have been reported in the literature.

In an early study, it was shown that CXCR1 treatment with diamide, a bifuctional sulfhydryl reagent that oxidizes thiol groups and leads to formation of disulfide bonds, resulted in a functionally inactive receptor and reduced CXCL8 (IL-8) binding [39]. Approaches using alkylating agents also pointed to the existence of free thiols in the extracellular domains of CXCR1 [39] and in the N-terminus and ECL3 of CCR6 [40].

The results from numerous other studies however strongly put forward the role of the N-terminus–ECL3 cysteine pair in receptor functions. Indeed, the mutation of one or both cysteines from the N-terminus and ECL3 decreased chemokine CCL2 (MCP1), CCL5 (RANTES), CXCL12 (SDF1), CXCL8 (IL-8) binding and chemokine-induced signaling in CCR2, CCR5, CXCR4, CXCR1 and CXCR2, respectively [37,41–44] as well as DARC interactions with chemokines [45]. However, it was shown for CCR2 that the cysteine present in the N-terminus is not directly involved in the interactions with CCL2 [37]. Similarly, a study with a constitutively active N119S-CXCR4 demonstrated that mutants carrying a salt bridge C28R/C274E or an aromatic pair C28F/C274F retained some of the activity of the receptor. It was further proposed that the N-terminus–ECL3 cysteine pair may stabilize the active state of CXCR4 [46]. Interestingly, the mutation of this cysteine pair in the two major HIV-1 co-receptors, CXCR4 and CCR5, seems to have little effect on the gp120 binding to the receptors [41,47].

The most compelling and direct evidence of the existence of a disulfide bridge between the N-terminus–ECL3 cysteines arises from one of the recently resolved CXCR4 X-ray structures (PDB ID: 3OEO), in which the N-terminus cysteine at position 28 is linked to ECL3 cysteine at position 274 (Fig. 1B). Interestingly, the helix VII of CXCR4 is two turns longer than in other GPCR structures, allowing the optimal positioning of C274 for this interaction [16]. Moreover, the proline residue directly preceding C28, also present in many other chemokine receptors, may play a crucial role in orienting the N-terminus regions in the vicinity of this cysteine to facilitate the disulfide bridge formation. Long-time molecular dynamics simulation confirmed the continuous presence of the disulfide bridge and suggested that its formation may be favored by the interactions between other residues from the M–C part and ECL3 (unpublished results, see Supplementary data [159,160]). The constraint imposed by the disulfide bond may fashion the chemokine binding pocket and/or be of importance in the correct positioning of the M–C part for chemokine binding or for its further interactions with the receptor (site II) (see Section 3.1). Indeed, in the case of CXCR4, the disulfide bond delocalizes the M–C part from TM1 to the top of TM7 and facing the second extracellular loop, which is proposed to participate in the second step of the binding mechanism. Additionally, by linking the TM1 and TM7 the disulfide bridge may stabilize the three-dimensional structure of chemokine receptors by locking the transmembrane segments in a circular arrangement (Fig. 1). Moreover, since ECL3 connects TM6 and TM7, which are proposed to participate in conformational changes that trigger receptor activation, the N-terminus–ECL3 disulfide bridge was suggested to have a role in the coupling of ligand binding to receptor activation [48].

However, it still remains to be determined whether the disulfide bridge observed in CXCR4 structure is also present in other chemokine receptors and whether it is permanent or dynamic (i.e. formed upon ligand binding). In addition, the impact of the C–TM length differences as well as the presence of signatures among the newly identified families (see Section 2.1) on disulfide bridge formation and on the distance between TM1–TM7 need to be addressed.

2.2.2. Tyrosine sulfation

In addition to their high glutamate and aspartate content, all M–C parts of chemokine receptor N-termini display at least one tyrosine residue that may potentially be post-translationally modified by the addition of a negatively charged sulfate to their hydroxyl groups. The reaction of tyrosine O-sulfation is catalyzed by the Golgi tyrosylprotein sulfotransferases (TPST-1 and TPST-2) and has been shown to play important roles in the regulation of protein–protein interactions of many secreted and transmembrane proteins [49]. Studies with sulfated chemokine receptors however have proven to be difficult mainly due to the lability of the sulfate group. To date, the presence of sulfated tyrosines has been demonstrated for only six human chemokine receptors: CCR2b, CCR5, CXCR3, CXCR4, CX_3CR1 and DARC (see Table 1) [22,50–55]. By means of various approaches including site-directed mutagenesis, treatment with sulfation inhibitors or sulfatases, using both whole receptors and N-terminus-derived peptides, it could be

shown that O-sulfation of their N-termini is critical for high-affinity binding to chemokines as well as for the recognition of the HIV-1 gp120 protein [22,50–55]. Notably, all these chemokine receptors bear a sulfated tyrosine located approximately nine residues before the conserved cysteine. Sequence analysis indicates that this potential sulfation site ($_{p}$sY) is present in almost all the receptors, arguing for the existence of a common sulfotyrosine-dependent ligand binding mode. Although the exact importance of sulfotyrosines within the chemokine receptor N-termini is not fully understood, the distribution of highly polarizable electrons on both the sulfate and the phenyl group make sulfotyrosines perfectly suitable to be accommodated by the positively charged pocket at the surface of the receptor ligands [32,33,56,57]. Indeed, recent structural modeling and NMR measurements suggest that all chemokines harbor a conserved sulfotyrosine-binding pocket, providing a molecular basis for sulfotyrosine conservation observed among chemokine receptors (Fig. 4). The presence of such sulfotyrosine-binding pocket was experimentally determined for four chemokines representative of the different families (XCL1 (Lymphotactin), CCL5 (RANTES), CXCL12 (SDF1) and CX3CL1 (Fractalkine)) [58]. In particular, for CXCL12, structural data demonstrated that the sulfotyrosine-binding pocket is defined by the residues V18^{CXCL12}, R47^{CXCL12} and V49^{CXCL12} located near the hydrophobic groove delimited by the N-loop and the third beta-strand (see Section 3.1, Fig. 4B).

However, besides the presence of the conserved potential sulfation site, many chemokine receptors bear multiple tyrosine residues whose post-translational modification is not equally important for ligand recognition [53,59,60]. These sulfotyrosines however also seem to contribute to the high-affinity chemokine binding as illustrated for the CXCR4/CXCL12 interactions, in which sulfation of the receptor tyrosine 7 and 12 in addition to the conserved sY21 increases the affinity for the chemokine over six-fold (K_DsY$_{21}$ ≈1 .3 μM versus K_DsY$_{7/12/21}$ = 0.2 μM) [61]. However, while the interacting partner of sY12, the K27^{CXCL12}, is well identified on the monomeric form of the chemokine, the interaction site of sY7 is not clearly defined and may involve a pocket formed upon chemokine dimerization or interaction with other receptor extracellular domains (Fig. 4B and C). Interestingly, the involvement of K27^{CXCL12} in heparin binding may also suggest that the N-terminus negatively charged residues and in particular sulfotyrosines play a role in heparin displacement prior to receptor binding [29]. Sulfation of tyrosines may additionally favor an extended conformation of the M–C part of the N-terminus. Indeed, we performed long time molecular dynamics for CXCR4, with or without sulfate groups at position 7, 12 and 21 and demonstrated that repulsive interactions caused by the negative charges of the sulfate groups prevent the internal collapse of the N-terminal domain thereby maintaining it in an open conformation accessible for ligand binding (Fig. 2) (see Supplementary data).

The prediction of protein tyrosine sulfation sites remains problematic. Nevertheless, although a specific signature could not be clearly identified among the proteins that are O-sulfated, several consensus features seem to be required for TPSTs activity. (a) Acidic residues are generally found in the vicinity of sulfated tyrosines, whereas basic amino acids abolish the reaction [62,63]. Another possible determinant for TPST activity is (b) a certain degree of flexibility of the peptide chain, as small or turn-inducing residues are often present close to sulfation sites [62,63]. Moreover, (c) disulfide bridges and N-glycosylation sites have been proposed to interfere with tyrosine sulfation [63,64]. Similarly, in silico identification of modified tyrosines remains challenging as sulfation prediction algorithms are often very restrictive. The sulfation prediction tool Sulfinator [65] for instance fails to identify the sulfation of tyrosines 7 and 12 of CXCR4, which has been determined experimentally. Moreover, in vitro sulfation of N-terminus peptides derived from receptors bearing multiple sulfotyrosines was shown to be sequential but also incomplete, giving rise to products displaying a variety of sulfation patterns that differentially affect the binding to chemokines. These observations point to the existence of a mechanism for regulation of ligand affinity/specificity towards sulfated receptors [55]. Moreover, TPST-1 and 2 show different tissue expression patterns and play distinct but overlapping biological roles [64,66–68]. The two isoenzymes also display different kinetic properties and show differences in substrate specificities as well as pH optima, which strengthens their possible involvement in chemokine-receptor network regulation [69,70].

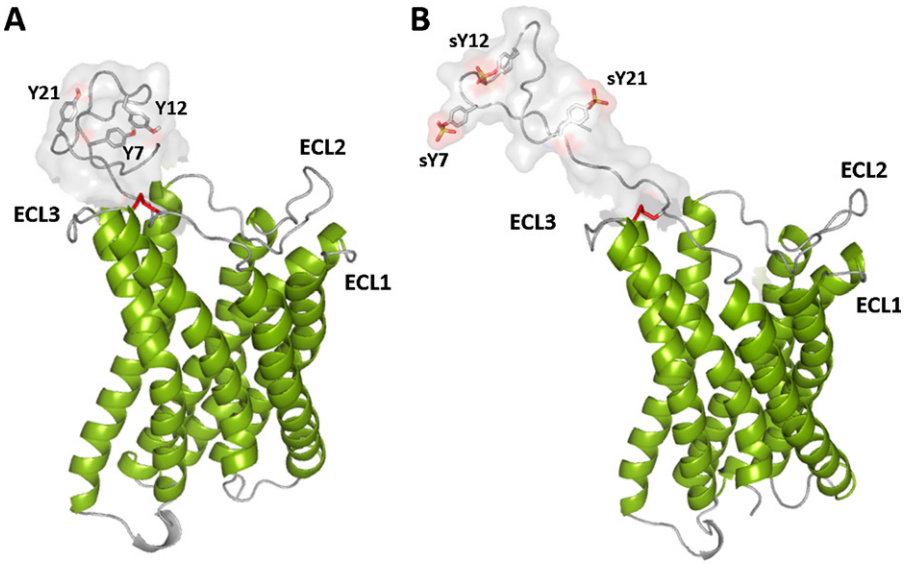

Fig. 2. Impact of tyrosine sulfation on CXCR4 N-terminus conformation. CXCR4 N-terminus with non-sulfated tyrosines (A) and CXCR4 N-terminus with sulfotyrosines (B) derived from the last snapshot (20 ns) of MD simulation carried out with the whole receptor. Receptor helical structures are shown in green; ECLs, ICLs and N-terminus are represented in gray; tyrosine and sulfotyrosine residues are displayed as sticks and the disulfide bond between the N-terminus and ECL3 is colored in red. Guided MD simulations suggest that in absence of sulfate groups the N-terminus tends to collapse forming a condensed structure, whereas tyrosine sulfation creates repulsive interactions promoting the adoption of a an extended structure largely accessible for chemokine binding (see Supplementary data).

Table 1
Sequence, length, charge and post-translational modifications of C chemokine receptor N-terminus.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco
XCR1	XCL1 XCL2	RA	MESSGNPESTTFFYYDLQSQPC-ENQAWVFAT	31 (-4)	22 (-3)	9 (-1)	2	0

2.2.3. Glycosylation

Like other transmembrane receptors, chemokine receptors may also be post-translationally modified by the addition of sugar moieties either to the amide group of asparagine residues (N-glycosylation) or to hydroxyl groups of serine or threonine residues (O-glycosylation). N-glycosylation occurs at the consensus sequence N-X-S/T, where X is any amino acid except proline, while O-glycosylation sites are less well characterized and generally comprise serine/threonine-rich regions. These post-translational modifications occur in the Golgi and are catalyzed by a series of glycosyltransferases and glycosidases that shape the carbohydrate chains. Most chemokine receptors bear one or two putative N-glycosylation sites as well as serine/threonine doublets or triplets within their M–C part. While no specific position or molecular signature can be defined for N-glycosylation, clusters of serine or/and threonine residues are generally found about two to four amino acids on either side of the conserved sulfated tyrosine (see Section 2.2.2). Experimental data on human chemokine receptor glycosylation are however scarce and only five receptors have been shown to carry N-linked (CCR2B, CXCR2, CXCR4 and DARC [50,55,71–73] or O-linked (CCR5 [74]) carbohydrate moieties in their N-terminus (see Tables 1–5). The exact role of N-terminal domain glycosylation remains unclear. Similarly to other GPCRs, glycosylation of the extracellular domains of chemokine receptors has been proposed to increase their flexibility or to directly participate in ligand binding. Indeed, depending on the nature of the carbohydrate chains, glycosylation may provide additional negatively charged moieties for electrostatic interactions with the positively charged chemokines. While the presence of sialyted O-glycans in CCR5 N-terminus (S6 and S7) was shown to be important for high-affinity binding to CCL3 (MIP1α) and CCL4 (MIP1β) [74], N-glycosylation of CXCR2 (N17), CXCR4 (N11) and DARC (N16) appears to have no influence on CXCL7 (NAP2), CXCL12 and CXCL8 interactions, respectively [73,75,76]. CXCR2 glycosylation was however shown to be crucial for receptor maintenance on the cell surface, chiefly by protecting it against protease degradation. Furthermore, N-glycosylation patterns have been suggested to have an impact on the subcellular distribution of CXCR2 [73]. Additionally, although in the case of CCR5 it has been shown that O-glycosylation at S6 and S7 does not impair sulfation of Y10 [74], the vicinity of carbohydrate chains was proposed to negatively influence tyrosine sulfation [64]. It was also postulated that differential CXCR4 N-glycosylation may contribute to the presence of structurally and functionally distinct receptor isoforms [77]. Therefore, glycosylation of the receptor N-terminus is likely to be of greater importance than initially appreciated and in particular cell-dependent glycosylation patterns may represent an additional level in the finely tuned regulation of the chemokine network. In addition, glycosylation of the CXCR4 N-terminus was shown to influence HIV-1 co-receptor usage (see Section 3.2).

Table 2
Sequences, lengths, charges and post-translational modifications of CC chemokine receptors N-termini.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CCR1	CCL3 CCL5 CCL7 CCL8 CCL13 CCL14 **CCL15** CCL16 **CCL23**	AR AS AT CA COPD HIV MS PS RA	METPNTTEDYDTTTEFDYGDATPC-QKVNERAFGAQLLPPLYS	42 (-6)	24 (-7)	18 (+1)	2	1	
CCR2	**CCL2** CCL7 CCL8 CCL11 CCL13 CCL16	AS CA COPD HIV LP MS RA	MLSTSRSRFIRNTN*ESGEEVTTFFDY*DYGAPC-HKFDVKQIGAQLLPPLYS	50 (-0)	32 (-2)	18 (+2)	2	1	(50)
CCR3	CCL2 CCL5 CCL7 CCL8 **CCL11** CCL13 CCL15 **CCL24** **CCL26** CCL28	AS CA COPD HIV	MTTSLDTVETFGTTSYYDDVGLLC-EKADTRALMAQFVPPLYS	42 (-4)	24 (-4)	18 (0)	2	0	(59, 60)
CCR4	CCL17 CCL22	AD AS CA DI IBD PS	MNPTDIADTTLDESIYSNYYLYESIPKPC-TKEGIKAFGELFLPPLYS	47 (-4)	29 (-4)	18 (0)	4	0	
CCR5	CCL3 to CCL5 CCL8 CCL11 CCL14 CCL16	AR AS AT CA CH COPD HIV IBD MS PS RA	MDY*QVS*S*PIY*DINY*Y*TSEPC-QKINVKQIAARLLPPLYS	38 (0)	20 (-3)	18 (+3)	4	0	(51, 74, 163)
CCR6	CCL20	CA IBD PS	MSGESMNFSDVFDSSEDYFVSVNTSYYSVDSEMLLC-SLQEVRQFSRL	47 (-6)	36 (-7)	11 (+1)	3	2	
CCR7[w]	CCL19 CCL21	CA IBD MS	QDEVTDDYIGDNTTVDYTLFESLC-SKKDVRNFKA	34 (-4)	24 (-7)	10 (+3)	2	1	
CCR8	**CCL1** CCL4 CCL16 CCL17	AD AS	MDYTLDLSVTTVTDYYYPDIFSSPC-DAELIQTNGKL	36 (-5)	25 (-4)	11 (-1)	4	0	
CCR9	CCL25	CA IBD	MTPTDFTSPIPNMADDYGSESTSSMEDYVNFNFTDFYC-EKNNVRQFASHFLPPLYW	56 (-5)	38 (-7)	18 (+2)	3	1	
CCR10	CCL27 CCL28	AD CA PS	MGTEATEQVSWGHYSGDEEDAYSAEPLPELC-YKADVQAFSRA	42 (-6)	31 (-7)	11 (+1)	2	0	

Table 3
Sequences, lengths, charges and post-translational modifications of CXC chemokine receptors N-termini.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CXCR1	CXCL1 CXCL6 **CXCL8**	AS CA COPD IBD PPP RA	MSNITDPQMWDFDDLNFTGMPPADEDYSPC-ML*E*TETLN*K*Y	40 (-8)	30 (-7)	10 (-1)	1	2	(27)
CXCR2	CXCL1 to CXCL3 CXCL5 to CXCL8	AS AT CA COPD IBD PS RA	MEDFNMESDSFEDFWKGEDLSN*Y SYSSTLPPFLLDAAP C-EP*E*SLEIN*K*Y	49 (-10)	39 (-8)	10 (-2)	2	1	(50)
CXCR3	CXCL9 to CXCL11	AR AS AT CA CH COPD DI IBD LP MS PS RA	MVLEVSDHQVLNDAEVAALLENFSSSY*DY*GENESDSC CTSPPC-PQ*D*FSLNFD*R*A	54 (-9)	43 (-8)	11 (-1)	2	2	(22, 54)
CXCR4	CXCL12	AS AT CA HIV RA	MEGISIY*TSDN*Y*TEEMGSGDY*DSMKEPC-FREENANFN*K*I	39 (-6)	28 (-6)	11 (0)	3	1	(29, 52, 61)
CXCR5	CXCL13	CA LP	MNYPLTLEMDLENLEDLFWELDRLDNYNDTSLVENHL C-PAT*E*GPLMASF*K*A	51 (-8)	38 (-8)	13 (0)	2	1	
CXCR6[ψ]	CXCL16	CA MS	MAEHDYHEDYGFSSFNDSSQEEHQDFLQFS*K*V	32 (-4)	32 (-4)	-	2	1	
CXCR7	CXCL11 CXCL12	CA	MDLHLFDYSEPGNFSDISWPC-NSS*D*CIVVDTVMCPNMPN*K*S	41 (-4)	21 (-3)	20 (-1)	1	3	

Table 4
Sequence, length, charge and post-translational modifications of CX3C chemokine receptors N-terminus.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CX3CR1	CX3CL1	AT CA IBD PS	MDQFPESVTENFEY*DD LAEAC-YIGDI	26 (-8)	21 (-7)	5 (-1)	1	0	(53)

3. Ligand binding mode

3.1. Binding of chemokines to chemokine receptor N-terminus

Numerous studies conducted with whole receptors [17–23,36,37] or receptor-derived synthetic peptides [24–33,78] have demonstrated that the N-terminal domain of chemokine receptors holds an important role in ligand binding. Based on some of these results and the observation that chemokine binding and receptor activation are separable events driven by distinct molecular mechanisms and involving different structural determinants, a general two-site model was proposed by different authors to describe the interaction of chemokines with their cognate receptors [11,12] (Fig. 3A–C). In this model, the receptor

Table 5
Sequences, lengths, charges and post-translational modifications of decoy receptors N-termini.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
D6	CCL2 to CCL8, CCL11 to CCL14 CCL17 CCL22	CA	MAATASPQPLATEDADAENSSFYYYDYLDEVAFMLC-RK*D*AVVSFG*K*V	47 (-6)	36 (-7)	11 (+1)	4	1	
DARC	CCL2 CCL5 CCL7 CCL11 CCL13 CCL14 CCL17 CXCL1 CXCL3 CXCL5 CXCL6 CXCL8 CXCL11	PL PS	MGNCLHRAELSPSTEN*SSQLDFEDVWN*SSYGVN*DSF PDGDYGANLEAAAPC-HSCNLLDDSALPFF	65 (-9)	51 (-8)	14 (-1)	2	2	(45, 55, 71, 72, 75)
CCX-CKR	CCL19 CCL21 CCL25 CXCL13	CA	MALEQNQSTDYYYEENEMNGTYDYSQYELIC-IKE*D*VREFA*K*V	42 (-7)	31 (-7)	11 (0)	6	2	

Tables present the length and (charge) for the complete N-terminal domains (N-term), M–C and C–TM parts. M–C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulfide bridge with the third extracellular loop (ECL3). C–TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimits the M–C part from the C–TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M–C part that are potentially sulfated ($_{p}$sY) are highlighted in gray. Potential N-glycosylation sites (NxS/T) are underlined. *Denotes post-translational modifications that were experimentally demonstrated. Double underlined italic residues highlight (1) the conserved K-K/R-PPLYS/W motif located in the C–TM parts at positions C+2, C+6 and C+13 respectively or (2) the negatively and positively charged residues conserved at positions +3/4 and +10/11. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. [ψ]CCR7 N-terminal sequence presents a signal peptide of 24 residues. Processing prediction indicates Gln25 as the amino terminal residue of CCR7 N-terminus. [ψ]CXCR6 does not present a cysteine in its N-terminal domain. AD: atopic dermatitis, AR: allograft rejection, AS: asthma, AT: atherosclerosis, CA: cancers, CH: chronic hepatitis, COPD: chronic obstructive pulmonary disease, DI: type I diabetes, HIV: human immunodeficiency virus infection, IBD: inflammatory bowel disease, LP: lupus, MS: multiple sclerosis, PL: plasmodium infection, PPP: palmoplantar pustulosis, PS: psoriasis, RA: rheumatoid arthritis. [a]Based on [161] the principal endogenous agonists are represented in bold. [b]Adapted from [162].

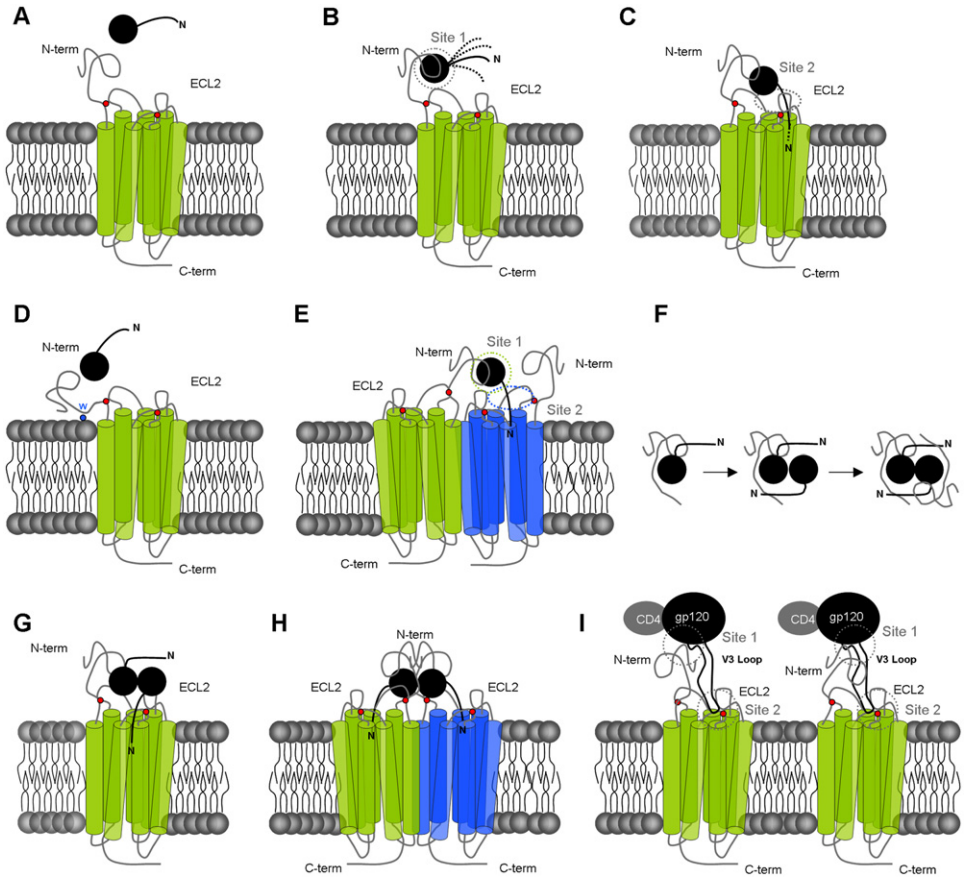

Fig. 3. Interaction models for chemokine receptors. (A–C) Putative two-site mechanism for the interaction between chemokines and chemokine receptors. (A and B) First step: interactions between the N-loop (site I) of the chemokine and the N-terminal domain of the receptor. (B and C) Step two: interactions between the flexible N-terminus (site II) of the chemokine and the extracellular loops as well as the transmembrane segments of the receptor. The disulfide bridges between N-term/ECL3 and ECL1/ECL2 are depicted as red dots. (D) Anchoring of the N-terminal domain of CXCR1 into the membrane through hydrophobic contacts mediated by an aromatic residue (W) (blue dot). (E) Receptor trans-activation. Chemokine binds the N-terminal domain (site I) of receptor 1 (green) and trans-activates receptor 2 (blue) through its binding at site II. (F) Induction of CXCL12 dimerization upon binding of a sulfated peptide corresponding to the N-terminus of CXCR4 (residues 1–38). A chemokine monomer binds to one N-terminal peptide which increases the interaction interface and facilitates the binding of the second chemokine monomer. The second N-terminal peptide binds to the second monomer leading to the formation of a symmetric 2:2 complex [29]. (G and H) Possible stoichiometries of CXCR4 interactions with CXCL12 dimer (G) Monomeric CXCR4 binds a dimer of CXCL12. (F) Dimeric CXCR4 binds a dimer of CXCL12. (I) Putative two-site binding mode describing the interactions between the gp120 protein and CCR5 [135]. Site I: the N-terminal domain of the receptor binds to the bridging sheet and the base of the V3 loop of the gp120 protein trough electrostatic interactions. Site II: the crown of the V3-loop interacts with the second extracellular loop of the receptor. Representation of the putative orientation of the N-terminus with respect to two different docking models based on NMR studies of gp120 associated to synthetic peptides derived from CCR5 N-terminus [136,137].

N-terminus plays a crucial role in the initial recognition of the chemokine through the binding of its N-loop (site I). This primary interaction is likely to contribute to correct chemokine orientation, promoting the binding of its flexible N-terminus (site II) to the extracellular loops and the transmembrane segments of the receptor, triggering its activation.

To date, little information about the structure of chemokine receptor N-termini is available. The N-terminal domains of chemokine receptors, especially their M–C part, are generally proposed to be highly flexible, showing an extended form when unbound and only adopting a fixed structure upon chemokine binding [27,48]. This hypothesis is in line with the lack of clear electron density for the M–C part in the CXCR4 X-ray structures [16].

The N-termini are the most variable extracellular domains of chemokine receptors in terms of sequence and length and this diversity is most probably an important determinant dictating the specificity of the receptor. The chemokine receptor N-termini display net negative charges and their binding to chemokines is proposed to be typically driven by electrostatic but also hydrophobic interactions. There exists a considerable amount of data on the importance of many individual residues within the N-terminus, obtained mainly from binding studies with mutated receptors [17,18,42,79,80]. These residues are however rarely conserved among the receptors with the exception of a tyrosine found approximately nine residues before the C–TM part (see Section 2.2.2). These observations suggest the existence of a common mechanism for N-terminus binding involving the conserved sulfotyrosine but also relying on non-conserved residues that may determine the selectivity of the receptors. In accordance with this hypothesis, NMR studies conducted with labeled chemokines in the presence of receptor N-terminus-derived peptides identified a groove delimited by the N-loop and the β-sheet as the receptor N-terminus binding site. Although not identical, this binding site seems highly conserved among different chemokines [32,33,56].

In particular, for CXCL8, Skelton et al. demonstrated that a small modified peptide covering residues 9–29 (M_9WDFDD$_{14}$-linker-M_{20}PPADEDYSP$_{29}$) of the CXCR1 N-terminus (K_i = 13 μM) occupies a cleft between the N-loop and the third β-strand in an extended fashion and with only a limited number of contact residues (in bold: **P$_{21}$PADEDYSP$_{29}$**) (Fig. 4A) [27]. In the complex, P21 and P22 formed hydrophobic interactions with L43^{CXCL8} and L49^{CXCL8} residues while P29 preceding the conserved cysteine wrapped

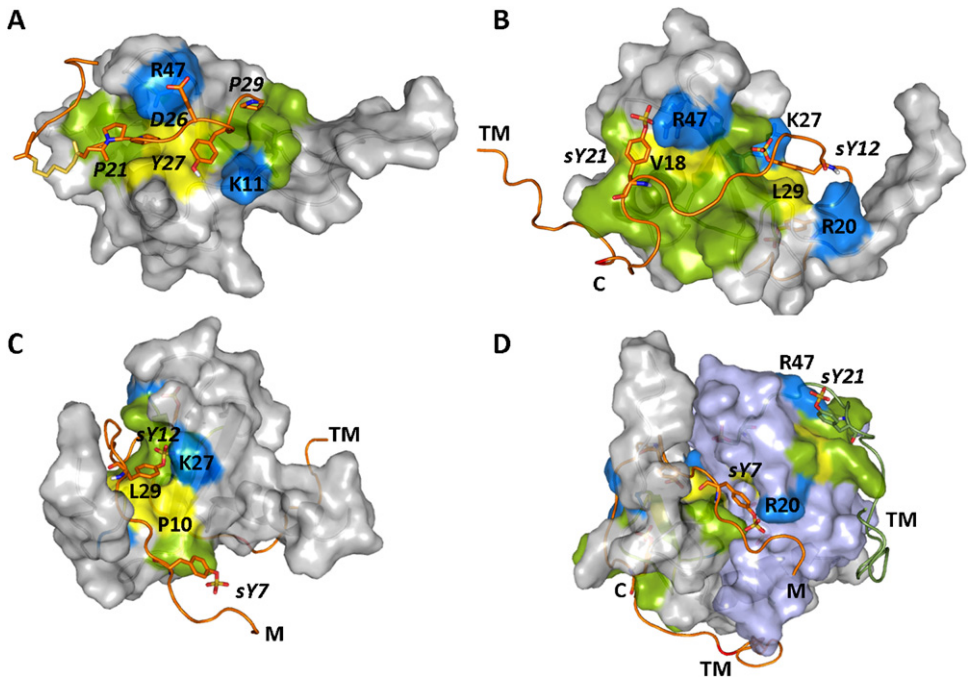

Fig. 4. Structures of chemokine/N-terminus derived peptide complexes. Chemokines are represented as surface and colored in gray. The hydrophobic N-terminus binding groove is colored in green and yellow. N-terminus-derived peptides are represented as cartoon, colored in orange and annotated in Italic. (A) NMR structure of the CXCL8–CXCR1 N-terminus complex [27]. Tyrosine 27-binding site includes residues I10, Y13, L49 (yellow) and K11 (blue). D46 of CXCR1 forms electrostatic interactions with R47 of CXCL8. N-terminus P21, P22 and P29 residues form hydrophobic interactions with the groove of the chemokine. (B–D) NMR structures of CXCL12 in complex with full-length CXCR4 N-terminus bearing sulfotyrosines at positions 7, 12 and 21 [29]. (B) Recognition sites for sulfotyrosines sY12 and sY21. Conserved sY21 binds a pocket defined by V18 and V49 (yellow) and overhung by residue R47 (blue) while sY12 interacts with a similar pocket formed by residues L29, P10 (yellow) and K27 (blue). (C) Binding of sulfotyrosine sY7 and sY12 to a CXCL12 monomer (60° rotation relative to B). sY12 occupies a defined binding pocket while sY7 points in the opposite direction making no clear interaction with the chemokine monomer. (D) Binding of sulfotyrosine sY7 to a dimer of CXCL12. sY7 occupies the cleft at the interface between two chemokine monomers and interacts with residues V24 and R20 of the second monomer. The second N-terminus peptide binding to the second monomer is represented as cartoon and colored in dark green.

around the chemokine β-sheet making hydrophobic contacts ($I10^{CXCL8}$ and $I40^{CXCL8}$). Y27, conserved in almost all chemokine receptors and most probably O-sulfated in the native CXCR1 receptor (see Section 2.2.2), interacts with a pocket delimited by $I10^{CXCL8}$, $K11^{CXCL8}$, $Y13^{CXCL8}$ and $L49^{CXCL8}$. The binding is stabilized by an additional electrostatic interaction between D26 and chemokine $K11^{CXCL8}$. The importance of these residues was confirmed by site-directed mutagenesis of the complete CXCR1, indicating that the binding mode deduced from the NMR study most likely reflects the interaction of the chemokine with the complete receptor [43].

More recently, Veldkamp et al. reported the NMR structure of a strictly dimeric form of CXCL12 in complex with a full-length CXCR4 N-terminal domain peptide [1–38] bearing sulfotyrosines at positions 7, 12 and 21 [29]. This study provided the first structural evidence of the existence of sulfotyrosine recognition sites and demonstrated that the CXCR4 N-terminal peptides adopt an extended conformation with sulfotyrosines 12 (sY12) and 21 (sY21) binding to one chemokine monomer and sulfotyrosine 7 (sY7) interacting with the second monomer (Fig. 4B–D). Interestingly, in the complex, sY21 is orientated in the opposite direction compared to the equivalent Y27 in CXCR1 and interacts with a hydrophobic pocket defined by $V18^{CXCL12}$ and $V49^{CXCL12}$ and with the overhanging basic residue $R47^{CXCL12}$, which in CXCR1 is occupied by P21 (Fig. 4A and B). It is noteworthy that a residue equivalent to $R47^{CXCL12}$ is also present in CXCL8 ($R47^{CXCL8}$) but is involved in stabilizing electrostatic interactions with E25 of CXCR1. Similarly, a positively charged residue equivalent to $K11^{CXCL8}$ is also present in CXCL12 ($R20^{CXCL12}$) but does not interact with any of the CXCR4 N-terminus residues. Furthermore, this study also provided structural data on the binding mode of the two other CXCR4 sulfotyrosines, sY7 and sY12, that are not strictly conserved in other receptor N-termini. In particular, sY12 was shown, just like sY21, to bind a hydrophobic pocket defined by $P10^{CXCL12}$, $L29^{CXCL12}$ and $K27^{CXCL12}$, whereas sY7 had no interacting partners on the first chemokine monomer and occupied a cleft delimited by the interface of the dimer forming an electrostatic interaction with $R20^{CXCL12}$ of the second monomer.

Altogether these data demonstrated that sulfotyrosine recognition, critical for high affinity interactions with chemokines, occurs at particular binding sites sharing a similar architecture and that a given chemokine can display several sulfotyrosine-binding sites. Moreover, other interactions supported by non-conserved residues scattered along the N-terminal domains most probably also play essential roles in sulfotyrosine recognition and in further stabilization of the chemokine-receptor complexes, possibly providing the molecular basis for the differences in affinity and selectivity observed among the different receptors.

However, while the N-terminus plays the predominant role in the initial chemokine binding, other extracellular parts have also been shown to participate in chemokine binding, in which case the combination of multiple low-affinity interactions provides high-affinity binding energy in chemokine-receptor interactions. Consistent with this assertion is the observation that by simultaneously grafting peptides corresponding to the CCR2 N-terminus and ECL3 on a stabilized variant of the protein G B1 domain, the affinity for CCL2 is 100 times as high as when only the N-terminus is present on the scaffold [35]. Similar results were obtained for CCR3 [34]. Other extracellular parts such as ECL2, which is involved in the formation of site II, could be of importance for the overall affinity of the receptor.

Moreover, accumulating data suggest that the mechanism underlying chemokine binding to their receptors is likely to be more complex than a simple two-site model. It has been proposed that site I and site II interactions may be far from independent. Indeed, conformational changes in both the chemokine and the receptor that follow the initial chemokine binding to the N-terminus of the receptor may energetically influence the subsequent interactions at site II [81]. This model may for instance explain why while CXCL8 binds CXCR1 with a significantly higher affinity than CXCL1, both chemokines bind the N-terminus of the receptor with similar affinities [24]. Therefore, in contrast to CXCL8, the changes resulting from the binding of CXCL1 to site I would negatively affect further interactions of the chemokine at site II. Such coupling between the two binding steps may thus have a major role in the regulation of chemokine affinity and selectivity for their receptors, providing yet another molecular basis for the complexity of chemokine–chemokine receptor network [82]. It is also conceivable that upon binding of the chemokine at site I, the area for further interactions with site II increases, either as a result of conformational changes in the chemokine/receptor [82] or by complementation of sites that are partly present on the receptor N-terminus and partly on the chemokine.

Recently, the possible role held by the vicinity of the cell membrane in the regulation of receptor N-terminus interactions with the ligand has also been put forward. Indeed, it has been demonstrated that the CXCR1 N-terminal peptides interact with membranes or membrane-mimicking micelles in extended but constrained conformation that may energetically facilitate the interactions with the chemokine [24,83]. NMR studies using a phospholipid bilayer-embedded CXCR1 receptor or an N-terminus–TM1 construct suggested that the CXCR1 N-terminal domain may be anchored to the membrane via a tryptophan residue at position 10 (Fig. 3D). The release of the N-terminus from the membrane upon strong interactions with the chemokine may thus be considered, at least for CXCR1, as the earliest step of the ligand binding mechanism [36]. Such hydrophobic association of the N-terminus with the cell membrane may have a great impact on its binding properties. Indeed, the affinity of the CXCR1 N-terminal fragment for CXCL8 was shown to be 20-fold higher in detergent micelles than in solution ($K_D \approx 1$ μM versus 20 μM). Moreover, membrane-like environment has been reported to influence the binding selectivity of the receptor N-terminal domains [24].

Another poorly understood aspect is the stoichiometry of chemokine-receptor interactions. Since many chemokine receptors are known to form homo- or heterodimers, the possibility of a cross-talk in which site I and site II interaction would take place on separate receptors should not be excluded (Fig. 3E). In accordance with this hypothesis, Monteclaro et al. demonstrated that the CCL2 binding to the CCR2 N-terminus fused to CD8 can activate in trans a chimeric CCR2 that carries an irrelevant N-terminus [37]. Besides the receptors, many chemokines as well have been shown to exist in different oligomeric states. Furthermore, in the case of CXCL12, it has been demonstrated that binding to the N-terminus of CXCR4 induces its dimerization in a symmetric 2:2 complex in which the dimerization interface is shared by both the residues from the N-terminal domain and the chemokine [30] (Figs. 4D and 3I). While CXCL12 dimerization has been suggested to be physiologically irrelevant [33], recent data recorded with strictly dimeric chemokine demonstrated distinct signaling pathways and differential chemotactic effect depending on the oligomeric state of CXCL12 [84]. Moreover, structural data showed that the CXCR4 N-terminus binds differentially to CXCL12 monomers and dimers [84]. In particular, while residues 4–9 of the CXCR4 N-terminal peptide make strong interactions with CXCL12 monomers, they are only weakly associated with the chemokine in its dimeric form. Similar 2:2 interactions were proposed for CXCL8 and CXCR1 N-terminus but remain controversial [26,81]. One cannot rule out the possibility that such dimerization may reflect the experimental setup, where in the absence of other possibilities of interactions with the receptor, chemokine dimerization is energetically favored. On the contrary, it has been proposed for CXCL8 that the binding of the N-terminal domain of CXCR1 to the chemokine dimer could promote its dissociation [81].

In an emerging concept, chemokine receptor response specificities underlie the differences in receptor trafficking. Particularly, it has been proposed that determinants of receptor internalization rates following ligand binding may be harbored by the N-terminus of chemokine receptors [23]. By swapping the N-termini of CXCR1 and CXCR2, two chemokine receptors that share 77% of sequence identity but show different binding and signaling profiles towards CXCL8, it was demonstrated that the trafficking profiles of the chimeric receptors were defined by the N-terminus and translated in temporal differences in activation of ERK1/2 signaling pathways, which are important for different signaling specificities. However, these determinants remain hitherto unidentified.

3.2. Binding of pathogen proteins to chemokine receptor N-terminus

To subvert the host immune system and promote their pathogenesis, viruses such as herpesviruses, poxviruses and retroviruses have evolved various strategies to interfere with the host chemokine network, for instance by expressing chemokine analogs (for review see [85,86]).

The Human herpes virus 8 (HHV-8), also named Kaposi's sarcoma-associated herpesvirus, expresses three viral macrophage inflammatory proteins (vCCL1, vCCL2 and vCCL3) that share homologies with CCL3 and CCL4 [87]. The characterization of these proteins revealed that vCCL2 has the unique ability to cross-bind to various CC and CXC chemokine receptors [88,89]. In particular, vCCL2 binds to CXCR4 and CCR5 and is capable of inhibiting the interaction with their cognate chemokine ligands as well as infection of host cells by HIV-1 [88,89]. Although vCCL2 displays a typical chemokine structure [90], very little information about its binding mode is currently available. Whether its ability to interact with chemokine receptors of both subfamilies involves the same or distinctive determinants remains to be elucidated.

Chemokine receptors can also be hijacked by pathogens to allow their entry into specific cell types. Two striking examples of such piracy are the malaria parasites (*Plasmodium vivax* and *Plasmodium knowlesi*) and the human immunodeficiency virus (HIV-1).

P. vivax and *P. knowlesi* belong to the five *Plasmodium* species responsible for human malaria, a mosquito-borne infectious disease causing fever and headache and progressing in the most severe cases to coma and death. *P. vivax* and P. *Knowlesi* infect human erythrocytes by using the decoy chemokine receptor DARC (Duffy blood group antigen) [91–93] that binds various CC and CXC chemokines [94]. *Plasmodium* interaction with DARC was shown to be mainly mediated by a conserved cysteine-rich domain present in the parasite Duffy binding proteins (PvDBP and PkDaBP) [95] and by a modified 35-amino acid fragment (residues 8–42) of the receptor N-terminus [96]. The tyrosine residues at position 30 and 41 of the N-terminus of DARC are sulfated although only the second one was reported as critical for PvDBP and PkDaBP binding [55]. Interestingly, erythrocytes interaction with PvDBP-expressing cells can be inhibited by a peptide derived from DARC N-terminus (IC_{50} = 1 μM) [96] while sulfation of tyrosine 30 and 41 in the peptide results in a more efficient inhibition (IC_{50} = 5 nM) [55]. Recent data point to the existence of a sulfotyrosine-binding pocket for DARC N-terminus on the interface of DBP dimer of *P. vivax* [97].

The human immunodeficiency virus (HIV-1), the causative agent of AIDS, uses chemokine receptors CCR5 and CXCR4 to specifically infect monocytes and macrophages (M-tropic) or T-cells (T-tropic), respectively [9,98–101]. This multi-step process is mainly mediated by envelope glycoproteins gp120 and gp41 organized in heterotrimer spikes on the outer surface of the viral membrane [102,103]. Gp120 is constituted of an alternation of five constant domains (C1–C5) and five variable loops (V1–V5). The domains C1, C2 and C4 form a four-stranded antiparallel β-sheet called the bridging sheet. Upon binding to CD4, its primary receptor, HIV-1 envelope glycoprotein gp120 undergoes conformational changes resulting in the spatial reorientation of the bridging sheet and the variable V1/V2 and V3 loops exposing specific binding sites for the co-receptors [104–107]. Binding of gp120 to CXCR4/CCR5 leads to a rearrangement of gp41, bringing together the cellular and the viral membranes and allowing their fusion [108].

Interactions between gp120 and chemokine receptors CCR5 and CXCR4 have been investigated using different approaches including chimeric receptors [109–115], site-directed mutagenesis [41,79,116–120] and other biochemical and immunological methods [9,121–127]. All these studies point to the importance of the co-receptor extracellular domains in gp120 binding, especially the receptor N-terminus and ECL2, although their relative contribution depends on the HIV-1 strain [128]. Discrimination between CCR5 and CXCR4 has been shown to mainly depend on the determinants present in the V3 loop (±35 aa) of gp120 such as positively charged amino acids at positions 11, 24 and 25, the overall charge and the distribution of the electrostatic potential [129,130]. However, co-receptor usage has also been shown to be affected by amino acid composition and glycosylation of the V1/V2 stem [131,132].

In particular, the interaction between the V3 loop and the N-terminus of CCR5 has been shown to depend on a cluster of negatively charged and tyrosine residues (D2, Y3, Y10, D11, Y14, Y15, E18) and suggested to be driven by electrostatic interactions [18,113,116,117,119]. Besides, the co-receptor function of CCR5 was also associated to other determinants such as S6, S7, I9, N13, Q21 and K22 [18,117,119]. Like for chemokines, sulfation of tyrosine residues, in particular Y10 and Y14, was identified to critically affect the binding of gp120 while O-glycosylation of serine residues had little effect [51,74,119,125].

Together with structural analyses of the V3 loop [106,107,133,134], these studies revealed the role of spatially distinct domains of gp120 in CCR5 interaction and led to the development of a two-site binding model, similar to that proposed for chemokines [135]. In this model, the conserved four-stranded bridging sheet (C4) and the base of the V3 loop bind to the CCR5 N-terminus (residues 2–15) (site I) through electrostatic interactions, while the crown of the V3 loop interacts with the co-receptor ECL2 (site II) (see Fig. 3I).

In the absence of high-resolution structures, new insight into the molecular details of gp120-coreceptor interactions arose from NMR studies of gp120 bound to synthetic peptides derived from specific co-receptor domains [136,137]. NMR study of a sulfated CCR5 N-terminus peptide (sY10–sY14 CCR5 2–15) in complex with gp120 revealed a well-defined structure for residues 7–15. The docking of this peptide into the crystal structure of gp120–CD4 suggested that CCR5 N-terminus binds to gp120 at the intersection of the bridging sheet and the V3 loop (Fig. 3I, left panel) [136]. Residues S7 and P8 bind to the V3 stem while sY10, N11, Y15 interact with $R327^{gp120}$, $R440^{gp120}$, $I439^{gp120}$, respectively. The pocket between the bridging sheet and V3 encircles sY14 and rigidifies the V3 stem into a β-hairpin structure. A more recent study performed with a longer sulfated peptide (sY10–sY14 CCR5 1–27) showed that residues 7–23 bind to gp120–CD4 with P8–S17 and A20–I23 forming helical structures [137]. This study also provided a clearer picture of the main CCR5 binding determinants, emphasizing the importance of the previously identified residues D2, Y3, sY10, D11, sY14, Y15, E18 while contradicting the results regarding V5, I9, I12 and T16. The integration of these data in a gp120 structural model suggested the interaction of residues 2–22 with the fourth constant domain as well as the stem of the V3 loop (site I). In contrast to the previous docking model, here, the peptide is flipped by 180° with sY14 fitting into a binding pocket and strongly interacting with $R440^{gp120}$ while sY10 binds to $R32^{gp120}$ (Fig. 3I, right panel).

The binding of gp120 to CXCR4 probably occurs via a similar mechanism, although the N-terminus seems less important for infection by certain isolates [114,138,139]. In contrast to CCR5, no precise cluster of residues critically affected virus entry. Mutagenesis studies however revealed the role of individual residues (Y7, N10, Y12, N20, Y21, N22, S23 and E26) for the co-receptor function of CXCR4, although the extent of their contribution was strain-dependent [47,79,140]. The sulfation of tyrosine residues, in particular Y21, only had a minor effect on the entry of X4-tropic HIV-1 [52], while controversial data were obtained regarding the impact of glycosylation. Mutation of the N-glycosylation site of CXCR4 N-terminus (N11) was initially shown to slightly facilitate R5 [141] or R5X4 [142] virus entry while having no effect on X4 viruses [79,118,142]. In another study however, the replacement of N11 with Q11 enhanced the binding and entry of X4 and R5 viruses [143].

4. Therapeutic discoveries targeting N-terminus interactions

Since their discovery twenty years ago, chemokines and their receptors have emerged as fundamental regulators of human physiology. The interest in chemokine biology also arises from their key roles in such pathologies as cancer, inflammatory and autoimmune diseases as well as HIV-1 infection (see Tables 1–5). Therefore much effort has been put into exploring ways to interfere with these processes, by either targeting the receptors or their ligands.

Therapeutic strategies directed against chemokine receptors have already proven efficacious in clinic. Two small molecules are currently on the market, namely the CXCR4 antagonist, AMD3100 (plerixafor, Mozobil™ developed by Genzyme) used for hematopoietic stem cell mobilization prior to autologous transplantation in patients with lymphoma and multiple myeloma and Maraviroc (Selzentry™, developed by Pfizer) for the treatment of CCR5-tropic HIV-1 infection [144,145]. These compounds, like the vast majority of chemokine receptor inhibitors, bind pockets in the transmembrane regions and do not interact with the N-terminal domain. However, because of their key roles in ligand recognition, the N-termini of chemokine receptors may also represent highly relevant targets for drug discovery. To the best of our knowledge, small molecules specific to the N-terminus have never been reported and this is certainly due to the unstructured, highly flexible nature of this domain. These characteristics however can also be regarded as advantageous for the generation of therapeutic antibodies able to block the initial site I-interaction of intact receptors. Immunization with synthetic N-terminus derivatives allows for instance to circumvent the need for receptor purification or avoid elicitating antibodies against irrelevant epitopes in whole-cell antigens but may overlook the post-translational modifications often present in the extracellular domains [146,147]. Antibodies recognizing linear or conformational epitopes exclusively or partly present in the N-terminus are commonly used in research and may also be exploited for therapeutic applications. Indeed, given that receptors which share ligands can at the same time have very distinct N-termini (see Tables 1–5) it is conceivable that highly specific, clinically relevant antibodies can be raised against these fragments.

To date, there are no anti-chemokine receptor antibodies approved for clinical use. However, clinical trials for at least two anti-N-terminus mAbs are in progress. This includes the anti-CCR5 mAb PRO140 currently evaluated against HIV infection as well as the CCR4-specific mAb KW-0761 for the treatment of adult T-cell leukemia–lymphoma and peripheral T-cell lymphoma [148,149].

Other rather encouraging results from studies with antibodies recognizing the chemokine receptor N-terminus have been published. Recently, a dromedary-derived VHH, CA52, directed against the N-terminus of DARC, efficiently inhibiting *P. vivax* invasion and able to displace CXCL8 from the receptor was described [150]. Similarly, sera from rabbits immunized with the first seven CCR5 N-terminus amino acids fused to T-helper cell epitope from tetanus toxoid were shown to inhibit HIV-1 infection of primary macrophages [147].

An attractive alternative to receptor inhibition consists of neutralizing the ligand, in particular by blocking the N-terminus-recognition site [151]. The report on the human mAb 10F8 whose epitope overlaps with the binding pocket of CXCR1 N-terminal domain (see Section 3.1, [27]), illustrates well the feasibility of such a strategy [152]. This antibody was shown to interact with CXCL8 with picomolar affinity and to inhibit its binding to neutrophils (IC_{50} 0.3 nM) as well as chemokine-induced neutrophil activation and chemotaxis. In addition, it proved relatively efficient in treating palmoplantar pustulosis, an inflammatory disease in which CXCL8 plays a predominant role.

Moreover, a considerable therapeutic potential can be expected from approaches targeting specifically sulfotyrosine-binding pockets, as sulfotyrosine-mediated interactions seem widely exploited not only in chemokine biology but also by pathogen proteins. In line with this assumption is the report of a sulfated peptide corresponding to the first 60 residues of DARC N-terminus and blocking at low nanomolar concentration the association of plasmodium PvDBP and PkDaBP with the receptor [55]. Attempts to neutralize chemokines or the HIV-1 envelope protein gp120 using N-terminus-derived peptides have so far proven unsuccessful mainly due to their low affinity and poor stability. It may however be possible to improve the affinity and pharmacokinetic properties of these peptides for example by incorporating in the sequence non-natural residues such as D-amino acids or chemical derivatives like the acid-stable sulfotyrosine mimic, (p-sulfomethyl)-phenylalanine [153,154]. Interestingly, recent high-throughput in silico screening of small molecules targeting the $sY21^{CXCR4}$ sulfotyrosine-binding pocket on CXCL12 identified several lead compounds of which one (ZINC 310454) bound CXCL12 with an affinity of 64 μM [155]. Extending the screening target to larger parts of the chemokine/N-terminus interaction surface may provide molecules of higher specificity and/or affinity.

5. Discussion

Chemokines are a family of small highly basic proteins that display a common fold but share little sequence similarities. By binding to chemokine receptors, they participate in many vital processes. The chemokine-receptor network is characterized by an apparent redundancy and many chemokines can bind to several receptors, while a chemokine receptor usually has multiple ligands. This overlapping selectivity reflects however sophisticated regulation mechanisms that are still not fully elucidated.

The N-terminus of chemokine receptors has a critical role in the initial step of chemokine binding as well as in determining the specificity and affinity of this interaction. At first sight, the N-termini vary remarkably between different receptors in terms of length and amino acid sequence. However, on closer examination several common characteristics and signatures can be discerned.

One such feature is the conserved cysteine residue involved in a disulfide bond that links the N-terminus and ECL3 and delimits two distinct regions within the N-terminus, the M–C and C–TM parts. While this disulfide bridge has been shown to be important for chemokine receptor biology, the exact way of how it exerts its function remains unclear [37,41–44]. It is likely that by linking the N-terminus to ECL3, this disulfide bridge participates in the positioning of the M–C part above TM7 in an arrangement favoring the presentation of the chemokine to site II. Moreover, as a large part of chemokine binding relies on the receptor extracellular domains, the C–TM "pseudo-extracellular loop" is perhaps an important additional feature shaping the ligand interaction interface in receptors having relatively short, compared to other protein-binding GPCRs, N-terminus. This supposition may be substantiated by the observation that CXCR4 structure differs from other GPCRs in the location and the form of the ligand binding pocket, which is situated closer to the extracellular surface [16]. In this context, the existence of different C–TM sizes (11 or 18 residues) identified here that bear distinct signatures (K-X_3-K/R-X_7-PPLYS/W and E/$D_{+3/+4}$-K/$R_{+9/+10}$ respectively) is rather intriguing and the potential impact of these elements on the receptor functionality should be addressed in the near future. Furthermore, the conservation of these motifs may open new perspectives for phylogenetic studies of chemokine receptors and allow their alternative classification that, in contrast to the current system, would not be merely based on the recognized ligands.

More information is available on the flexible M–C part of the N-terminus since it had early been demonstrated to be directly involved in ligand binding. There has been growing interest in the post-translational modifications present in this region and one of the current central areas of concern in chemokine receptor interactions with ligands is sulfation of their N-terminal domains. For several receptors this post-translational modification has been demonstrated to be important for high-affinity binding to chemokines. Most chemokine receptors bear a potentially sulfated tyrosine about nine residues before the conserved cysteine, which may therefore interact with the sulfotyrosine-binding pocket suggested to be present on the surface of all chemokines [58]. Complementation between the negatively charged receptor N-terminus and the positive charges within this conserved binding site as well as hydrophobic interactions were proposed to facilitate the binding by proper positioning of the sulfotyrosine-bearing N-terminus and stabilization of the interaction. Although sulfotyrosine-driven binding mechanism seems to be shared by many chemokine-receptor pairs, the auxiliary residues involved in this interaction are highly variable and might have co-evolved in the binding partners determining, at least in part, their specificity. In several chemokine receptors, many other potentially sulfated tyrosines are present in the N-termini and appear to participate in high-affinity interactions with ligands. For CXCR4, their binding was shown to follow a mechanism similar to that proposed for the conserved sulfotyrosine indicating that other sulfotyrosine binding sites may exist at the surface of chemokines [29]. Sulfotyrosine-mediated recognition appears to be exploited not only in chemokine interactions but also by pathogen proteins and therefore sulfotyrosine-binding pockets represent valuable targets for drug development.

Furthermore, we propose that the presence of the multiple sulfate groups may provide repulsion forces that energetically favor an extended conformation of the N-terminus, exposing the residues that are critical for ligand binding.

Other features commonly found in the M–C part are the putative N-glycosylation sites. The presence of sugar chains has been experimentally determined in only a few receptors and their exact role has yet to be further investigated. It is nevertheless highly plausible that similarly to tyrosine sulfation, cell-dependent

glycosylation patterns result in structurally and functionally different receptor isoforms, like those observed for CXCR4 [77]. Such differences in the post-translational modifications may thus represent an additional level in the fine-tuning of the complex chemokine-receptor network.

Unfortunately, the recent resolution of the X-ray structure of CXCR4 failed to provide details on the flexible M–C part [16]. Nevertheless, alternative approaches exploiting chimeric, mutated or truncated receptors produced a compelling set of information on the critical roles of these N-terminus parts in ligand binding and receptor function [17–23]. In particular, NMR analyses of chemokines or viral proteins in complex with synthetic peptides derived from the receptor N-termini were a considerable steppingstone in the understanding of the receptor N-terminus biology and provided the first insights on the structural basis for site I interactions [27,29]. However, data from these studies should be interpreted with some caution. Among the problems to be taken into consideration is the fact that the peptides used do not always cover the full N-terminus sequence and often bear no post-translational modifications that are normally present in this receptor domain. Although studies with sulfated N-terminal fragments have been reported (mainly for CXCR4 and CCR5) the addition of this group is not a straightforward task [30,61,156,157].

To date, many questions on chemokine receptors remain under debate. It has become clear that post-translational modifications of the N-terminus should not be underrated in the role they play in receptor function but investigating it is somewhat challenging. The exact stoichiometry of chemokine-receptor interactions, including such aspects as receptor–receptor cross-talk, chemokine oligomerization and the biological relevance of receptor N-terminus-induced chemokine dimerization as observed for CXCR4–CXCL12 couple, also need to be further examined. Although, the development of chemokine receptor antagonist still remains a major challenge, the efforts made to unravel and characterize the structural and functional properties of chemokine binding mode will probably, in the future, enable the development of new specific chemokine-neutralizing molecules or N-terminus-targeting antibodies with high therapeutic potential [151,155,158].

Acknowledgments

This manuscript is supported by the "Fonds National de la Recherche", Luxembourg, grants: AFR-3004509 (CX-CRP project), C09/BM/20 (PROMETAVIR project) and 10/BM/822389 (3D-SPCAD project) and the Centre de Recherche Public-Santé, Luxembourg, grant 20100708 (GPCR project). The authors thank Dr. Carole Devaux, Dr. Sylvie Delhalle, Dr. Marie-Eve Dumez, Aurélie Fischer and Rémi Crestani for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2012.08.008.

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GPCRS

Closing the Ring: A Fourth Extracellular Loop in Chemokine Receptors

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Chemokine receptors are heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCR) that play fundamental roles in many physiological and pathological processes. Typically, these receptors form a seven-transmembrane helix bundle, which is stabilized by a disulfide bond bridging the top of the third transmembrane segment (TM3) and the second extracellular loop (ECL2). Resolution of the three-dimensional structures of the chemokine receptors CXCR1, CXCR4, and CCR5 revealed the existence of a second disulfide bridge that links the N terminus of the receptor to the top of the seventh transmembrane segment (TM7), thereby closing the receptor into a ring. An important consequence of this second disulfide bond is the formation of an additional extracellular loop, which shapes the entrance of the ligand-binding pocket and adds rigidity to the overall surface of the receptor. Here, we discuss the features of these "pseudo-loops," the structural requirements for their formation, and the effects they may have on receptor function.

Chemokine receptors are rhodopsin-like, guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) and are present at the surface of various cell types. By binding to their ligands, chemokine receptors regulate vital cellular mechanisms, including migration and adhesion, as well as growth and survival, but they are also involved in pathological processes such as cancer and HIV-1 infection. Previously, knowledge about the structure of chemokine receptors was built on predictions based on other class A GPCRs and on functional studies. The resolution of the three-dimensional (3D) structures of three chemokine receptors by x-ray crystallography (for CXCR4 and CCR5) and nuclear magnetic resonance (NMR) analysis (for CXCR1) has provided more precise information on the conformations adopted by members of this receptor family (Fig. 1, A to D) (*1*–*3*).

Similar to other rhodopsin-like GPCRs, chemokine receptors consist of a flexible extracellular N terminus that is followed by a bundle of seven hydrophobic plasma membrane–spanning α helices [known as transmembrane (TM) domains] that are connected by three hydrophilic extracellular loops (ECLs) and three intracellular loops (ICLs). In addition to the canonical disulfide bond that links the top of the third TM domain (TM3, at the end of ECL1) to the middle of ECL2, all three currently available 3D structures of chemokine receptors demonstrate the presence of a second disulfide bridge between the N terminus of the receptor and the top of TM7, at the end of ECL3 (Fig. 1). As a consequence, the C-terminal residues of the N terminus of the receptor form an extracellular loop (which is termed "ECL4"), which connects TM1 and TM7 and closes the receptor into a ring-like conformation (Fig. 1A). This fourth loop consists of six (for CCR5) or eight amino acid residues (for CXCR1 and CXCR4) (Fig. 1G) and is thus comparable to ECL1 and ECL3, which contain between four and eight residues each. With the exception of CXCR6, all of the chemokine receptors have a cysteine in the last third of their N-terminal regions, which suggests that the additional disulfide bridge is conserved. Although the formation of this disulfide bridge is critical for the function of several chemokine receptors, the role of the additional loop in ligand recognition and receptor activation has been given less attention.

The formation of ECL4 at the surface of chemokine receptors requires structural adaptations and possibly has consequences on receptor function. In CXCR1, CXCR4, and CCR5, the transmembrane helix that forms TM7 is two turns longer than that in other GPCRs. Elongation of the chemokine receptor helix seems to be required to position the conserved cysteine toward the inner face of TM7, which favors its engagement in the disulfide bridge with the N terminus of the receptor (Fig. 1, A and E). The ECL4 pseudo-loop may play an important role in chemokine recognition. Chemokine receptors are thought to bind to their ligands through a two-step mechanism that involves successive interactions between the chemokine and both the flexible N terminus of the receptor [chemokine recognition site 1 (CRS1)] and a pocket located in the vicinity of the transmembrane segments and the extracellular loops (CRS2) (*4*, *5*). Note that ECL4 and the disulfide bond between the N terminus and TM7 reposition the remaining flexible part of the N-terminal region of the receptor from the top of TM1 to the top of TM7 alongside ECL3 (Fig. 1E). Such a delocalization is likely necessary for chemokine binding and would provide an optimal orientation of the flexible N terminus of the receptor (CRS1) with respect to CRS2. This repositioning may be further facilitated by the proline residue that often directly precedes the conserved cysteine, which forms a kink in CRS1 and brings CRS1 in front of the β hairpin of ECL2, a major determinant of CRS2 in CXCR4 (Fig. 1, A and E) (*6*). ECL4 also influences the shape, size, and charge of the entrance of the transmembrane binding pocket for endogenous ligands (CRS2) and small pharmacological modulators (Fig. 1, B to D). Similar to the canonical disulfide bridge between TM3 and ECL2, the bond between the N terminus and TM7 may also contribute to the overall stability and rigidity of the receptor, as well as to the conformational changes that occur upon chemokine binding. Finally, this loop may limit the diffusion of small molecules across the helix bundle, and it may participate in receptor-receptor interactions, type I dimerization, or both.

Despite difficulties in predicting the starting residue of TM1, and although there is little ECL4 sequence similarity among receptors, we identified three subfamilies of chemokine receptors that are characterized by different molecular signatures within their pseudo-loops (Fig. 1G) (*4*, *7*). The receptors CCR1, CCR5, and CCR9 share conserved, positively charged residues at positions C_{+2} and C_{+6} (family A), whereas CCR6, CCR8, CCR10, all of the CXC receptors, CX3CR1, and the atypical chemokine receptor ACKR2 (D6) have a negatively charged residue at position C_{+3} or C_{+4} (family B). The side chains of the residues that define family A (Lys^{26}, C_{+6}) and family B (Glu^{32}, C_{+4}) are well aligned in the superposed x-ray structures of CXCR4 and CCR5 (Fig. 1F) and point toward the inner face of the receptors, suggesting that this

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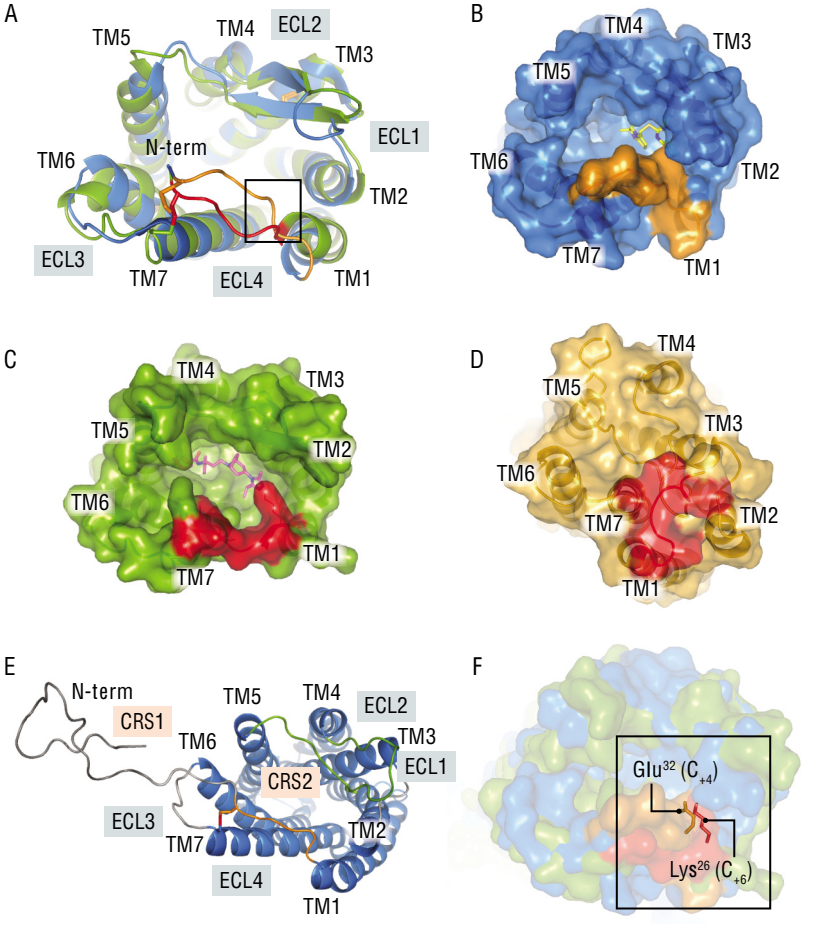
position may be of importance for receptor function. This observation is consistent with data demonstrating that the Asp^{25} of CX3CR1 (C_{+4}) is critical for binding to its ligand CX3CL1 (fractalkine) (*8*) and that Glu^{32} of CXCR4 (C_{+4}) is predicted to interact with the N-terminal lysine of CXCL12 (also known as SDF1-!), which accounts for its agonist activity (*9*). Other receptors, such as CCR2, CCR3, CCR4, CCR7, and ACKR4 (CCX-CKR) bear both types of signatures (family A/B). The receptors XCR1, ACKR1 (DARC), and ACKR3 (CXCR7) display no feature that enables their classification into one of the two families (family C). In contrast to sequences preceding the conserved cysteine, no posttranslational modifications are predicted among the different ECL4s, except for that of ACKR3, which displays a putative N-glycosylation site (NKS) at position C_{+5}.

The presence of a fourth ECL and its molecular signatures may not be restricted to chemokine receptors. Indeed, the additional cysteine residues in the N terminus and TM7 (ECL3) are also found in ~30% of receptors that belong to the rhodopsin fam-



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Receptor	Sequence	Family
XCR1	P-CENQAWVF	C
CCR1	P-CQKVNERA	A
CCR2	P-CHKFDVKQ	A/B
CCR3	L-CEKADTRA	A/B
CCR4	P-CTKEGIKA	A/B
CCR5	P-CQKINVKQ	A
CCR6	L-CSLQEVRQ	B
CCR7	L-CSKKDVRN	A/B
CCR8	P-CDAELIQT	B
CCR9	Y-CEKNNVRQ	A
CCR10	L-CYKADVQA	B
CXCR1	P-CMLETETL	B
CXCR2	P-CEPESLEI	B
CXCR3	P-CPQDFSLN	B
CXCR4	P-CFREENAN	B
CXCR5	L-CPATEGPL	B
CXCR6*	S-SQEEHQDF	B
CX3CR1	A-CYIGDIVV	B
ACKR1	P-CHSCNLLD	C
ACKR2	L-CRKDAVVS	B
ACKR3	M-CPNMPNKS	C
ACKR4	I-CIKEDVRE	A/B

Fig. 1. Overall structure of the extracellular surface of chemokine receptors and the location of ECL4 pseudo-loops. (**A**) Comparison between the x-ray structures of CXCR4 (blue) and CCR5 (green) showing the conserved overall arrangement of the extracellular features (ECL1, ECL2, and ECL3), as well as the location and shape of the ECL4 pseudo-loops of CXCR4 (orange) and CCR5 (red). Note that the ECL4 pseudo-loop of CXCR4 points more toward the inside of the ligand-binding pocket than does that of CCR5. The black square highlights the position of family-defining residues presented. (**B**) Ligand-binding pocket of CXCR4 [based on Protein Data Bank (PDB) structure 3ODU] in complex with the CXCR4 antagonist IT1t (yellow). ECL4 is colored in orange. (**C**) Ligand-binding pocket of CCR5 (PDB 4MBS) in complex with the small allosteric CCR5 inhibitor Maraviroc (pink). ECL4 is colored in red. (**D**) NMR structure of CXCR1 in a liquid crystalline phospholipid bilayer (orange) (PDB 2NLN). ECL4 is colored in red. In the absence of the small ligand, ECL2 lies on top of the ligand-binding pocket, blocking its access, and TM1 is off-centered from the TM circle in comparison with ECL2 of CXCR4 and CCR5. (**E**) Arrangement and position of CRS1 and CRS2 in CXCR4. The N terminus, ECL2, and ECL4 are colored in gray, green, and orange, respectively. The complete structure of CXCR4 was generated by molecular dynamic simulation after engraftment of the 28 N-terminal residues to the resolved x-ray structure (PDB: 3ODU) (*4*). (**F**) Positional conservation of residues at positions C_{+6} in CCR5 (family A, red) and C_{+4} in CXCR4 (family B, orange). (**G**) Sequences and classification of chemokine receptor ECL4 pseudo-loops. The ECL4 pseudo-loop of chemokine receptors encompasses residues from the cysteine (C), involved in a disulfide bridge with the top of TM7 (at the end of ECL3), to the first amino acid residue of TM1. ECL4 classification is based on the presence of two conserved positively charged residues at positions C_{+2} and C_{+6} (family A) or a negatively charged residue at positions C_{+3} or C_{+4} (family B). ECL4s containing both signatures are classified as family A/B, whereas those presenting none of the conserved residues are classified as family C. Residues belonging to incomplete signatures are underlined. The asterisk indicates that no cysteine is present in the N terminus of CXCR6.

ily, including receptors for lysophospholipid (LPA), bradykinin (B1-2), endothelin (ETA-B), melanocortin (MC1-5), serotonin (5-HT), purinergic (P2Y), and orphan receptors. The structure of the latest resolved rhodopsin-like receptor, $P2Y_{12}$, revealed the presence of a pseudo-loop equivalent to that found in the chemokine receptors (*10*); however, the conservation of these residues does not necessarily imply the formation of a pseudo-loop, as is shown by the structures of the dopamine receptor D_3 and the serotonin receptor $5\text{-}HT_{1B}$, which lack a disulfide bridge between the two conserved cysteines (*11*, *12*). Therefore, in the near future, the presence and the exact role of ECL4s in ligand-binding, signal transduction, and receptor interactions will need to be addressed in more detail, not only for chemokine receptors, but also for other receptor families.

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Acknowledgments: The authors thank C. Seguin-Devaux, J. Hanson, and P. Lambert for fruitful discussions and critical reading of the manuscript and K. Arumugam for molecular modeling. **Funding:** This manuscript was supported by the Centre de Recherche Public de la Santé (CRP-Santé), Luxembourg, grant 20100708; the Fonds National de la Recherche, Luxembourg, grants AFR-3004509 and CORE C11/BM/1209287; and Télévie, grant 7.4568.14.

10.1126/scisignal.2005664

Citation: M. Szpakowska, D. Perez Bercoff, A. Chevigné, Closing the ring: A fourth extracellular loop in chemokine receptors. *Sci. Signal.* **7**, pe21 (2014).

Epub ahead of print December 23, 2015 - doi:10.1189/jlb.2MR0815-383R

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Mini-Review

vCCL2/vMIP-II, the viral master KEYmokine

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RECEIVED AUGUST 27, 2015; REVISED NOVEMBER 19, 2015; ACCEPTED NOVEMBER 20, 2015. DOI: 10.1189/jlb.2MR0815-383R

ABSTRACT

Viral CC motif chemokine or viral macrophage inflammatory protein-II is 1 of the 3 chemokines encoded by the human herpesvirus-8 to interfere with the host chemokine receptor network, facilitate the immune escape, and promote its survival. Viral CC motif chemokine 2 binds to a broad spectrum of viral and human chemokine receptors of all 4 classes and, depending on the receptor, acts either as an agonist or an antagonist, inducing or blocking the recruitment of specific immune cell subsets. These atypical binding and signaling properties make this viral chemokine not only a useful tool to investigate the complexity of the chemokine–receptor interaction network or the virus–host interplay but also for the development of receptor inhibitors. This mini-review summarizes the knowledge currently available on viral CC motif chemokine 2 binding, signaling, and structural mimicry and discusses its role and importance for the virus, the therapeutic potential, and the open questions regarding the biology of this fascinating chemokine. J. Leukoc. Biol. 99: 000–000; 2016.

Introduction

Chemokines are small (8–14 kDa) secreted proteins that play a central role in guiding directional migration (chemotaxis) of leukocytes in immunosurveillance, and immune responses and are important mediators of cell adhesion, growth, and survival [1]. They exert these functions by interacting with chemokine receptors that are 7-transmembrane-domain GPCRs. To date, 47 chemokines and 19 receptors, forming a highly intricate and precisely regulated network, have been identified in humans. Based on conserved cysteine motifs, chemokines are divided into 4 subfamilies—CC, CXC, XC, and CX3C—and the receptors are named according to the subfamily of chemokines they bind (CCR, CXCR, XCR, and CX3CR, respectively). In addition, other receptors referred to as ACKR (ACKR1–4) can recognize chemokines and act as scavengers or signal through alternative pathways, further contributing to the complexity of the chemokine network [2].

Abbreviations: ACKR = atypical chemokine receptor, AIDS = acquired immune deficiency disorder, CCL = chemokine (CC motif) ligand, CXCL = chemokine (CXC motif) ligand, CX3CL = chemokine (CX3C motif) ligand, GAG = glycosaminoglycan, GPCR = G protein-coupled receptor, HCMV = human cytomegalovirus, HHV-6 = human herpesvirus-6, KSHV = Kaposi's sarcoma-associated herpesvirus, NK = natural killer, NMR = nuclear

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Among a myriad of mechanisms that viruses use to evade the immune system or exploit various biologic processes of the host cell to promote their survival, large DNA viruses, such as herpesviruses and poxviruses, have evolved strategies to interfere with the chemokine-receptor network by encoding their own chemokine and receptor homologs or chemokine-binding proteins capable of sequestering a broad range of chemokines [3–6].

vCCL2/vMIP-II, A VIRAL CHEMOKINE HOMOLOG ENCODED BY HHV-8

vCCL2 (also known as vMIP-II) is a viral CC chemokine encoded by HHV-8, known as the Kaposi's sarcoma–associated herpesvirus (KSHV), the causative agent of Kaposi's sarcoma, a disease generally linked with immunodeficiency, but also to 2 rare proliferative disorders: primary effusion lymphoma and multicentric Castleman disease [7–9].

vCCL2 was initially identified from a fragment of the HHV-8 genome isolated from a Kaposi's sarcoma biopsy [10]. Among the viral ORFs present in the HHV-8 genome, 3 (K6, K4, and K4.1) were predicted to encode the CC chemokine homologs vCCL1/vMIP-I, vCCL2/vMIP-II, and vCCL3/vMIP-III, respectively, and 1 (ORF74), a CXC chemokine receptor homolog [11]. vCCL2 is produced as a 94-aa precursor with a 23-aa N-terminal signal peptide and a C-terminal arginine, which are cleaved to yield the mature 70-aa chemokine (7.9 kDa) (Fig. 1A) [11, 12].

vCCL2 was most likely captured from a cluster on the human chromosome 17 (17q11-32), which contains genes encoding most of the CC chemokines. Indeed, it shows high sequence identity with the human chemokines CCL3 (47.1%), CCL18 (44.1%), CCL15 (43.4%), and CCL4 (40.6%), which bind CCR1, -3, -5 and -8. vCCL2 also has a high sequence identity with vCCL1 (55.7%) which acts as an agonist of CCR8 [13, 14], but much less identity with vCCL3 (24.5%), which activates CCR4 and XCR1 [15, 16].

The tridimensional structure of vCCL2 has been resolved by both x-ray crystallography [17] and NMR [18], and it has been shown to adopt a fold typical of human chemokines characterized by a flexible and disordered N terminus of 10 residues followed by the cysteine motif (C_{11} and C_{12}), an N-loop, 3

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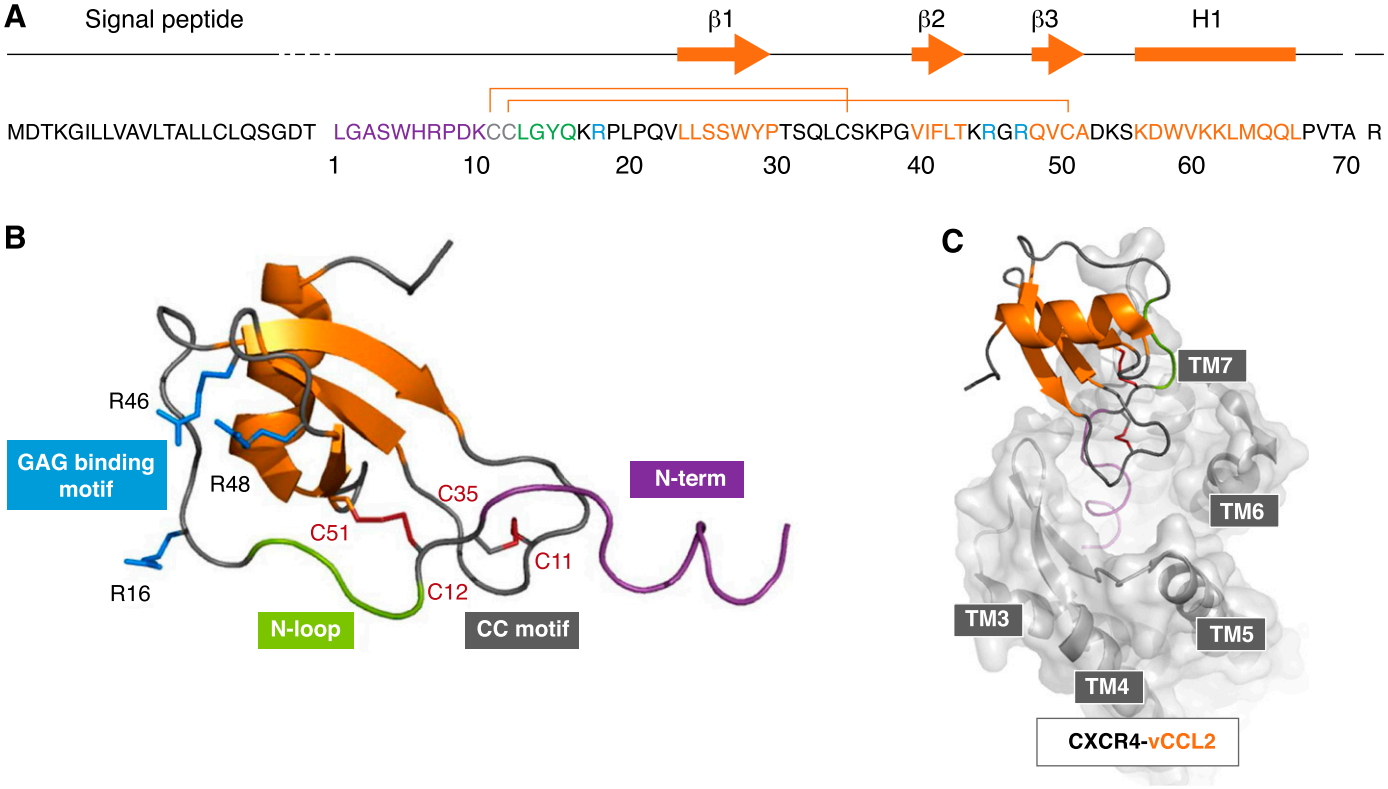

Figure 1. Structures and N-terminal features of vCCL2. (A) Primary sequence of the vCCL2 precursor encoded by HHV-8 ORF K4. The vCCL2 precursor (94-aa) presents an N-terminal signal peptide and a C-terminal arginine (R), which are cleaved to yield the 70-aa mature chemokine. The exible N terminus (residues 1–10), the cysteine motif (C_{11}-C_{12}), and the N-loop (residues 13–21) are shown in purple, gray, and green, respectively. The 3 b-strands and the C-terminal a-helix are represented in orange and the disul de bridges connecting the cysteine motif to the core of the chemokine are indicated by red lines. Arginine residues involved in GAG binding (R16, R46, and R48) are blue. (B) Tridimensional structure of vCCL2 resolved by x-ray crystallography (PDB 4RWS) showing the structural arrangement of vCCL2 features including the highly disordered N terminus (purple), the N-loop (green), the core of the chemokine with the 3 b-strands and the C-terminal a-helix (orange), and the conserved cysteines (red). (C) Tridimensional structure of the complex between CXCR4 and vCCL2 (PDB 4RWS). The receptor is shown as a cartoon/surface and is gray. The chemokine is represented as a cartoon and is purple (N-term), green (N-loop), and orange (core). The structure-stabilizing disul de bridges are red. vCCL2 makes substantial contacts with the receptor with the N terminus inserted deep in the transmembrane (TM) cavity.

anti-parallel b-strands, and a C-terminal a-helix. The N terminus and the b-sheet of vCCL2 are connected by 2 disul de bridges linking the cysteine residues 11–35 and 12–51 (Fig. 1B). However, in contrast to its closely related human CC chemokine homologs, in solution, vCCL2 exists exclusively as a monomer [18, 19], although an engineered dimeric variant (L13F) has been reported [20].

Ever since its discovery almost 2 decades ago, vCCL2 has been intensively investigated and demonstrated to have quite atypical binding and signaling properties that re ect the complex and sophisticated mechanisms that HHV-8 has evolved to control its life cycle and modulate the host immune response.

BINDING AND ACTIVITY OF vCCL2 TOWARD VIRAL AND HUMAN CHEMOKINE RECEPTORS

vCCL2 binds with nanomolar af nity to a broad spectrum of viral and human chemokine receptors and, depending on the receptor, acts either as an antagonist or an agonist. It is the only chemokine identi ed so far that is capable of binding to chemokine receptors of the 4 families (CCR, CXCR, XCR, and CX3CR) and may be seen as a "master KEYmokine", a master key for chemokine receptors (Fig. 2).

Being a CC chemokine, vCCL2 recognizes mostly receptors that belong to the CC family. vCCL2 was shown to act as an antagonist toward CCR1, -2, -5 [12], and -10 [21], competing with the endogenous chemokine binding and signaling, but also as an agonist of CCR3 [22] and -8 [23], triggering G protein-mediated intracellular calcium release and activation of the ERK and PI3K/AKT signaling pathways, or chemotaxis [24]. Several ligands for these receptors, including CCL3, -4, -5, and -15 have high sequence identity with vCCL2 (Fig. 2).

(continued from previous page)

magnetic resonance, ORF = open reading frame, PEL = primary effusion lymphoma, PET = positron emission tomography, Th1/Th2 = Type 1 or 2 T helper cells, vCCL2/vMIP-II = viral CC motif chemokine 2/viral macrophage inflammatory protein-II, XCL = chemokine (C motif) ligand

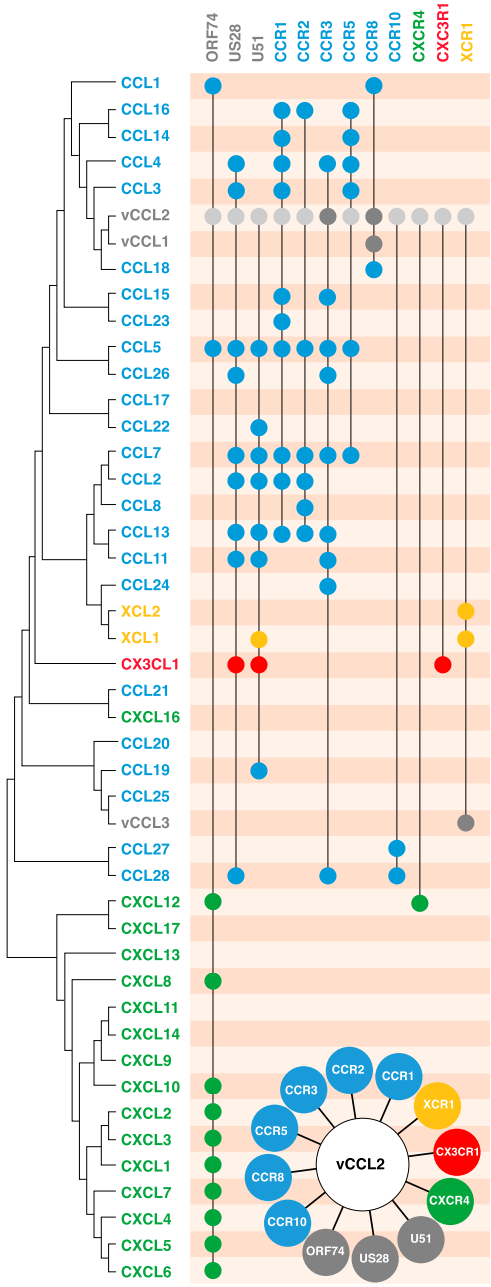

Figure 2. Similarity between HHV-8-encoded and human chemokines and overview of vCCL2-binding receptors. Amino acid sequence similarity of HHV-8 and human chemokines and their binding speci city. Only chemokine receptors targeted by vCCL2 are shown. Left: sequences were aligned with ClustalW and the similarity tree was built with Omega6. The names of chemokines are colored according to their classes: CC (blue), CXC (green), XC (yellow), and CX3C (red). HHV-8 chemokines are gray. Right: chemokine binding speci city of receptors targeted by vCCL2. Agonist and antagonist activities of vCCL2 toward the different receptors are represented by dark and light gray circles, respectively. Inset: overview of the diversity of human and viral chemokine receptors targeted by vCCL2. Human receptors are colored according to the class of chemokine they bind: CCR (blue), CXCR (green), XCR (yellow), and CX3CR (red). Viral chemokine receptors are gray.

Apart from CC receptors, vCCL2 is an antagonist ligand for the only 2 representatives of the XCR and CX3CR families, XCR1 [25, 26] and CX3CR1 [27, 28], and for only 1 CXC receptor, CXCR4 [12] (Fig. 2, inset). The recently resolved crystal structure of vCCL2 in complex with CXCR4 [29] revealed a 1:1 stoichiometry interaction with extensive contact surface between the 2 partners. In addition, the study provided a molecular explanation for the speci city of CC and CXC chemokines toward their respective receptors and for the cross-family interaction of the vCCL2–CXCR4 pair. It suggested that, on the one hand, vCCL2 bears CXC chemokine-like features that are involved in the interaction with CXC receptor-conserved residues of CXCR4. On the other hand, some residues present in CXCR4 are signatures of CC rather than CXC receptors and therefore may contribute to the binding of vCCL2 [29].

vCCL2 also binds to several viral chemokine receptor homologs. It was shown to downregulate the constitutive activity of the HHV-8-encoded receptor ORF74, a viral homolog of the human CXCR2, binding to many CXC and CC chemokines [30, 31]. Similarly, vCCL2 is described to inhibit the constitutive activity of US28 [12] and U51 [32], which are homologs of CX3CR1 and CCR7, encoded by HCMV and HHV-6, respectively.

Besides its interactions with chemokine receptors, it is proposed that the binding of vCCL2 to GAGs present on the surface of endothelial cells may also play an important role in vivo. Although their interaction modes are similar, vCCL2 binds to GAGs much more tightly than most endogenous chemokines [20]. Therefore, by binding to GAGs vCCL2 may build up its own chemokine gradient to interact more ef ciently with leukocytes that express its target receptors but also to compete with the binding of the endogenous chemokines to GAGs, interfering with the normal leukocyte recruitment. In addition, binding to GAGs may protect vCCL2 against proteolysis [20] (Fig. 3).

ROLE OF vCCL2 IN HHV-8 BIOLOGY

HHV-8 can establish life-long asymptomatic infections in immunocompetent individuals but, as stated earlier, it is most notorious for its association with Kaposi's sarcoma, often affecting AIDS patients, as well as 2 other proliferative diseases: primary effusion lymphoma and multicentric Castleman disease [7–9]. HHV-8 infects mainly endothelial and B cells but also monocytes and dendritic cells [33–35]. As in other herpesviruses, its life cycle consists of 2 stages characterized by different gene expression programs. During the latent or dormant phase, only a limited number of proteins is expressed, whereas in the course of the lytic or productive phase, most of the genes are transcribed and the replication with viral progeny production takes place [36, 37]. A ne regulation between the latent and the productive cycle allows the virus to propagate, to persist for a long period in the host, and to avoid clearance by the immune system. HHV-8 has also evolved complex mechanisms, including the use of viral chemokines and receptors as a means of exploiting the host chemokine system to favor its own survival. vCCL2 is expressed as an early lytic gene [38, 39] and plays an important role in modulating the activity of HHV-8 and host chemokine receptors through autocrine and paracrine effects.

One of the strategies the virus uses is the skewing of the host immune system away from the Th1 cytotoxic response,

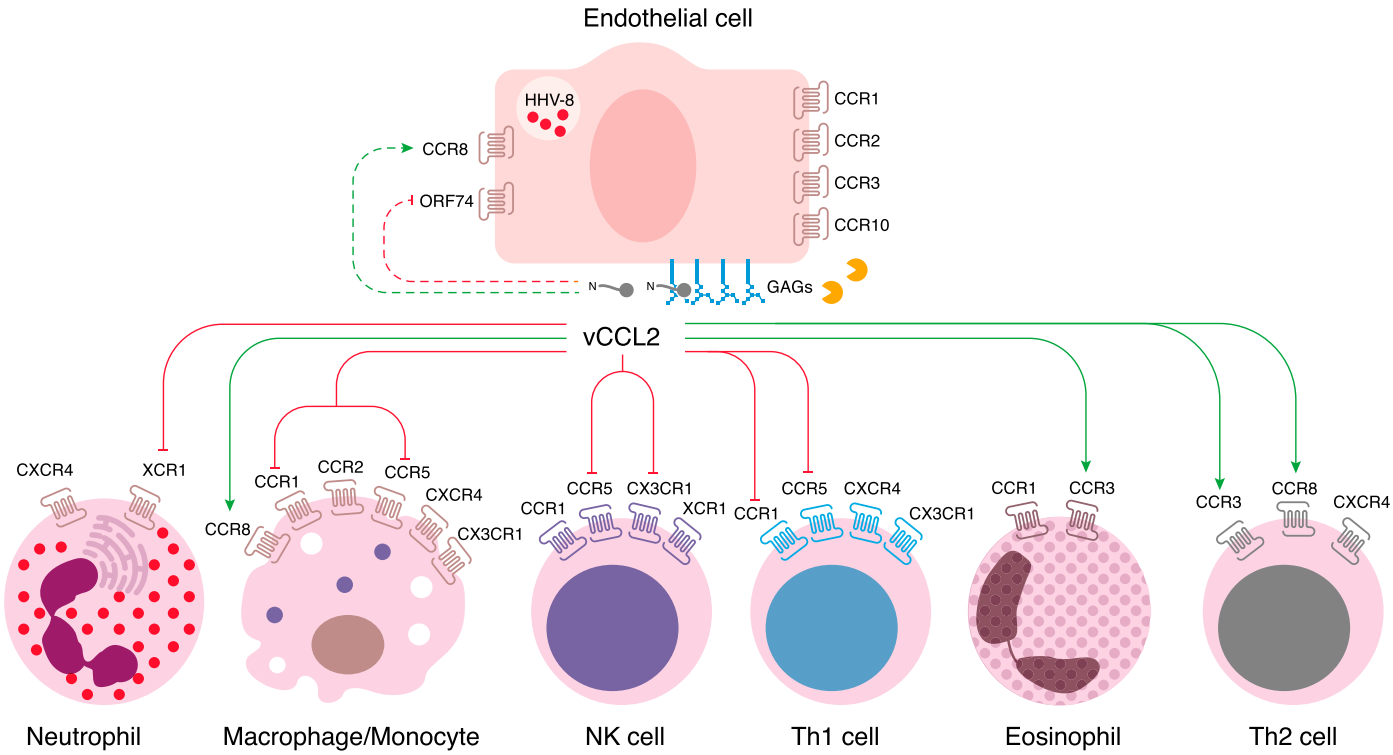

Figure 3. Presumed interplay between vCCL2 and chemokine receptors expressed by infected and immune cells. Receptor antagonist (red) and agonist (green) activity of vCCL2 is represented by solid (paracrine) and dashed (autocrine) lines. Only the interactions for which functional experimental data have been reported are presented. vCCL2 expressed by HHV-8 infected endothelial cells interacts with the human chemokine receptor CCR8 and ORF74 to promote cell survival and limit ORF74-driven tumorigenesis (autocrine activity). vCCL2 targets chemokine receptors expressed by different immune cells to limit antiviral cytotoxic responses by blocking CCR1 and -5 expressed by Th1 cells and macrophages, whereas it induces the recruitment of Th2 cells and eosinophils by acting on CCR3 and -8. For clarity, the chemokine receptor expression patterns of the different cell subsets include only vCCL2-binding receptors.

undesirable for the virus-infected cells, toward a Th2 response, which is less effective against intracellular pathogens. vCCL2 is proposed to strongly contribute to this mechanism of immune evasion through its selective antagonist action on Th1-speci c chemokine receptors (CCR1, -2, and -5 and CX3CR1) and agonist effect on Th2-related CCR3 and -8 (Fig. 3).

Numerous studies have reported an antagonist effect of vCCL2 on CCR1, CCR2, CCR5, CXCR4, and CX3CR1 that inhibits endogenous ligand-induced calcium responses and cell migration in both cell lines overexpressing the receptor of interest or lymphocytes isolated from peripheral blood [12, 22, 23, 27, 28]. Owing to its large spectrum of receptors, vCCL2 is also able to block the recruitment of immune cells at different stages of their activation. It has been shown, for instance, that, despite the differences in the patterns of chemokine receptors expressed, vCCL2 can inhibit the migration of both naïve and activated NK cells through interaction with CX3CR1 and CCR5, respectively [40]. vCCL2 is often referred to as a broad-spectrum antagonist chemokine, but its agonist activity toward CCR3 and -8 is also documented. It has been shown to induce CCR3-dependent calcium release and eosinophil chemotaxis [41] and arrest in shear ow [22]. Similarly, CCR8-transfected cells have been reported to migrate in response to vCCL2 [23].

In addition to facilitating the evasion from cytotoxic immune responses, vCCL2 plays a role in blocking the defense mechanisms from within the cells. It has been shown to inhibit lytic cycle-induced proapoptotic signals in infected cells, thereby prolonging their survival and favoring the virus-productive replication and propagation. This antiapoptotic effect of vCCL2 is suggested to operate, at least in part, through CCR8 in both an autocrine and a paracrine manner, contributing to viral persistence and latency maintenance [24].

Moreover, vCCL2 may participate in controlling the 2 gene expression programs of HHV-8. vCCL2 acts as an inverse agonist toward the HHV-8-encoded GPCR, ORF74. This constitutively active receptor is expressed during the early lytic stage of the virus and confers a highly proliferative potential to the cells. The down-regulation of ORF74 by vCCL2 may temporally control the activity of HHV-8 by repressing its reactivation, thereby helping to escape the host immune surveillance [30, 31].

vCCL2 is also proposed to facilitate virus dissemination through its proangiogenic effect. It has been shown by different approaches, including an in ovo chick embryo chorioallantoic membrane–based assay and in vivo lentivirus-delivered vCCL2, that it has the potential to enhance blood vessel formation and survival and may therefore also contribute to Kaposi's sarcoma or PEL-linked pathogenesis [41, 42].

THERAPEUTIC POTENTIAL OF vCCL2 AND ITS DERIVED PEPTIDES

Since the first description of its antagonist properties toward several receptors binding to proinflammatory chemokines and mediating HIV entry, vCCL2 and peptides derived from its N terminus have been explored for their immunomodulatory potential in acute and chronic inflammatory diseases or for their anti-HIV and antitumoral activity.

vCCL2 has been expressed as a full-length native recombinant protein or as a fusion protein [43, 44] in various eukaryotic [44, 45] and prokaryotic systems [18, 20] or with different gene delivery approaches [42, 46, 47]. Fully synthetic vCCL2, with or without nonnatural amino acids, has also been produced [17, 48, 49]. Because of its specific inhibitory properties toward many receptors for proinflammatory chemokines such as CCL2, -3, -4, and -5, vCCL2 has been tested in vitro and in mice and rats for its ability to block leukocyte recruitment and infiltration to reduce Th1-driven inflammation after ischemic brain and spinal cord injuries [50–52] to limit cardiac, corneal, or renal allograft rejection [46, 47, 53], glomerulonephritis [27] and cutaneous hypersensitivity reaction [45]; and to promote posttransplantation angiogenesis [42]. In addition, vCCL2 labeled with [^{64}Cu]-DOTA has been shown to be a sensitive probe to detect by PET imaging the up-regulation of different chemokine receptors involved in atherosclerosis [54]. Because of its unique ability to bind to CCR5, CXCR4, and CCR3, vCCL2 has been evaluated as an HIV-1 inhibitor, to block viral entry through several coreceptors [12, 41, 55]. vCCL2 showed moderate inhibition of HIV entry through the main coreceptors CXCR4 and CCR5, but seemed more potent in blocking CCR3, which is essential for HIV infection of microglia [12]. This higher potency may be partly explained by the agonist activity of vCCL2 toward CCR3 demonstrated in other studies [22, 41], which could affect HIV infection by triggering specific cellular responses and subsequent receptor internalization.

In parallel to studies performed with the full-length chemokine, the possibility of reducing the size of vCCL2 to a peptide level (down to as few as 9 residues), while maintaining parental activity and selectivity, has also been investigated. Because of their small size, such peptides are easier to produce and modify and have been proposed to hold a great potential for the design of novel therapeutics [56]. In accordance with the generally accepted chemokine–receptor 2-step binding mode [57], peptides encompassing the flexible N terminus, the cysteine motif, and the N-loop of vCCL2 (1-LGASWHRPDKCCLGYQKRPLP-21) have been shown to act as CXCR4 inhibitors that block the entry of HIV 34 strains [58–61] or modulate inflammation [58–62] with potency in the micromolar range. Notably, binding of peptides derived from the first 21 residues of vCCL2 was shown to be maintained for its all-D-amino-acid analog, revealing the unsuspected high permissivity of CXCR4 to stereoisomer replacement and offering the interesting possibility of designing peptides with higher resistance to proteolysis [58, 63–65]. These studies also pointed out the importance of the residues Leu^{1}, Arg^{7}, and Lys,^{9} for CXCR4 binding and demonstrated that dimerization improves the potency of the peptides to nanomolar range, providing one of the first indications of the high propensity of CXCR4 to form homodimers [66]. The peptide corresponding to residues 1–21 did not show any HIV-1 inhibitory ability against R5 viruses, suggesting that vCCL2 most likely interacts with CXCR4 and CCR5 according to a slightly different binding mode or using different determinants, with a higher contribution of the N-terminal fragment in CXCR4 binding. No information is available on the binding of vCCL2 peptide derivatives to other receptors targeted by the parental chemokine.

Cyclic peptides bearing homologies with the three-residue segment Trp5-His6-Arg7 present in vCCL2 N-terminus have also been explored for their antitumoral activity in in vivo models of lung metastases and growth of renal cell xenografts. These peptides showed significant reduction of tumor spread and expansion with potency in the micromolar range [67].

Finally, D-peptides derived from the flexible N terminus of vCCL2 (residues 1–10) have also been explored as a vehicle to specifically target and deliver molecules, such as small drugs or DNA to CXCR4-overexpressing cancer cells [68].

DISCUSSION

vCCL2 is an atypical and a fascinating chemokine. It binds to a broad spectrum of both viral and human chemokine receptors across the 4 classes, showing antagonist or agonist activity at both the autocrine and paracrine levels. These unique properties reflect a sophisticated strategy of molecular mimicry and receptor piracy that HHV-8 has evolved to turn the host chemokine receptor network to its own advantage.

Despite the number of receptors that vCCL2 binds, it should not be regarded as a nonselective chemokine. Rather, its broad-spectrum binding properties should be seen as tightly linked to its ability to precisely modulate multiple facets of host chemokine-mediated defenses. vCCL2 has been shown to favor the survival of infected cells by skewing the host immune response away from the deleterious Th1 type and toward the Th2 type. Although vCCL2 has initially been described as an antagonist of numerous receptors—notably, those acting as HIV entry coreceptors [12]—it has since been demonstrated to activate CCR3 and -8, triggering direct G-protein signaling. This dual activity and tailored specificity makes the unique character of vCCL2.

The binding of vCCL2 to its receptors does not appear to be dictated by their degree of promiscuity. vCCL2 binds several multiple-ligand receptors, such as CCR1, -2, -3, and -5, but also interacts with receptors that have a narrow ligand spectrum, such as CCR8, CCR10, XCR1, CX3CR1, and CXCR4 (Fig. 2). Moreover, the agonist or antagonist activity of vCCL2 toward a particular receptor cannot be easily predicted or explained based on vCCL2 sequence or its similarity with human chemokines. Among the CCR family, vCCL2 activates CCR3 and -8 but antagonizes CCR1 and -5, the 4 receptors being activated by CCL3, -4, -15 or -18, the human chemokines closest to vCCL2. At the same time, vCCL2 interacts with CCR2 and -10, which are activated by endogenous chemokines that have poor sequence identity with vCCL2.

The ability of vCCL2 to bind receptors outside the CCR family is unusual, but was probably acquired to achieve more efficient polarization of the immune response by blocking XCR1, CX3CR1, and CXCR4. Reprogramming vCCL2 to target these receptors was most likely less difficult, as XCL1, XCL2, and

CX3CL1 cluster with the CC chemokines on the similarity tree, and CXCL12 is one of the CXC chemokines that displays the highest sequence similarity with the CC family (Fig. 2). vCCL2 binding and signaling properties are even more remarkable, given that it shares high sequence identity with vCCL1, which binds to only 1 receptor, and that the 2 chemokines are proposed to have evolved by gene duplication within the virus.

The atypical binding properties of vCCL2 appear to be the result of a multiconstraint compromise in molecular reshaping of both its N terminus (addressing and message) and its core (addressing). It has been shown, for instance, that speci c modi cations in vCCL2 can improve its af nity for a particular receptor class, at the expense, however, of binding to other receptors [28] illustrating the fragility of this compromise. It cannot be excluded that vCCL2 binding to some receptors may simply be a side effect of its intrinsic promiscuity. The resolution of additional structures of human receptors in complex with vCCL2 and further studies of the role of its core and N terminus are nevertheless needed to unravel the molecular basis of its unusual behavior.

It is uncertain whether all host receptors identi ed in vitro for vCCL2 are used in vivo. Considering the intricacy and the spatiotemporal expression variability of the chemokine-receptor network, the relevance of these interactions to HHV-8 in physiologic conditions remains unclear. Moreover, although the functional consequences of the binding of vCCL2 are well described for several receptors, its activity toward others, such as CCR2 or -10, remains less well documented.

Among the 3 chemokines encoded by HHV-8, vCCL2 is the only one able to bind and reduce the constitutive activity of ORF74. Although blocking the activity of its own receptor may seem counterproductive for the virus, it probably plays an important role in controlling the potential deleterious tumorigenic effect of ORF74. vCCL2 was also shown to bind U51 and to display subnanomolar af nity toward US28, the chemokine receptors expressed by 2 other herpesviruses, HHV-6 and HCMV, respectively. The biologic relevance of these observations remains to be investigated. However, because HHV-6 and HCMV can infect similar cell types as HHV-8, including monocytes or endothelial cells, and because both viruses have also been found in Kaposi's sarcoma lesions, one could speculate that vCCL2 is involved in some aspects of their infection [69–71]. In a similar manner, HHV-6 has been suggested to promote HHV-8 pathogenesis [39].

The signaling by human and viral chemokine receptors in response to vCCL2 has been mostly studied in the context of their canonical G protein pathways, mainly by monitoring intracellular calcium mobilization. However, in light of the recent observations that chemokine receptors can also trigger alternative pathways dependent on b-arrestin or other G-protein subtypes, it cannot be excluded that vCCL2 signaling may also be more complex than initially thought [72, 73]. Furthermore, vCCL2 may activate different signaling pathways through the same receptor but depending on the cell type (tissue or cell bias) or which receptors are coexpressed [40], adding a level of complexity to its interplay with chemokine receptors. These aspects, together with the use of different cellular models and assays, may also explain some of the discordant results obtained for the agonist vs. antagonist activity of vCCL2 toward several human receptors such as CCR5 [43], -8 [13, 74] and -10 [26].

In addition, the binding and activity of vCCL2 toward recently deorphanized receptors such as CXCR8 or the 4 representatives of the ACKR family considered as silent/scavenger or arrestin-signaling receptors have not been documented yet. Because some of these receptors are expressed either by cells susceptible to HHV-8 infection or cells of the immune system, their interaction with vCCL2 should be given more attention in the future.

Beyond the better comprehension of HHV-8 biology and the complex interplay that this virus has evolved to in ect the host immune response, the investigation conducted on vCCL2 has allowed to signi cantly improve our understanding of the human chemokine receptor network and especially of the molecular basis of human receptor recognition and activation. The atypical binding properties of this viral master KEYmokine have also been shown to be instrumental in taking up several technical challenges and have opened some promising therapeutic avenues for immune modulation. Nevertheless, many aspects of vCCL2 biology and of its molecular mimicry remain to be elucidated.

AUTHORSHIP

M.S. and A.C. wrote and revised the publication.

ACKNOWLEDGMENTS

This study was supported by Luxembourg Institute of Health (LIH) Grants 20100708 and 20150415, the "Fonds National de la Recherche" (FNR) Luxembourg, grants AFR-3004509. The authors thank J. Hanson for fruitful discussions and a critical reading of the manuscript.

DISCLOSURES

The authors declare no con icts of interest.

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KEY WORDS:
chemokine receptor · GPCR · HHV-8 · KSHV · CXCR4

Biochimica et Biophysica Acta 1843 (2014) 1031–1041

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1 infection

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article info

Article history:
Received 15 July 2013
Received in revised form 23 December 2013
Accepted 17 January 2014
Available online 27 January 2014

Keywords:
CXCR4
CXCR7
Extracellular loops
HIV-1
CXCL12
GPCR

abstract

The chemokine receptor CXCR4 interacts with a single endogenous chemokine, CXCL12, and regulates a wide variety of physiological and pathological processes including in ammation and metastasis development. CXCR4 also binds the HIV-1 envelope glycoprotein, gp120, resulting in viral entry into host cells. Therefore, CXCR4 and its ligands represent valuable drug targets. In this study, we investigated the inhibitory properties of synthetic peptides derived from CXCR4 extracellular loops (ECL1-X4, ECL2-X4 and ECL3-X4) towards HIV-1 infection and CXCL12-mediated receptor activation. Among these peptides, ECL1-X4 displayed anti-HIV-1 activity against X4, R5/X4 and R5 viruses (IC_{50} = 24 to 76 M) in cell viability assay without impairing physiological CXCR4–CXCL12 signalling. In contrast, ECL2-X4 only inhibited X4 and R5/X4 strains, interfering with HIV-entry into cells. At the same time, ECL2-X4 strongly and speci cally interacted with CXCL12, blocking its binding to CXCR4 and its second receptor, CXCR7 (IC_{50} = 20 and 100 M). Further analysis using mutated and truncated peptides showed that ECL2 of CXCR4 forms multiple contacts with the gp120 protein and the N-terminus of CXCL12. Chemokine neutralisation was mainly driven by four aspartates and the C-terminal residues of ECL2-X4. These results demonstrate that ECL2 represents an important structural determinant in CXCR4 activation. We identi ed the putative site for the binding of CXCL12 N-terminus and provided new structural elements to explain the recognition of gp120 and dimeric CXCR4 ligands.

1. Introduction

The chemokine receptor CXCR4 is a class A G-protein-coupled receptor (GPCR) expressed at the surface of a large variety of cells including T lymphocytes, monocytes, neutrophils, dendritic and endothelial cells [1–3]. The interaction of CXCR4 with its unique endogenous ligand, the chemokine CXCL12, also named SDF1 , plays a crucial role in various processes such as hematopoietic stem cell [4,5] and leukocyte traf cking [6,7], vascular and neuronal development as well as in ammation and immune-modulation [5,7,8]. In addition to its physiological role, CXCR4 is involved in several pathologies including in ammatory diseases, WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelokathexis) syndrome, cancer and HIV-1 infection [9–13]. CXCR4 and another chemokine receptor, CCR5, act as co-receptors for the entry of HIV-1 into host cells by interacting with the viral envelope protein gp120 after its engagement with CD4 [11,14–21]. Viruses enter via CCR5 or CXCR4 (termed "R5 viruses" and "X4 viruses"), or use both co-receptors (termed "R5/X4" or dual tropic viruses). Viruses using CCR5 are believed to be preferentially transmitted as they infect effector memory CD4 + T-cells as well as macrophages and dendritic cells, which are abundant underneath the epithelial layer where infection occurs. Viruses utilising CXCR4, preferentially infecting naïve CD4 T-cells, generally appear later during infection and are associated with a decline of the immune response and with the onset of AIDS [22–27].

CXCR4 is also expressed on a large number of cancer cells and its interaction with CXCL12 has been demonstrated to favour tumour cell survival, proliferation and mobility leading to metastasis development [13,28–30]. Besides CXCL12, CXCR4 also interacts with the broad-spectrum human herpes virus 8-encoded chemokine vCCL2, which acts as an antagonist [31]. In 2005, CXCR7 was identi ed as the second CXCL12-binding chemokine receptor [32,33]. Similar to CXCR4, CXCR7 promotes cancer metastasis and its over-expression is often associated with more aggressive tumour phenotypes and bad prognosis [34–36]. Importantly, the biology and regulation of the activity of CXCR4, CXCR7 and their common ligand CXCL12 were suggested to be interdependent [37].

CXCR4 was shown to adopt a typical GPCR fold consisting of a seven-transmembrane helix bundle. However, the location and shape

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http://dx.doi.org/10.1016/j.bbamcr.2014.01.017

of its ligand-binding pocket differs from that of other protein-binding GPCRs and is situated closer to the receptor surface [38] suggesting a greater implication of the N-terminus and the three extracellular loops (ECL1, ECL2 and ECL3) in ligand binding and receptor activation [39–41].

The tridimensional structure of chemokines consists of (1) an elongated and exible N-terminus, (2) a cysteine motif, (3) a loop of approximately ten residues, often referred to as the N-loop, (4) a single-turn of 3_{10} helix, (5) three antiparallel -strands and (6) a C-terminal -helix. These secondary structures are connected by turns known as the 30s, 40s, and 50s loops, which re ects the numbering of residues in the mature protein [42,43]. The chemokine structure is further stabilised by two disulphide bridges connecting the cysteine residues of the N-terminus with those located on the 30s and 50s loops [42].

On the basis of the large amount of information available for CXCL12 and CXCR4, a general two-step mechanism was proposed to describe the interaction of chemokines with their cognate receptors [44,45]. The initial step of this model corresponds to the anchoring of the chemokine to the receptor's N-terminus (Chemokine Recognition Site 1, CRS1) and is followed by the binding of the exible N-terminus of the chemokine to a second site (CRS2) located in the vicinity of the transmembrane segments (TMs) and the extracellular loops of the receptor. In line with this model, studies using sulfated peptides derived from the N-terminal domain of CXCR4 demonstrated that peptide corresponding to CRS1 binds the surface of CXCL12 in an extended conformation close to the chemokine N-loop [46,47]. Furthermore, these studies highlighted the importance of sulfotyrosines present on CRS1 and sulfotyrosine-binding pockets present on the chemokine. Binding of the chemokine N-terminus to CRS2 was suggested to induce conformational changes in the receptor and in its subsequent activation. In agreement with this model, short peptides derived from the exible N-terminus of CXCL12 were shown to be suf cient to speci cally bind CXCR4, and displayed agonist activity [48–51]. Further analyses conducted with af nity puri ed CXCR4 identi ed several amino acids located on the CXCL12 -sheet and 50s loop as additional receptor interacting residues [52]. Although all these results corroborate the two-step binding model, the exact stoichiometry of the CXCR4–CXCL12 interaction as well as the receptor determinants forming the CRS2 remain to be clari ed [38,46].

The critical role of CXCR4 in cancer biology and HIV-1 infection makes this receptor and its ligands valuable targets for drug development. To date, several small CXCR4 antagonists including AMD3100, T140 or CTCE-9908 have been described [53–57]. Although these molecules are very potent in blocking HIV-1 infection and metastasis development, they are often associated with important side effects and/or inverse action on other chemokine receptors [53,58,59]. Therefore, other inhibition strategies need to be explored. Over the last few years, ligand neutralisation by small molecules, peptides and antibody fragments has emerged as an interesting alternative to the classical receptor inhibition [60–67]. However, peptidic derivatives of receptor extracellular loops have never been reported as potential chemokine neutralisers. In the context of CXCR4 and CXCR7, targeting their common chemokine, CXCL12, would allow the simultaneous interference with its binding to both receptors [68]. On the other hand, the development of molecules neutralising the HIV-1 envelope protein gp120 rather than the receptor would confer T-cell protection against viral infection without impeding the physiological functions of CXCR4.

In the present study, we investigated the neutralising properties of individual peptides corresponding to the rst, second and third extracellular loops (ECL1, ECL2, ECL3) of CXCR4 towards CXCL12 binding and HIV-1 infection. Analyses with mutated and truncated peptides provided new insights on the molecular basis of receptor–ligand recognition opening new perspectives for the development of CXCR4 ligand neutralisers.

2. Materials and methods

2.1. Peptides, proteins and cells

Peptides corresponding to the extracellular loops of CXCR4 (ECL1-X4, ECL2-X4 and ECL3-X4) were designed based on the receptor topology predicted prior to its X-ray structure resolution [39] (Table 1). All peptides including scrambled control peptides ECL1-$X4_{scrbl}$ (FNYSGAKFVNDLWA) and ECL2-$X4_{scrbl}$ (DVQDPRVLDWRNDVYSFYAFQFVCINE) were purchased from JPT and contained an amide group at the C-terminus to avoid additional negative charges. Peptide ECL2-X4 was also purchased biotinylated at its N-terminus and the biotin moiety was separated from the peptide by a Ttds linker ([N1-(9-Fluorenylmethoxycarbonyl)]-1,13-diamino-4,7,10-trioxatridecan-succinamic acid). The CXCL12 N-terminal peptide comprised the rst 17 residues of the chemokine (KPVSLSYRCPCRFFESH). Control peptide (SPAPERRGYSGYDVPDY) (Ctrl) corresponded to a HCDR3 sequence binding to an antibody directed against human in uenza haemagglutinin [69]. Chemokines CXCL12 (SDF1), vCCL2 (vMIP-II), CCL5 (RANTES), CCL3 (MIP-I) and CCL4 (MIP-I) were purchased from Peprotech. Alexa Fluor 647-labelled CXCL12 was purchased from Almac. MT-4, Cf2Th-CXCR4, $CEM.NK^R$, $CEM.NK^R$-CCR5 and U87.CD4.CXCR4 cell lines were obtained through the NIH AIDS programe from Dr. D. Richman, Dr. J. Sodroski and Dr. A. Trkola [70–72]. Cells stably expressing CXCR7 were obtained by transfecting U87.CD4 cells with pBABE-CXCR7 vector.

2.2. HIV-1 infection inhibition assay

Inhibition of HIV infection of MT-4 cells and peptide cytotoxicity were measured as previously described [73,74]. MT-4 cells ($6 \cdot 10^4$ cells/well) were incubated with or without X4 (III_B), R5/X4 (89.6) or R5 (Ba-L) viruses (100 $TCID_{50}$) for ve days in the presence of three-fold dilutions of ECL-X4 and control peptides starting at a concentration of 100 M. Viral entry inhibition and peptide cytoxicity were evaluated by monitoring the absorbance at 540 nm (Abs) corresponding to MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) reduction by mitochondrial enzymes using a Multiskan Ascent spectrophotometer (Thermo- sher). Protection (%) was calculated using the following equation: $(Abs_{cells + virus + peptide} - Abs_{cells + virus}) / (Abs_{cells} - Abs_{cells + virus}) \times 100$.

Luciferase-tagged recombinant viruses harbouring the NL4.3 Env and VSV-g pseudovirions were produced as previously described [75–77]. U87.CD4.CXCR4 cells (10,000 cells/well) were infected in 96-well plates with Env-recombinant viruses (200 pg p24, quanti ed by Perkin Elmer kit) for 48 h at 37 °C. Medium was replaced and cells were cultured for another 48 h, after which luciferase activity was assayed using the Promega Luciferase kit (Promega) and read on a Polarstar Omega microplate reader (BMG labtech). All infections were performed in triplicate. Peptide cytotoxicity was determined in uninfected cells using the MTT method as described above.

2.3. Binding of uorescently labelled CXCL12 to CXCR4

Alexa Fluor 647-labelled CXCL12 (100 ng/ml) was incubated for 30 min at room temperature with CXCR4 ECL-X4 peptides (50 M) prior addition to Cf2Th-CXCR4 cells. After 90 min incubation at 4 °C, cells

Table 1
Sequence and length of peptides derived from CXCR4 extracellular loops.

Name	Length	Position	Sequence
ECL1-X4	14	97–110	DAVANWYFGNFLCK
ECL2-X4	27	176–202	NVSEADDRYICDRFYPNDLWVVVFQFQ
ECL3-X4	21	262–282	DSFILLEIIKQGCEFENTVHK

Residues in bold are solvent exposed in the CXCR4 X-ray structure.

were washed, incubated with an amine reactive cell viability dye (LIVE/DEAD® Fixable Dead Cell Stain, Lifetechnologies) for 30 min at 4 °C and analysed on a BD FACS Canto cytometer (BD Biosciences) using BD FACS Diva software. Unlabelled CXCL12 chemokine (100 fold excess) was used as positive control for Alexa Fluor 647-labelled chemokine displacement, while the LIVE/DEAD stain allowed the simultaneous determination of peptide cytotoxicity.

2.4. Surface plasmon resonance measurements

Surface plasmon resonance (SPR) allows sensitive real time measurement of protein–protein interactions [78]. This technology is based on the evaluation of changes in the refractive index upon binding of an analyte to a ligand immobilised at the surface of a sensor chip. Biotinylated ECL2-X4 peptide (1 M) was immobilised on a streptavidin chip (GE Healthcare) by injection at a ow rate of 5 l/min for 20 min in 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20 (HBS-EP) on a BIAcore 3000. Typically, a signal ranging from 1000 to 1500 RU was obtained. For all sensorgrams, signal obtained with an irrelevant peptide (Ctrl) was subtracted from signal obtained with ECL2-X4. Binding analyses were performed by injecting 200 nM of CXCL12, vCCL2, CCL5, CCL3, and CCL4 for 3 min at a ow rate of 30 l/min. All binding measurements were performed in triplicate and were presented as average ± standard deviation. Kinetic analyses were performed by injecting various concentrations of CXCL12 and vCCL2 (7 to 500 nM) in HBS-EP at a ow rate of 30 l/min. Association and dissociation times of 2 min and 20 min, respectively were recorded. Measurements were carried out in duplicate. Surface regeneration was performed by a single injection of 10 l of 10 mM glycine buffer pH 1.5. The presence of mass transfer phenomena and linked reactions was excluded by performing the control assays as recommended by the manufacturer. Kinetic data analysis was performed using the BIAevaluation 4.1 software. The overall dissociation constant (K_D) values and on (ka) and off (kd) rates for the complexes were obtained after global ts of the experimental data using a simple model for 1:1 (Langmuir) binding.

2.5. cAMP assay

Primary intracellular cyclic AMP (cAMP) production upon CXCL12 binding in the presence or absence of ECL-X4 peptides was evaluated on MT-4 cells using the TR-FRET LANCE cAMP assay (Perkin Elmer) adapted for a 96-well plate format. MT-4 cells ($2 \cdot 10^4$ cells/well) were diluted in HBSS stimulation buffer (5 mM HEPES, 0.1% BSA, 0.5 mM IBMX pH 7.4) containing Alexa Fluor 647-labelled anti-cAMP antibody. Cells were incubated with forskoline (FSK) and CXCL12 was preincubated with ECL-X4 or control peptides for 30 min at room temperature. cAMP production was measured by adding europium-labelled streptavidin and biotin-cAMP for 1 h at room temperature. LANCE signal was recorded at 665 nm in a TECAN Genios Pro uorimeter and compared with cAMP standard curves (10^{-6} to 10^{-11} M).

2.6. Intracellular calcium release

Intracellular calcium release induced by CXCL12 was measured using indo-1-acetoxymethyl ester (Interchim) as calcium-responsive uorescent probe. Calcium release was monitored in MT-4 cells in 20 mM HEPES buffer containing 2.5 mM probenecid and 0.1% BSA. All measurements were performed at 37 °C in a 1 ml stirred cell using wavelengths of 355 nm for excitation and 475 and 405 nm for emission in a PTI QM-4 QuantaMaster uorimeter. For inhibition experiments, CXCL12 (7.5 nM) was incubated for 10 min in the presence of CXCR4-derived peptides (100 M).

2.7. Internalisation of CXCR4, CXCR7 and CCR5 receptors

Internalisation of CXCR4, CXCR7 and CCR5 receptors from the cell surface was monitored by ow cytometry. Phycoerythrin-conjugated monoclonal antibodies (mAb) 12G5 (BD Pharmingen) and 4G10 (Santa Cruz Biotechnology) were used to follow CXCR4 internalisation from the surface of MT-4 cells. Phycoerythrin-conjugated monoclonal antibodies T21/8 (EBiosciences) and 11G8 (R&D Systems) were used to monitor CCR5 and CXCR7 internalisation from CEM.NK^R-CCR5 and U87-CXCR7 cells, respectively.

Cells were incubated for 30 min in the presence of CXCL12 (50 nM), CCL5 (20 nM) or vCCL2 (400 nM). For neutralisation experiments, chemokines were pre-incubated 30 min at 37 °C with ECL-X4 and control peptides. Internalisation was stopped after 30 min by addition of NaN_3 (0.1%) and placing cells on ice. Cells were then stained with the adequate antibody for 30 min at 4 °C. Cell viability and potential cytotoxic effect of peptides was monitored using the LIVE/DEAD® Fixable Dead Cell Stain. Samples were analysed on a BD FACS Canto cytometer (BD Biosciences) using BD FACS Diva software.

2.8. Chemotaxis

Chemotaxis assays were performed in ChemoTx 96-well cell migration systems (Neuro Probe) equipped with a 5 m-pore polycarbonate membrane lter as recommended by the manufacturer. Brie y, migration buffer (RPMI 1640) containing CXCL12 (12 nM) and two-fold dilutions of ECL-X4 and control peptides (3 M to 100 M) were loaded in the lower chamber. Calcein-AM loaded Jurkat cells ($2.5 \cdot 10^5$ cells) were added to the upper chamber. Migration was allowed for 2 h and 15 min at 37 °C. Cells in upper and lower chambers were counted by measuring uorescence (E_{ex} 485 nm, E_{em} 525 nm) using a Tecan Genios Pro uorimeter.

3. Results

3.1. Inhibition of HIV infection by ECL-X4 peptides

The ECL-X4 peptides (Table 1) were analysed for their ability to inhibit the infection of MT-4 cells with the laboratory-adapted CXCR4-using (X4) HIV-1 strain III_B (Fig. 1A). Peptides ECL1-X4 and ECL2-X4 fully protected cells from virus cytopathic effect (IC_{50} = 24 and 31 M, respectively) whereas peptide ECL3-X4 displayed 10% protection at a concentration of 100 M. No inhibition was observed with the control peptide or the scrambled ECL1-X4 and ECL2-X4 peptides (ECL1-$X4_{scrbl}$ and ECL2-$X4_{scrbl}$) (Fig. 1A, B and D). None of these peptides were toxic within the concentration range tested as demonstrated in Fig. 1SD. Interestingly, peptide ECL1-X4 also protected MT-4 cells against infection with dual-tropic (R5/X4) virus (89.6) as well as against the CCR5-using (R5) virus (Ba-L) with IC_{50} values of 69 M and 76 M, respectively (Fig. 1B). In contrast, peptide ECL2-X4 failed to protect cells against R5 virus and displayed a reduced protection against dual-tropic virus (Fig. 1D).

To de ne the minimal length and crucial residues of ECL1-X4 and ECL2-X4 involved, truncated and mutated peptides were analysed in MT-4 infection assays (Fig. 1C, D and E). While progressive truncation of the rst two N-terminal residues of ECL1-X4 (ECL1-$X4_{98–110}$ and ECL1-$X4_{99–110}$) reduced protection (IC_{50} = 43 M and 67 M), deletion of the C-terminal lysine (Lys110) (ECL1-$X4_{97–109}$) abrogated its antiviral properties (Figs. 1C, 7A). In contrast, mutation of the cysteine at position 109 to an alanine (C109A) did not modify the antiviral potency (IC_{50} = 22 M) indicating that protection is independent of peptide dimer formation.

Truncated 18-mer ECL2-X4 analogues (ECL2-$X4_{176–193}$, ECL2-$X4_{181–198}$ and ECL2-$X4_{185–202}$) failed to inhibit infection of MT-4 cells by X4 virus III_B (Figs. 1D, 7C) demonstrating the necessity of full length ECL2-X4 peptide for viral inhibition. Alanine scanning of peptide

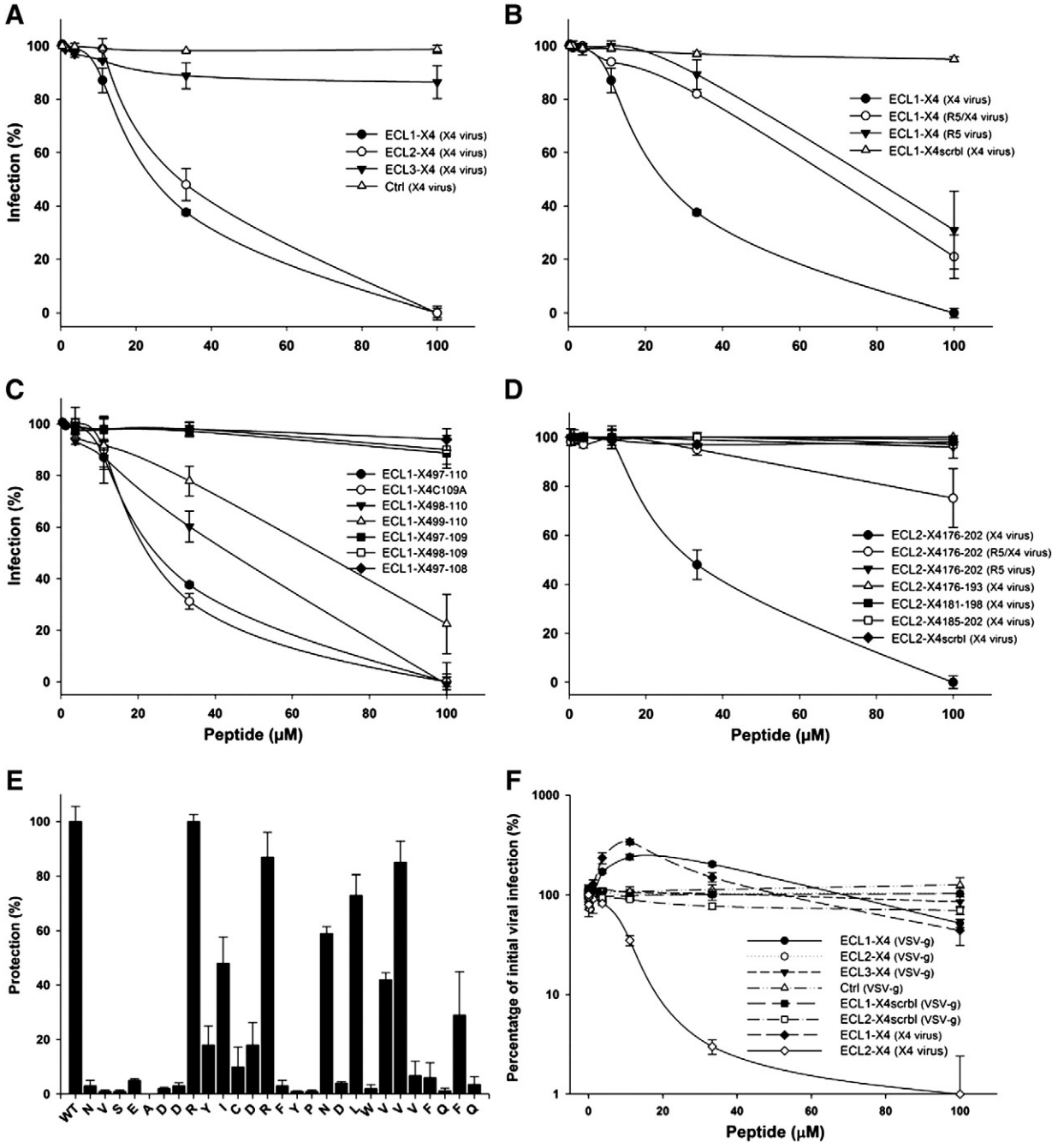

Fig. 1. Inhibition of HIV-1 infection by ECL-X4 peptides. Protection of MT-4 cells against HIV-1 infection by ECL-X4 peptides was evaluated by determining cell survival after 5 days of infection in an MTT assay. Panel A: Inhibition of X4 HIV-1 (III_B) infection by ECL-X4 peptides. Panel B: Inhibition of X4 (III_B), R5/X4 (89.6) and R5 (Ba-L) HIV-1 infection by peptide ECL1-X4. Panel C: Inhibition X4 HIV-1 (III_B) infection by full-length (ECL1-$X4_{97–110}$), truncated (ECL1-$X4_{98–110}$, ECL1-$X4_{99–110}$, ECL1-$X4_{97–109}$, ECL1-$X4_{97–108}$, and ECL1-$X4_{98–109}$), mutated (ECL1-$X4_{C109A}$) and scrambled (ECL1-$X4_{scrbl}$) peptides (Fig. 7A). Panel D: Inhibition of X4 (III_B) R5/X4 (89.6) and R5 (Ba-L) HIV-1 infection by full-length (ECL2-$X4_{176–202}$), truncated (ECL2-$X4_{176–193}$, ECL2-$X4_{181–198}$ and ECL2-$X4_{185–202}$) and scrambled (ECL2-$X4_{scrbl}$) peptides (Fig. 7C). Panel E: Inhibitory properties of alanine mutated ECL2-X4 analogues. Data are presented as percentage of protection obtained at a concentration of 200 M. All experiments were performed in triplicate and are presented as average ± standard deviation. Panel F: Inhibition of NL4.3 recombinant virions and VSV-g pseudoparticles by ECL-X4 peptides. U87.CD4.CXCR4 cells were infected with recombinant HIV particles harbouring the CXCR4-using envelope protein (NL4.3) and of VSV-g pseudovirions in the presence of serial 3-fold dilutions of ECL-X4, scrambled (scrbl) and control (ctrl) peptides. Viral infection was evaluated by measuring luciferase activity in cell lysates after 4 days. Data are presented as percentage of the infection obtained in the absence of peptide. All experiments were performed in triplicate and are presented as average ± standard deviation.

ECL2-X4 revealed different inhibitory behaviour of alanine analogues and identi ed three clusters of residues important for ef cient protection (Figs. 1E, 7B). While mutations R183A (IC_{50} = 68 M) or R188A (IC_{50} = 50 M) slightly affected the antiviral properties of the peptides, mutations Y184A, I185A, C186A, D187A, N192A (IC_{50} = 176 M), L194A (IC_{50} = 83 M), V196A and V197A (IC_{50} = 123 M) resulted in less active peptides and mutations N176A, V177A, S178A, E179A, D181A, D182A, F189A, Y190A, P191A, D193A and W195A, V198A, F199A, Q200A and Q202A abolished HIV inhibitory properties.

ECL-X4 peptides and controls were further analysed for their ability to interfere with viral entry using an Env recombinant virus harbouring CXCR4-using envelope (NL4.3) and VSV-g pseudovirions. Whereas ECL2-X4 inhibited only NL4.3 viral entry with an IC_{50} value of 9.6 M, it displayed no activity against VSV-g pseudoparticles indicating that

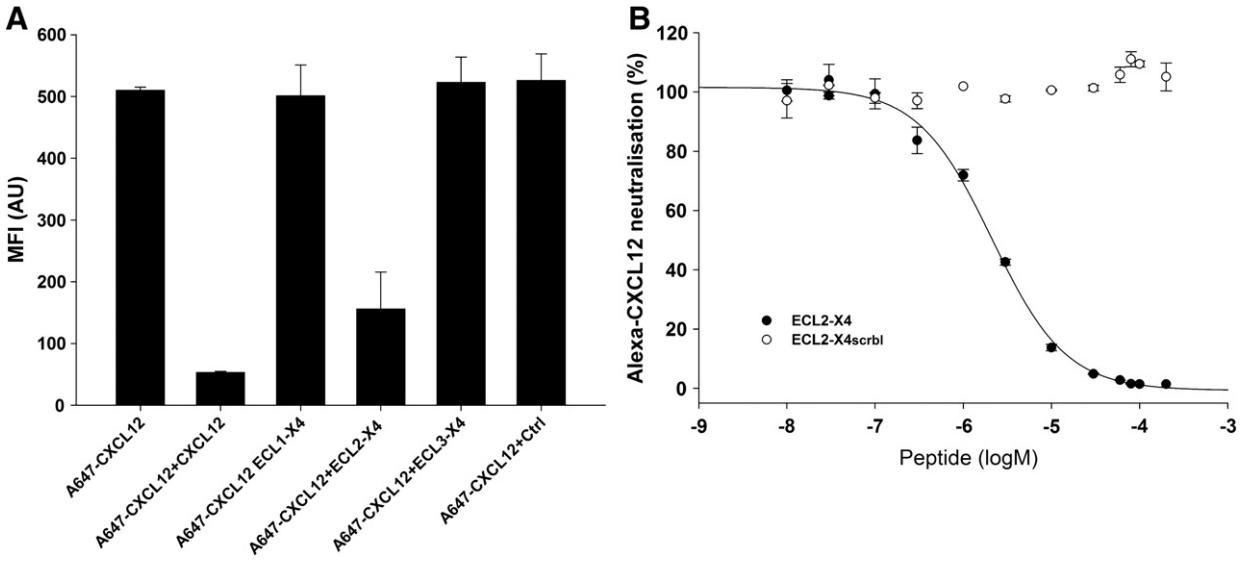

Fig. 2. Inhibition of CXCL12 binding to CXCR4 by ECL-X4 peptides. Panel A: Inhibition of CXCL12 binding to Cf2Th cells expressing CXCR4 by ECL1-X4, ECL2-X4, ECL3-X4 and control (Ctrl) peptides (50 M). Panel B: Comparison of CXCL12 inhibitory properties of ECL2-X4 and ECL-$X4_{scrbl}$ peptides (200 M to 10 nM). Alexa Fluor 647-labelled CXCL12 (AF647-CXCL12) (100 ng/ml) was pre-incubated with CXCR4 or control peptides for 30 min at RT before addition on Cf2Th–CXCR4 cells for 90 min at 4 °C. All experiments were performed in duplicate and are presented as average ± standard deviation.

the antiviral activity of ECL2-X4 is speci c to HIV-1 and due to a block of viral entry (Fig. 2). In contrast, at a concentration of 100 M ECL1-X4 peptide partially interfered with both NL4.3 and VSV-g mediated entry. At concentrations of 33 to 11 M, the peptide favoured viral entry, while at lower concentrations it had no effect. These observations suggest that part of the antiviral activity observed with ECL1-X4 peptide on MT-4 cells can be related to non-speci c effects.

3.2. Chemokine binding and neutralisation by ECL-X4 peptides

ECL-X4 peptides were also analysed for their ability to inhibit the binding of uorescently labelled CXCL12 to CXCR4. This labelled chemokine speci cally bound to Cf2Th cells overexpressing CXCR4 and not to the parental Cf2Th cells (Fig. 2SD). Only peptide ECL2-X4 inhibited binding of uorescently labelled CXCL12 to CXCR4 in a dose-dependent manner (Fig. 2) (IC_{50} = 2 ± 1 M). No inhibition was observed with ECL1-X4, ECL3-X4, scrambled ECL2-X4 (ECL2-$X4_{scrbl}$) and Ctrl peptides (Fig. 2). Surface plasmon resonance experiments demonstrated a strong and speci c binding of ECL2-X4 to CXCR4-related chemokines in solution (CXCL12 K_D = 22 ± 0.5 nM, and vCCL2 K_D = 27 ± 0.5 nM). This data indicated that the reduced binding of uorescent CXCL12 in the presence of ECL2-X4 was related to chemokine neutralisation and not receptor–peptide interactions (Fig. 3).

3.3. Inhibition of CXCL12-induced CXCR4/CXCR7 signalling and internalisation by ECL-X4 peptides

The effect of ECL-X4 peptides on the CXCR4 G-protein signalling was further investigated (Fig. 4A and B). In agreement with the binding data, in cAMP assay, only peptide ECL2-X4 inhibited the CXCL12-induced CXCR4 activation (IC_{50} = 35 ± 0.5 M) whereas ECL1-X4, ECL3-X4 and control peptide had no effect (Fig. 4A). In the absence of CXCL12, none of the ECL-X4 peptides acted as a CXCR4 agonist (data not shown). Similar results were obtained with CXCL12-induced calcium release measurement (Fig. 4B). ECL1-X4, ECL3-X4 and control peptide did not exert any effect whereas ECL2-X4 peptide abolished the calcium response induced by CXCL12 with an IC_{50} of 5.7 M (Fig. 4B, inset). The inhibitory properties of ECL2-X4 peptide were con rmed in a receptor internalisation assay using the 4G10 mAb directed against the CXCR4 N-terminus (Fig. 4C). Using this antibody avoided interference between mAbs recognising the CXCR4 ECLs. As previously observed, only peptide ECL2-X4 inhibited CXCL12-induced CXCR4 internalisation (IC_{50} = 19 ± 2 M) (Figs. 4C and 5). The ability of peptide ECL2-X4 to inhibit the CXCL12-induced migration of leukaemia-derived T cells was also monitored using a Transwell system. Peptide ECL2-X4 abolished chemotaxis of Jurkat cells at concentrations higher than 50 M while only partial or no inhibition was observed with peptides ECL3-X4 and ECL1-X4, respectively (Fig. 4D). Finally, the inhibitory properties of peptide ECL2-X4 towards CXCL12-induced CXCR7 internalisation were evaluated (Fig. 5). Peptide ECL2-X4 inhibited CXCR7 internalisation albeit with less potency than was observed for CXCR4 (IC_{50} = 100 ± 24 M vs. IC_{50} = 19 ± 2 M).

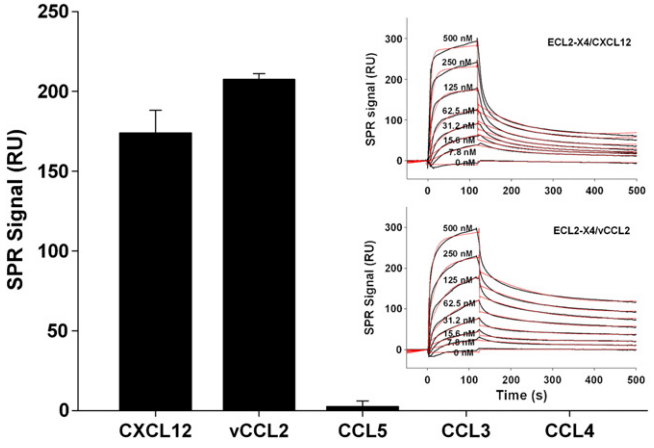

Fig. 3. Binding speci city of peptide ECL2-X4. Binding was evaluated by surface plasmon resonance (SPR) using a biotinylated ECL2-X4 peptide immobilised on a SA-chip. CXCL12, vCCL2, CCL5, CCL3, and CCL4 chemokines were injected at 200 nM. Binding intensities corresponding to SPR signals recorded at the end of the association phase are presented as average values ± standard deviation of triplicate experiments. Insets: Kinetic analysis of the binding of peptide ECL2-X4 to chemokines CXCL12 (upper panel) and vCCL2 (lower panel). Measurements were performed with two-fold dilutions of chemokine starting at 500 nM. Kinetic rate constants (ka and kd) for both complexes were tted globally according to a Langmuir 1:1 model using BIAevaluation 4.1 software. The tting results were ka = 4.01 ± 0.05 × 10^5 M^{-1} s^{-1}, kd = 8.84 ± 0.07 × 10^3 s^{-1}, K_D = 22.1 ± 0.5 nM for ECL2-X4/CXCL12 and ka = 1.86 ± 0.02 × 10^5 M^{-1} s^{-1}, kd = 5.05 ± 0.04 × 10^3 s^{-1}, K_D = 27.1 ± 0.5 nM for ECL2-X4/vCCL2, respectively.

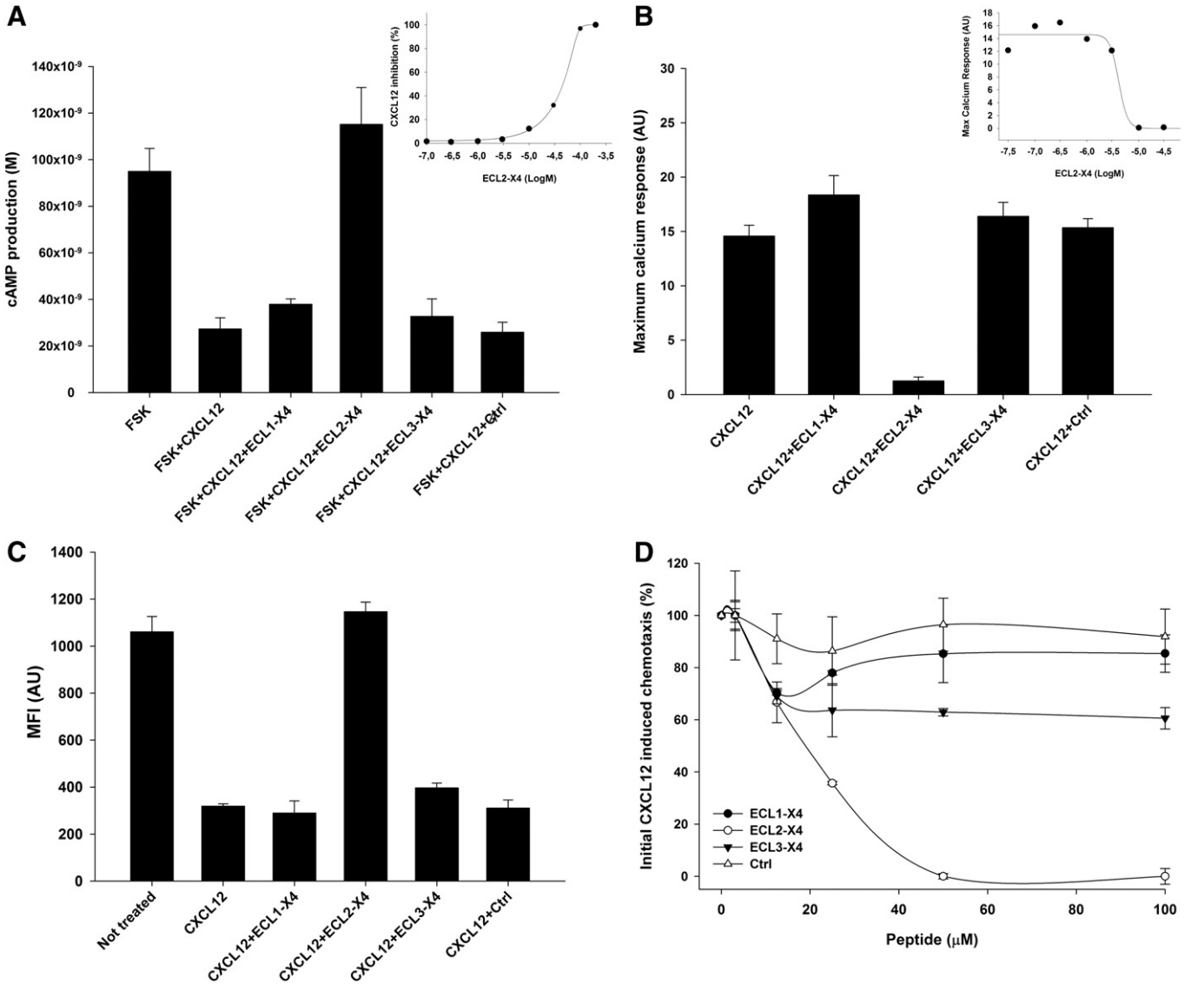

Fig. 4. Inhibition of the CXCL12-CXCR4 signalling pathways by ECL-X4 peptides. Panel A: Modulation of cAMP production by peptides ECL1-X4, ECL2-X4, ECL3-X4 and control. Modi cation of the forskolin-induced cAMP production was monitored using the TR-FRET-based LANCE assay. CXCL12 (30 nM) was pre-incubated with peptides (100 M) for 30 min at 37 °C before addition on MT-4 cells. Inset: Dose-dependent inhibition of CXCL12 by peptide ECL2-X4 (10 nM to 200 M). Panel B: Inhibition of CXCL12-induced calcium release by ECL-X4 peptides. Antagonist properties were monitored in the presence of CXCL12 (7.5 nM) by measuring maximum calcium response using Indo-1 uorescence. Inset: Dose-dependent inhibition of CXCL12-induced calcium release by peptide ECL2-X4 (100 M to 10 nM). Panel C: Inhibition of CXCL12-induced CXCR4 internalisation in MT-4 cells. CXCL12 (50 nM) was pre-incubated with ECL-X4 peptides (100 M) for 30 min at 37 °C before addition on MT-4 cells for 30 min at 37 °C. CXCR4 surface expression was monitored by ow cytometry using the 4G10 antibody. Panel D: Inhibition of CXCL12-induced migration of leukaemia-derived T-cells by ECL-X4 peptides. All experiments were performed in duplicate and are presented as average ± standard deviation.

3.4. Inhibition of vCCL2 binding by peptide ECL2-X4

The ability of peptide ECL2-X4 to neutralise vCCL2 was also evaluated (Fig. 7C). This chemokine binds to CXCR4 as well as to other CXC and CC chemokine receptors including CCR5. As vCCL2 displays an inherent antagonist activity, its binding to chemokine receptors was monitored in competition experiments. To avoid any interference between ECL-X4 peptides and the competing mAbs, the binding of vCCL2 was monitored on CCR5-expressing cells using the anti-CCR5 T21/8 mAb. At concentration of 400 nM vCCL2 abolished the binding of the anti-CCR5 antibody to the receptor, while in the presence of peptide ECL2-X4, this binding was fully restored, demonstrating that peptide ECL2-X4 also neutralised vCCL2. This interaction was concentration-dependent with a potency equivalent to that observed for CXCL12 (IC_{50} = 29 ± 6 M vs. IC_{50} = 19 ± 2 M). Peptide ECL2-X4 had no effect on CCL5 binding to CCR5 con rming its speci city.

3.5. Inhibition of full-length and CXCL12 N-terminus-derived peptide by truncated and mutated ECL2-X4 analogues

To unravel the structural basis of CXCL12 and vCCL2 neutralisation by ECL2-X4, partially overlapping truncated 18-mer peptides covering the full-length sequence of ECL2-X4 (176–193, 181–198 and 185–202) were analysed in a CXCR4 internalisation assay (Figs. 5, 6 and 7C). Truncation of the C-terminal residues (ECL2-$X4_{176–193}$) abrogated the inhibitory properties of the peptide, while peptides ECL2-$X4_{181–198}$ and ECL2-$X4_{185–202}$ displayed only 30% of the full-length ECL2-X4 activity at the highest concentration tested (300 M) (Fig. 5). Similar pro les were observed for vCCL2, although the analogue ECL2-$X4_{181–198}$ displayed stronger inhibition potency (Fig. 6). To assess the importance of individual amino acids for the neutralising properties of ECL2-X4 towards CXCL12, the IC_{50} values of alanine mutants were determined (Fig. 8A). Only the P191A mutant almost completely lost its capacity to inhibit

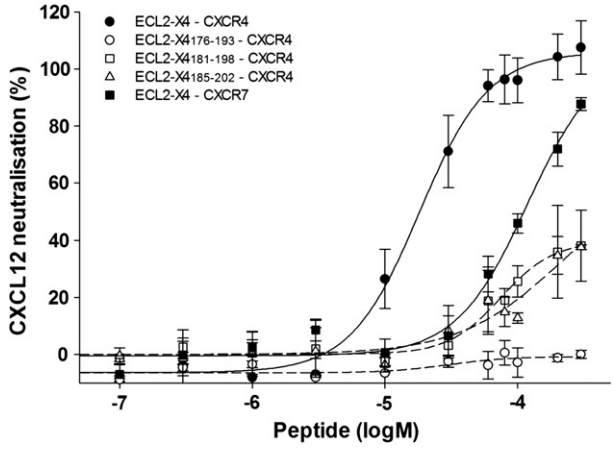

Fig. 5. Inhibition of the binding of CXCL12 to CXCR4 and CXCR7 by ECL2-X4-derived peptides. Neutralisation properties towards CXCL12 were evaluated in receptor internalisation assays. CXCL12 (50 nM) was pre-incubated with different concentrations (10 nM to 300 M) of ECL2-X4 and truncated analogues for 30 min at 37 °C prior addition on cells for 30 min at 37 °C. The surface expression of CXCR4 was monitored using the 4G10 antibody (– CXCR4) while CXCR7 expression was detected with the 11G8 antibody (– CXCR7). Full-length ECL2-X4 peptide covered positions 176 to 202. Truncated ECL2-X4 peptides (ECL2-$X4_{176–193}$, ECL2-$X4_{181–198}$ and ECL2-$X4_{185–202}$) are partially overlapping 18-mers covering the entire ECL2 sequence (Fig. 7C). Data represent the mean ± standard deviation of duplicate experiments.

the CXCL12-induced receptor internalisation (IC_{50} N 300 M) while replacement of residues Asp181 (D181A, IC_{50} = 38 ± 3 M), Asp182 (D182A, IC_{50} = 63 ± 7 M), Asp187 (D187A, IC_{50} = 46 ± 5 M), Asp193 (D193A, IC_{50} = 75 ± 9 M) and Cys186 (C186A, IC_{50} = 91 ± 8 M) as well as the hydrophobic residues Leu194 (L194A, IC_{50} = 38 ± 8 M), Trp195 (W195A, IC_{50} = 50 ± 5 M), Val196 (V196A, IC_{50} = 44 ± 11 M), Val197 (V197A, IC_{50} = 47 ± 6 M) and Phe201 (F201A, IC_{50} = 50 ± 15 M) located at the C-terminus of peptide ECL2-X4 resulted in more than two-fold reduction in CXCL12-neutralising property. In contrast, the removal of positively charged residues in mutants R183A (IC_{50} = 15 ± 3 M) and R188A (IC_{50} = 14 ± 3 M) slightly favoured chemokine binding.

To further pinpoint the CXCL12 region involved in the interaction with ECL2-X4, neutralisation of the short agonist peptide derived from the N-terminus of CXCL12 (residues 1–17) was evaluated in

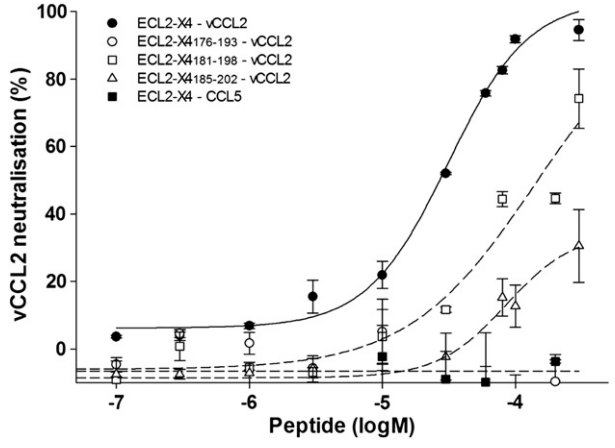

Fig. 6. Inhibition of vCCL2 binding to CCR5 by ECL2-X4-derived peptides. Neutralisation properties towards vCCL2 were evaluated in internalisation assay (– vCCL2). vCCL2 (400 nM) was pre-incubated with different concentrations of ECL2-X4 peptides (10 nM to 300 M) for 30 min at 37 °C before addition on CCR5-expressing cells for 30 min at 37 °C. Control experiment was performed using CCL5 (20 nM) (– CCL5). CCR5 surface expression was detected with the T21/8 antibody. Full-length ECL2-X4 peptide covers positions 176 to 202. Truncated ECL2-X4 peptides (ECL2-$X4_{176–193}$, ECL2-$X4_{181–198}$ and ECL2-$X4_{185–202}$) are partially overlapping 18-mers covering the entire ECL2 sequence (Fig. 7C). Data represent the mean ± standard deviation of duplicate experiments.

internalisation assay as this peptide alone was shown to induce receptor internalisation with an EC_{50} of 50 M (Fig. 8B). As observed for full-length chemokine, peptide ECL2-X4 inhibited over 70% of CXCR4 internalisation induced by this agonist peptide indicating that ECL2-X4 neutralises CXCL12 predominantly by binding to its exible N-terminal extremity.

4. Discussion

4.1. Different properties of ECL1-X4 and ECL2-X4 against HIV infection

Among the ECL-X4 peptides, ECL1-X4 and ECL2-X4 displayed potent antiviral activity against replicative viruses without inducing any toxicity effects. These peptides were soluble in the concentration range tested excepted peptide ECL2-$X4_{scrbl}$ which was partially insoluble in the culture media at a concentration of 100 M. In infection inhibition assay using MTT readout ECL1-X4 protected cells from infection with X4, R5/X4 but also R5 virus strains. Peptides covering positions 99 to 110 were required to achieve antiviral properties. The ability of peptide ECL1-X4 to inhibit viruses of different tropisms may be partially explained by the high sequence similarity of ECL1 in CXCR4 and CCR5. Indeed, ECL1 of CXCR4 (DAVANWYFGNFLCK) and CCR5 (YAAAQWDFGNTMCQ) show 50% of sequence identity and signi cant homology at non-conserved positions (Fig. 7A). Antiviral properties have previously been reported for a CCR5 derived ECL1 peptide (ECL1-R5) using fusion assay [79]. This peptide inhibited the cell-to-cell fusion entry of R5 and R5/X4 envelopes with potency in the micromolar range but was inactive against X4 virus envelope. These ndings suggested an important role for the non-conserved residues in tropism determination. In agreement with this hypothesis, Asp97 of ECL1 of CXCR4 was shown to be critical for ef cient infection by CXCR4-using viruses [80].

However, it must be noted that in entry assays with VSV-g pseudotypes and HIV (NL4.3) recombinant particles, ECL1-X4 blocked 50% of the total infection at 100 M and increased cell infection by two to four-fold at 11 and 33 M. This suggests that the peptide may interact non-speci cally with other viral surface determinants or interfere with other steps of the virus cell cycle. Recently, the antiviral activities of a synthetic mimic of CXCR4 extracellular surface covering ECL1–ECL2–ECL3 or cyclic peptides corresponding to ECL1 and ECL2 were described [81,82]. The former mimic of the extracellular surface showed inhibition in the low micromolar range (IC_{50} = 10 M) and was active only against X4-virus. This observation may result from the

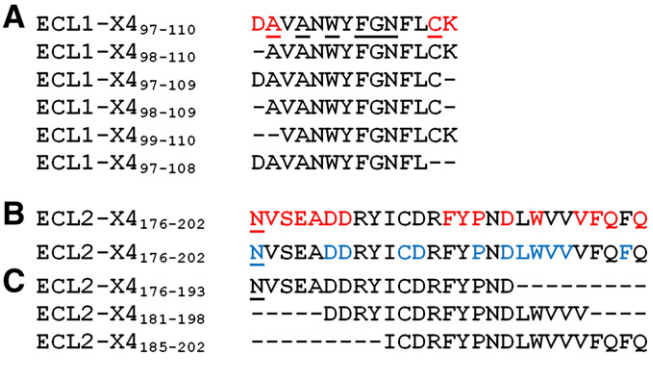

Fig. 7. Sequence alignment of full-length and truncated ECL1-X4 and ECL2-X4 peptides. Panel A: Sequences of full-length (positions 97 to 110) and truncated ECL1-X4 peptides. The N- and C-terminal extremities of peptide ECL1-X4 are important (DA) or critical (K) for HIV-1 inhibition and are coloured in red. Residues identical in ECL1 of CXCR4 and CCR5 are underlined. Panel B: Full-length ECL2-X4 peptide (positions 176 to 202). Residues within ECL2-X4 critical for HIV-1 inhibition (upper line) or important for CXCL12 neutralisation (IC_{50} values twice as high as the wild-type peptide) (lower line) are coloured in red and blue respectively. Panel C: Sequence alignment of truncated overlapping 18-mer ECL2-X4 peptides. Underlined asparagines (N) correspond to putative N-glycosylation sites (N_XS/T).

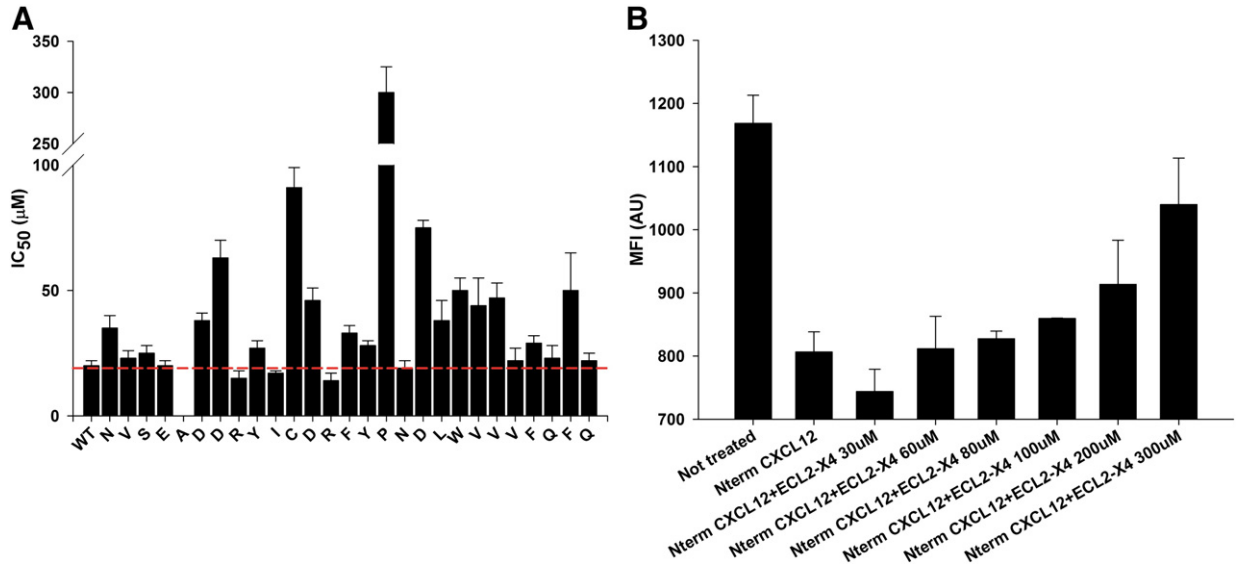

Fig. 8. Mutational scanning of peptide ECL2-X4 and its inhibitory properties towards CXCL12 and the agonist peptide derived from CXCL12 N-terminus. Panel A: Inhibition of CXCL12-induced CXCR4 internalisation by ECL2-X4 mutants monitored by ow cytometry. IC_{50} values are compared to that recorded with wild-type (WT) peptide ECL2-X4 (IC_{50} = 19 ± 2 M) (red dotted line). Panel B: Neutralisation of the agonist peptide derived from CXCL12 N-terminus (residues 1–17) by peptide ECL2-X4. CXCL12 N-terminus peptide (50 M) was pre-incubated with various ECL2-X4 concentrations (10 M to 300 M) and neutralisation was monitored as the decrease of CXCL12 N-terminus-induced CXCR4 internalisation. Experiments were performed in duplicate and are presented as average ± standard deviation.

presence of the ECL2 sequence, which possibly restricts the activity of the mimic towards X4 viruses. Cyclic ECL1 and ECL2 peptides displayed IC_{50} values of 35 M and 32 M, very similar to what we observed with individual linear peptides using the MTT readout. However, the activity of ECL1-derived peptides was observed indirectly by measuring cell viability and should also be further investigated using entry assays and Env of different viruses.

Peptide ECL2-X4 inhibited the CXCR4-using virus strain III_B with potency similar to ECL1-X4 (i.e. low micromolar range). Truncation and mutational analyses showed that full-length peptide is required for protection and the majority of the residues, with the notable exception of Arg183, Arg188, Val197 and Leu194, were important for ef cient inhibition (Fig. 1E). The critical residues were present as three clusters covering positions 176 to 182, 189 to 195 and 198 to 202, with the rst two including mainly solvent-exposed residues involved in the -hairpin structure (Figs. 7B and 9B). These results are in line with studies conducted on cells expressing mutated CXCR4, which demonstrated the importance of aromatic and negatively charged residues of ECL2 for HIV-1 entry [39–41,83]. In contrast to ECL1-X4, ECL2-X4 did not protect cells against infection by CCR5-using viruses, which could be explained by the poor sequence identity (10%) observed between the corresponding regions of CXCR4 and CCR5. It has been demonstrated that peptides derived from the second extracellular loop of CCR5 are also potent HIV-1 inhibitors [79,84]. These studies, however reported con icting results regarding the antiviral effect of ECL2-R5 against dual-tropic and CXCR4 viruses, which may be attributed to the differences in entry assays used (cell–cell fusion vs. single round infectivity assay). It is worth noting that a recent NMR study conducted using

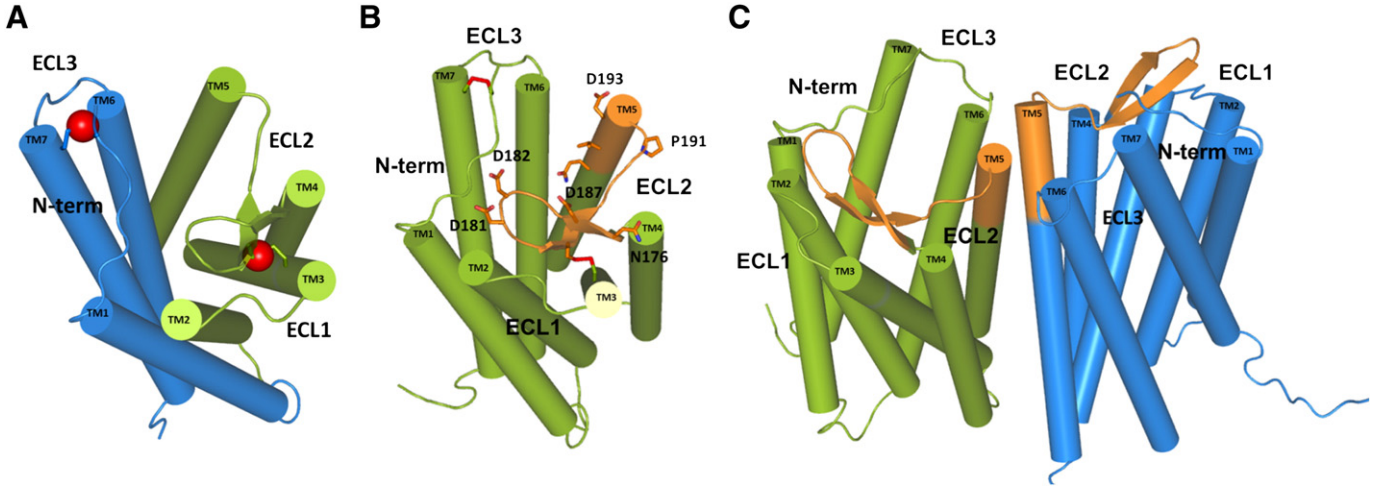

Fig. 9. Spatial arrangement of CXCR4 extracellular domains and positioning of ECL2 residues involved in interactions with CXCL12 and its N-terminus. Panel A: Top-down view of CXCR4 extracellular surface (PDB 3ODU). Disulphide bridges (red dots) of the extracellular parts of CXCR4 divide the receptor into two distinct domains (Nterm-ECL3 in blue and ECL1–ECL2 in green) potentially involved in different steps of ligand binding. Panel B: Overall CXCR4 receptor and localisation of ECL2. Residues located in ECL2/top of TM5, which in internalisation assay displayed IC_{50} values two fold higher than the wild-type peptide ECL2-X4 are represented as sticks and are coloured orange. Side chains of residues D181, D182, D187 and D193 point towards the inner face of the receptor. Panel C: Structure of CXCR4 dimer and location of CXCL12 N-terminus-binding sites (ECL2 and the top of TM5 coloured orange). CXCR4 monomers are coloured green and blue. Receptor dimerisation mainly involves the extracellular surface of TM5 and TM6 and brings two CXCL12 N-terminus-binding sites in close vicinity.

a small peptide corresponding to the C-terminal part of ECL2-R5 identi ed several gp120-interacting residues (underlined, 181-HFPYSQYQFW-190) corresponding to the second cluster pointed out in the present study (189-FYPNDLW-195) [84].

In CXCR4, the top of TM3 (end of ECL1) and ECL2 are linked by a disulphide bond. A second disulphide bond is present between the N-terminus of the receptor and the top of TM7 (end of ECL3) (Fig. 8A). Based on the positioning of these disulphide bridges and the overall receptor topology, the extracellular surface can be divided into two structural domains (Nterm–ECL3 and ECL1–ECL2) potentially involved in discrete steps of ligand recognition (Fig. 9A). As in the case of chemokines, the binding of gp120 to the co-receptors has been proposed to occur as a two-step process with the N-terminus forming the rst gp120-recognition site and the extracellular loops supporting the second binding step [85]. The recognition of the CXCR4 N-terminus seems however less important than the N-terminus of CCR5 for CCR5-mediated entry [86–88]. Furthermore, our results demonstrate that peptide ECL1-X4 and ECL3-X4 are less effective than ECL2-X4 in preventing HIV-1 entry, reinforcing the view that the latter is the key gp120-binding determinant of CXCR4.

4.2. ECL2-X4 neutralises CXCL12 by binding to its exible N-terminus — molecular basis for CRS2 interaction

Based on our data, ECL2 is most likely to be the major determinant of the CXCR4 CRS2. Indeed, only peptides derived from this loop were able to speci cally bind CXCL12 and inhibit its interactions with CXCR4 thereby preventing receptor activation. The binding of CXCL12 to CXCR4 is generally described as a two-step mechanism [44]. While the receptor N-terminus is commonly accepted as the major determinant of the initial chemokine recognition (CRS1), the residues constituting CRS2 involved in the subsequent receptor-activating interaction with the chemokine N-terminus are not precisely de ned. In silico predictions pointed out residues located in ECL2, ECL3 as well as the TM5 and TM6 regions [41,44,89]. While full-length ECL2-X4 peptide was needed for ef cient chemokine neutralisation, mutational analysis highlighted the crucial role of Pro191. Furthermore, Cys186, the four aspartate residues scattered along the peptide (Asp181, Asp182, Asp187 and Asp193) as well as the LWVV cluster and Phe200 located at the C-terminal part of ECL2 were important for neutralisation (Figs. 7B, 8A and 9B). In the CXCR4 crystal structure, all four aspartates are solvent-exposed and ideally positioned on the loop to interact with ligands (Fig. 9B). Pro191 was crucial for both CXCL12 and HIV-1 neutralisation, most probably by reducing the exibility of this region and introducing a kink at the C-terminus of ECL2 upstream the LWVVVFQFQ sequence (annotated as the top of TM5 in the X-ray structure). This kink may be necessary for correct positioning of the N- and C-terminal parts of the peptide for optimal ligand binding. Based on this structural arrangement, a plausible mechanism for the initial interactions of CXCL12 at CRS2 would rely on stabilising contacts of the four aspartates with the core and the N-terminus (Arg8 and Arg12) of the chemokine, ensuring the correct orientation of its exible N-terminus for receptor-activating insertion in the transmembrane cavity close to the top of TM5 (Fig. 3SD). This insertion would result in conformational changes in the TM5 and TM6 region, allowing the formation of new interactions between the N-terminal lysine of CXCL12 and Asp262 or Glu288 located at the inner segment of TM6 and TM7, respectively [44]. In accordance with the proposed mechanism, the agonist activity of the small peptide derived from CXCL12 N-terminus was inhibited by peptide ECL2-X4. The exible N-terminus of the chemokine has also been shown to represent a potential scaffold for development of drugs targeting CXCR4 [51]. Interestingly, dimeric peptides derived from CXCL12 and vCCL2 N-termini showed ten times stronger activity in inhibiting HIV-1 infection or CXCL12 binding than their monomeric counterparts [48–51]. The molecular basis for this increase in potency is not entirely understood. Nevertheless, it is noteworthy that in the crystal structure, CXCR4 is present in a dimeric form (Fig. 9C). The dimerisation, which takes place at the extracellular side of the TM5–TM6 region, brings the two CXCL12 N-terminus-binding sites closer together in a symmetric manner which could therefore account for more favourable bivalent interactions of the dimeric peptides with a dimeric form of the receptor.

To date, the exact stoichiometry of CXCL12–CXCR4 interactions remains to be clari ed [38]. As observed for CXCR4, CXCL12 can also form dimers and monomeric or dimeric forms of the chemokine were shown to have different effects on signalling and cellular responses [46,90]. Our data indicate that peptide ECL2-X4 binds to CXCL12 by forming multiple contacts with an important contribution of the hydrophobic and negatively charged residues, reminiscent of the interactions described for CXCR4 N-terminus peptides (CRS1) and CXCL12 [46]. Indeed, peptides corresponding to CRS1 bound the chemokine in an extended conformation and occupied the cleft delimited by N-loop and the -sheet leaving the exible N-terminus of the chemokine free for an interaction with ECL2 (CRS2). It is therefore conceivable that the interaction at CRS1 positions the chemokine, induces conformation changes or creates larger interaction interface facilitating the subsequent binding of the chemokine at CRS2. However, the binding of CRS1-derived peptides to CXCL12 has also been demonstrated to induce its dimerisation and the formation of a symmetric 2/2 complex in which the two receptor N-terminus-binding sites are located at opposing faces of the dimer [46]. Therefore, it is also plausible that the CRS1 and CRS2-derived peptides (N-term and ECL2) recognise equivalent sites on each monomer providing structural basis for the binding of dimeric CXCL12.

4.3. Therapeutic potential of ECL-X4-derived peptides

During the last two decades, peptides derived from the N-loop of CCL5, the N-termini of CXCL12 [49] and vCCL2 [50], the variable loop of gp120 (V3 loop) and the extracellular surface (N-term and ECLs) of CCR5 [79] were proposed as potential sources of antiviral molecules. In this study, peptide ECL2-X4 displayed antiviral properties towards CXCR4-using viruses and interacted strongly and speci cally with CXCL12, blocking its interactions with both CXCR4 and CXCR7. Considering the increasing number of studies reporting the implication of these receptors in the spread and survival of tumour cells, neutralising their common ligand may be a highly relevant therapeutic strategy [35,91–93]. Peptide ECL2-X4 was however less potent in inhibiting the binding of CXCL12 to CXCR7 than to CXCR4, which may be in part explained by the ten times higher af nity of CXCL12 towards CXCR7 (Fig. 4) [32,33]. In contrast to peptides derived from the N-terminus of chemokine receptors, peptides derived from the extracellular loops do not require tyrosine sulfation to be fully active [67]. The N-terminus of CXCR4 bears three sulfotyrosines at positions 7, 12 and 21, which were previously shown to be critical for CXCL12 binding [46,94]. We have previously observed that an unsulfated peptide derived from the N-terminus of CXCR4 (residues 1–40) displayed no CXCL12 or HIV-1 inhibition properties in the different assays presented above. This post-translational modi cation is usually dif cult to introduce at multiple sites of long synthetic peptides due to the lability of sulfate group often resulting in a heterogeneous mixture of sulfated peptide species. This observation emphasises the therapeutical potential of peptides derived from the receptor extracellular loops. However, their potency, af nity and stability (low micromolar range) as well as the pharmacokinetic properties remain to be largely improved through stabilisation in a protein scaffold or by incorporating non-natural residues such as D-amino acids or chemical derivatives. Recent modi cations of CCL5-derived peptides at hot spots demonstrated that such rational design allowed improving the overall peptide potency by more than 100 times to reach the nanomolar range [95,96]. Therefore, the mutational analyses conducted in this study provide valuable positional information for such further improvements.

In the near future, additional work will be needed to better understand the structural determinants of CRS1 and CRS2 in CXCR4 and CXCR7, as well as to elucidate the ligand–receptor stoichiometry and to determine if the chemokine neutralising properties observed with ECL2-derived peptides can be extended to other CXC receptors.

4.4. Conclusions

Linear peptides derived from CXCR4 extracellular loops are structural and functional mimics of the complete receptor surface and some display neutralising properties towards CXCL12 binding and HIV-1 infection.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.01.017.

Acknowledgements

This manuscript is supported by the Public Research Centre for Health (CRP-Santé), Luxembourg, grant 20100708 (GPCR47 project) and the "Fonds National de la Recherche", Luxembourg, grants: AFR-3004509 (CX-CRP-7 project), CORE C11/BM/1209287 (PROMETAVIR project) and CORE C09/BM/20 (Mimokine Project). The authors wish to thank Dr. Danielle Perez-Bercoff for thorough reading, helpful discussions and her technical advices regarding the recombinant viral assay and Nicolaas Brons for technical assistance in flow cytometry measurements as well as Dr. Carole Devaux and Dr. Sylvie Delhalle for critical reading of the manuscript.

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