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as Drug Targets

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WILEY-VCH Verlag GmbH & Co. KGaA

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Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at http://dnb.d-nb.de.

Cover Design Grafik-Design Schulz, Fußgönheim
Typesetting Thomson Digital, Noida, India
Printing and Binding Strauss GmbH, Mörlenbach

Printed in the Federal Republic of Germany
Printed on acid-free paper

ISBN: 978-3-527-32118-6

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ISBN: 978-3-527-32118-6

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ISBN: 978-3-527-32118-6

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Preface

This volume is dedicated to the family of chemokine receptors, belonging to the class of G protein-coupled receptors (GPCRs). Chemokine receptors are primarily expressed on leukocytes, but are also present on cells of nonhematopoietic origin, such as endothelial cells and neurons. The chemokine receptor system is known to orchestrate various aspects of the immune system but also appears to control a variety of physiological processes. Excessive expression of chemokines and chemokine receptors and deregulated chemokine receptor function results in various disease states, including chronic inflammatory and vascular diseases and oncogenesis. Viruses have also taken advantage of the chemokine receptor system, indicating a role of this class of receptors in viral infection. The chemokine receptors CCR5 and CXCR4 are the two major co-receptors for HIV-1 entry into host cells. The pox- and herpesviruses express chemokines, chemokine-binding proteins and/or chemokine receptors, which may contribute to viral pathogenesis.

Since GPCRs are one of the most favored drug targets and the role of chemokine receptors in disease is becoming apparent, chemokine receptors are considered prime targets in drug research. The first successful small molecule targeting the chemokine receptor system was the CCR5 antagonist, for the prevention of HIV infection, approved by the FDA in 2007. The second small molecule (a CXCR4 antagonist) was approved by the FDA at the end of 2008 for hematopoietic stem cell mobilization. In the mean time the understanding of the chemokine receptor system is growing and several promising drugs are currently being tested in late-stage clinical trials.

The present volume by Martine Smit, Sergio Lira, and Rob Leurs is organized into three main sections, addressing fundamental, pathophysiological and drug discovery aspects of chemokine receptors. Following the philosophy of this series, authors from the different chapters come from academic institutions and pharmaceutical industry, fostering an active exchange between these two communities. The first section introduces the field of chemokines and their receptors, particularly referring to structural aspects. The second section focuses on the relevance of human and viral chemokine receptors in various diseases, such as inflammation, CNS diseases and cancer. The final section gives an overview of the currently available chemokine

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

receptor ligands and their therapeutic impact. Six different chemokine receptor subtypes are particularly referred to. The last chapter comments on chemokine-binding proteins as therapeutics.

The series editors thank Martine Smit, Sergio Lira, and Rob Leurs for their enthusiasm to organize this volume and to work with such a fine selection of authors. We also express our thanks to Nicola Oberbeckmann-Winter, Waltraud Wüst, and Frank Weinreich from Wiley-VCH for their valuable contributions to this project and to the entire series.

August 2010

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A Personal Foreword

The chemokine receptors, belonging to the family of G protein-coupled receptors are considered attractive targets for therapeutic intervention. Chemokines and their receptors play a prominent role in the development, homeostasis and activation of leukocytes in the innate and adapative immune system. Expression of chemokine receptors is not confined to leukocytes but is also apparent on cells of nonhematopoietic origin such as endothelial cells and neurons. Their excessive activity or dysfunction, however, is associated with the establishment of inflammation and diseases such as multiple sclerosis, inflammatory bowel disorder, arthritis and atherosclerosis. There is growing evidence that chemokine receptors play a role in cancer, including cancer metastasis and angiogenesis. Virus-encoded chemokines, chemokine receptors and chemokine scavengers have been identified, underscoring the importance of the chemokine system in viral pathogenesis. Besides chemokine receptors, CCR5 and CXCR4 have been shown to play profound roles in HIV pathogenesis through their ability to act as co-receptors for viral entry. This has led to the first approval by the FDA for a chemokine receptor antagonist for the prevention of HIV infection.

In the past decades much insight has been obtained on the structure of chemokines, the identification of their receptors and the mechanisms underlying the complex biologies in which they participate. This volume addresses the fundamental, pathophysiological and drug discovery aspects of chemokine receptors. The first part includes chapters that describe the fundamental aspects of chemokines and chemokine receptors. First, overviews are provided of the structure of chemokines and their receptors in relation to their biology and structural insights for homology modelling of chemokine receptors. The latest insights into the molecular mechanisms underlying chemokine-directed migration, a key event induced upon chemokine receptor activation, are presented. Besides the "classical" chemokine receptors, the biochemistry and biology of "atypical" chemokine receptors are explored and outlined. Last, the functional consequences and implications of homo/hetero dimerization of chemokine receptors are discussed.

The second part of this volume includes chapters that provide a comprehensive description of the role of the chemokine receptors in various diseases. The roles of

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ISBN: 978-3-527-32118-6

various chemokines and chemokine receptors in chronic inflammatory diseases are outlined, including COPD, IBD, atherosclerosis and psoriasis. Thereafter, the role of the chemokine system in neurodegenerative diseases, including MS and EAE, brain ischemia and HIV-associated dementia, and in neuropathic pain is addressed. In addition, the latest insights into the role of chemokines and chemokine receptors in cancer metastasis are provided and potential roles of virus-encoded chemokine receptors are discussed. The final chapters discuss in detail different chemokine receptors, including CCR5, CXCR4, CXCR2, CXCR3, CCR1 and CCR3, as well as chemokine-binding proteins, with respect to therapeutical targeting and/or drug development.

Finally, we want to thank all authors of the different chapters from both academic institutions and the pharmaceutical industry for their valuable contributions. In addition, we want to acknowledge the pleasant collaboration with the series editor Dr. Raimund Mannhold as well as Dr. Frank Weinreich and Dr. Nicola Oberbeckmann-Winter from Wiley-VCH during the editing of this volume.

July 2010

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Part One
Fundamentals of Chemokines and Chemokine Receptors

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ISBN: 978-3-527-32118-6

1
Structural Aspects of Chemokines and their Interactions with Receptors and Glycosaminoglycans

Amanda E. I. Proudfoot, India Severin, Damon Hamel, and Tracy M. Handel

1.1
Introduction

Chemokines are a large subfamily of cytokines (~50 in humans) that can be distinguished from other cytokines due to several features. They share a common biological activity, which is the control of the directional migration of leukocytes, hence their name, *chemoattractant cytokines*. They are all small proteins (approx. 8 kDa) that are highly basic, with two exceptions (MIP-1α, MIP-1β). Also, they have a highly conserved monomeric fold, constrained by 1–3 disulfides which are formed from a conserved pattern of cysteine residues (the majority of chemokines have four cysteines). The pattern of cysteine residues is used as the basis of their division into subclasses and for their nomenclature. The first class, referred to as CXC or α-chemokines, have a single residue between the first N-terminal Cys residues, whereas in the CC class, or β-chemokines, these two Cys residues are adjacent. While most chemokines have two disulfides, the CC subclass also has three members that contain three. Subsequent to the CC and CXC families, two additional subclasses were identified, the CX_3C subclass [1, 2], which has three amino acids separating the N-terminal Cys pair, and the C subclass, which has a single disulfide.

The first chemokine, PF-4, was identified in 1977 [3] but it was not for almost a decade that other members of the family started to emerge, with the discovery of the proinflammatory chemokines: IP-10 was identified in 1985 as a protein showing homology to PF4 [4], while IL-8 and the MIP-1 proteins were isolated in the late 1980s as active protein from tissues or culture supernatants. The neutrophil chemoattractant, IL-8, was purified from culture supernatant of stimulated blood monocytes [5] and the monocyte chemoattractants MIP-1α and MIP-1β were purified from LPS-stimulated mouse macrophages [6]. The primary amino acid sequence of these chemokines rapidly led to the identification of the highly conserved four-cysteine motif described above and also allowed their classification into the two principal subclasses. The number of chemokines then grew rapidly

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ISBN: 978-3-527-32118-6

through homology cloning using the conserved motifs, but the real explosion in the identification of members came from EST database searches [7]. Initially, chemokines were given names usually associated with their activity; for example, the MIP-1 proteins were discovered as "*m*acrophage *i*nflammatory *p*roteins". Similarly, PF-4 (platelet factor IV) was a factor produced from platelets. However, since members of the family were often identified concomitantly by different laboratories resulting in different names, a systemic nomenclature was introduced in 2000 in order to introduce harmonization [8]. In this nomenclature, the ligands are named according to subclass (CC, CXC, C, CX3C) followed by L for ligand and a number. Under this nomenclature IL-8 became CXCL8 while MIP-1α became CCL3. This nomenclature was created for human chemokines based on their genomic localization, but was rapidly "pirated" for the mouse chemokines, since even prestigious journals insisted that the new nomenclature be applied to the mouse chemokines! Interestingly certain chemokines are not found in both the human and mouse systems. For instance CXCL8 does not exist in the mouse, and the equivalent of several mouse chemokines such as lungkine and MCP-5 (CXCL15 and CCL12, respectively), have not been identified in humans (as shown in Table 1.1), which shows the old and new nomenclatures for human chemokines. In the rest of the chapter, we refer to chemokines by their new nomenclature.

Initial support for the division of chemokines into the α (CXC) and β (CC) subclasses was not only structural, but also based on biological activity as it described leukocyte specificity. The discovery that chemokine receptors were seven transmembrane spanning G protein-coupled receptors (GPCR) in the early 1990s [9, 10] was extremely important for the pharmaceutical industry as it presented a novel target class in the GPCR family which represent up to 60% of the targets of marketed medicines. The initial hope was that individual leukocyte populations would express a single chemokine receptor, which held firm until the cloning of the third CXC receptor, CXCR3 [11]. Until this point, the CXC chemokines were thought to be responsible principally for neutrophil recruitment and were therefore implicated in acute inflammation, while CC chemokines recruited other leukocyte types and were thus involved in chronic inflammation. However CXCR3 is mainly expressed on activated T cells, and its ligands were initially identified as IFNγ inducible polypeptides and are therefore pivotal in chronic inflammatory disorders. The subsequent identification of CXCR4 and CXCR5, as well as several CC chemokine receptors and their respective ligands, then introduced yet another concept in chemokine biology – that chemokines could be further subdivided into two broad classes on the basis of: (i) those that are inducible and therefore involved in inflammation and (ii) those that are constitutively expressed and are involved in leukocyte homing.

This chapter concentrates on the structure of chemokines and their receptors and how these aspects may be related to their biology. Understanding the relationship between the structure and function of chemokines has lead to ideas of how chemokines can be modified to produce analogs that are useful for modifying disease, in animal models and perhaps in man in the future.

Table 1.1 The old names and the new systematic nomenclature of the human chemokines are listed side by side and grouped into their respective CXC, XC, CX3C and CC families. For example, I-309 (old) is now referred to as CCL1 (new). Mouse chemokines for which no human homologs has been identified are shown in parentheses.

Old name	Systematic nomenclature	Old name	Systematic nomenclature
Gro-α	CXCL1	I-309	CCL1
Gro-β	CXCL2	MCP-1/MCAF	CCL2
Gro-γ	CXCL3	MIP-1α	CCL3
PF4	CXCL4	LD78	CCL3L1
ENA-79	CXCL5	MIP-1β	CCL4
GPC-2	CXCL6	RANTES	CCL5
NAP-2	CXCL7	(C10, MRP-1)	(CCL6)
IL-8	CXCL8	MCP-3	CCL7
Mig	CXCL9	MCP-2	CCL8
IP-10	CXCL10	(MRP-2, CCF18, MIP-1γ)	(CCL9,CCL10)
I-TAC	CXCL11	Eotaxin	CCL11
SDF-1α/β/δ/γ/ε/ϕ	CXCL12	(MCP-5)	(CCL12)
BCA/BLC	CXCL13	MCP-4	CCL13
BRAK/bolekine	CXCL14	HCC-1	CCL14
(m Lungkine)	(CXCL15)	HCC-2/Lkn/MIP-1δ	CCL15
	CXCL16	HCC-4/Lec	CCL16
		TARC	CCL17
Lymphotactin-1	XCL1	DC-CK1/PARC	CCL18
Lymphotactin-2/SCIM-1β	XCL2	MIP-3b/ELC/Exodus-3	CCL19
		MIP-3a/LARC/Exodus-1	CCL20
Fractalkine/neurotactin	CX3CL1	6Ckine/SLC/Exodus-2	CCL21
		MDC/STCP-1	CCL22
		MPIF-1/Ckbeta-8	CCL23
		MPIF-2/Eotaxin-2	CCL24
		TECK	CCL25
		Eotaxin-3	CCL26
		CTACK/ILC	CCL27
		MEC	CCL28

1.2 Receptor–Ligand Interactions

The classification of chemokine receptors is based on the ligands they bind, in other words CXC receptors bind CXC ligands, CC receptors bind CC ligands and so on, as shown in Figure 1.1. Chemokine receptors have been identified that bind chemokines but do not signal. One of these, the Duffy antigen receptor for chemokines (DARC) is a promiscuous chemokine receptor expressed on erythrocytes that binds both CC and CXC ligands [12]. In contrast, the decoy receptor D6 only binds CC chemokines [13]. Thus, with some exceptions like DARC, the chemokine system is specific with respect to the binding pattern, in that chemokines in each class do not

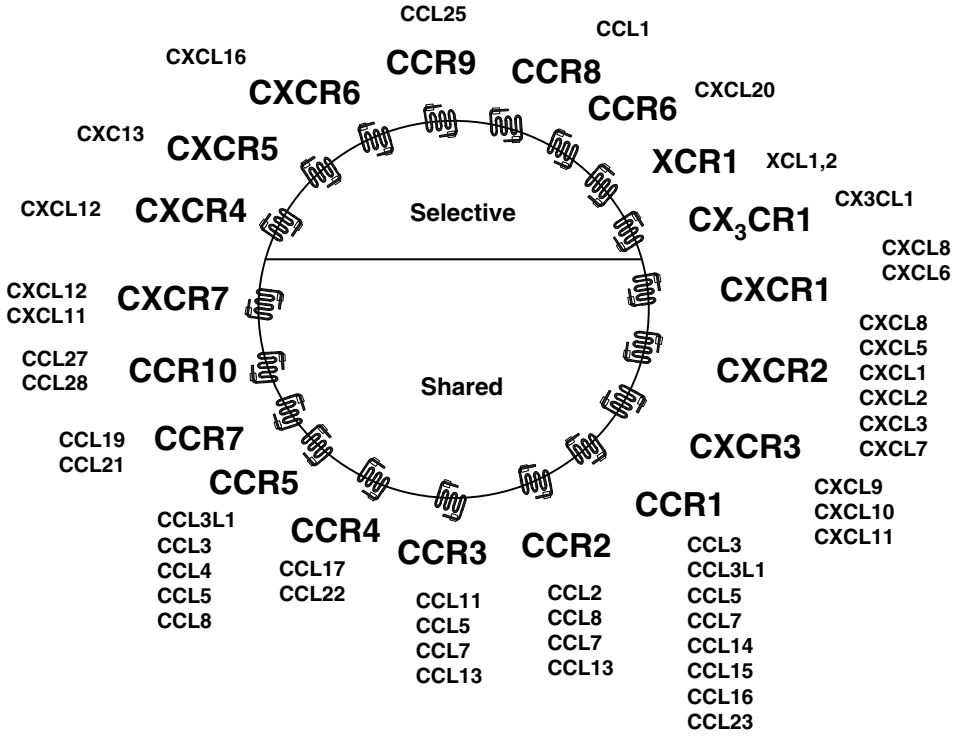

Figure 1.1 This diagram shows the pairing of chemokine receptors with their respective ligands. For example, CCL2, CCL8, CCL7 and CCL13 are all ligands of the receptor, CCR2. Some receptors like CXCR4 are much more selective and have a single ligand.

bind to receptors of another class. However, binding across classes has been demonstrated with antagonists. Thus CXCL9, CXCL10 and CXCL11, the agonists of CXCR3, also bind to CCR3 as antagonists and inhibit Th2 cell migration [14]. Interestingly a chimera consisting of the first eight residues of CCL11 (and the remainder consisting of CXCL11) bound CCR3 more strongly than the parental chemokines. CCR3 can also be antagonized by CCL18, despite the fact that the receptor for CCL18 remains unidentified to date [15]. Viruses have also adopted deviations from classical chemokine receptor pharmacology. For example the virally encoded chemokine receptor, US28, does not demonstrate reciprocal heterologous competition like most chemokines/receptors. Instead, one of its ligands, CX3CL1, cannot be competed by certain CC chemokines, whereas these CC chemokines are all displaced by CX3CL1 [16]. We believe that the study of the virally encoded members of the chemokine system will teach us a great deal about the intricacies involved in chemokine/receptor interactions, since viruses have produced chemokine ligands, such as vMIP-II, that can bind across chemokine receptor subclasses.

Beyond the basic rule of subclass selectivity (with the exceptions noted), the binding patterns of the chemokine system is far from simple! First, the assignment of

receptor-ligand pairs arises from *in vitro* assays, and one should be aware that the situation *in vivo* may be different due to factors that cannot be captured *in vitro*. Second, the majority of receptor/ligand interactions are not specific in that several receptors bind more than one ligand – in fact only about one-third are specific single ligand binders to date. Third, the reason that this statement is qualified by "to date," is that as the identification of new ligands continued, absolute specificity has tended to disappear, although the question remains as to whether there are additional ligands to be identified. CXCR1 was classified as a specific receptor for CXCL8 for seven years, until CXCL6 was identified as a ligand [17]. As an extreme example, CCR1 binds at least eight ligands. The situation is further complicated by the fact that certain chemokines are ligands of more than one receptor, which is best exemplified by CCL5 which binds to CCR1, CCR3 and CCR5.

However, the biology that has emerged over the past decade or so has identified a broad definition which supports the classification of selective versus shared receptors. The selective receptors have been shown to generally correspond to those which are constitutively expressed and are involved in development and homeostasis. In contrast, the shared receptors are those which are inducible and associated with inflammatory disease [18]. The fact that the shared receptors are the "villains" in disease makes the task of understanding how to target them a challenge, particularly if one is interested in using neutralizing antibodies against the ligands. Intuitively one would suggest that a small molecule inhibitor of the receptor would be the chosen strategy, or alternatively a neutralizing receptor antibody, but neither of these strategies is that simple. Therefore neutralization of a prominent ligand could be a successful strategy – one could suggest CCL2 for CCR2 or CXCL10 for CXCR3? However, it is not always easy to establish which ligand is the most potent and has the highest affinity for a certain receptor. This is well illustrated by CCR5, and a comparison of the rank order of the published potencies of its ligands. Using a calcium mobilization assay, the rank order potency of ligands on CCR5 expressed in CHO cells was reported as CCL5 > CCL4 > CCL3 [19] whereas in RBL cells stably transfected with CCR5, the rank order was CCL5 > CCL4 = CCL3 [20] and in HEK293/CCR5 transfectants the order was different again, with CCL3 > CCL5 > CCL4 [21]. However in the third example, the form of CCL3 used was the allelic variant, CCL3L1 (LD78β instead of LD78α), which has a Pro instead of a Ser residue at position 2 and two S/G switches (Figure 1.2a). Thus although CCL3 is often described as being a ligand for CCR5, its affinity is approximately 100 nM, whereas CCL3L1 has an affinity of 1 nM.

Another complexity arises from the fact that although certain chemokines can bind to several different receptors, the induced biological activity may differ significantly and can even vary depending on the cell type on which the receptor is expressed. CCL5 induced downregulation of three of its receptors and the ensuing recycling illustrates this phenomenon nicely. On incubation with CCL5 *in vitro*, the surface expression is reduced by approximately 80% in each case of CCR1 and CCR5 from the surface of PBMC [22, 23] and CCR3 from eosinophils [22, 24]. However on removal of the chemokine from the culture medium, very different patterns of receptor recycling are observed. In the case of CCR5, receptor density returns to that

(a)

```
                 *         20         *         40         *         60         *
CCL3    : ASLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA : 70
CCL3L1  : APLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPSVIFLTKRCRQVCADPSEEWVQKYVSDLELSA : 70
```

(b)

```
                    *         20         *         40         *         60         *
SDF1_alpha    : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_beta     : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_gamma    : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_delta    : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_epsilon  : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70
SDF1_theta    : MNAKVVVVLVLVLTALCLSDGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQV :  70

                         80         *        100         *        120         *        140
SDF1_alpha    : CIDPKLKWIQEYLEKALNK---------------------------------------------------- :  89
SDF1_beta     : CIDPKLKWIQEYLEKALNKRFKM------------------------------------------------ :  93
SDF1_gamma    : CIDPKLKWIQEYLEKALNKGRREEKVGKKEKIGKKKRQKKRKAAQKRKN---------------------- : 119
SDF1_delta    : CIDPKLKWIQEYLEKALNNLISAAPAGKRVIAGARALHPSPPRACPTARALCEIRLWPPPEWSWPSPGDV : 140
SDF1_epsilon  : CIDPKLKWIQEYLEKALNNC------------------------------------------------- :  90
SDF1_theta    : CIDPKLKWIQEYLEKALNKIWLYGNAETSR------------------------------------- : 100
```

Figure 1.2 Alignment of the allelic CCL3 variants (a) and the splice variants of CXCL12 (b).

observed initially [23]. With CCR3, only 70–80% of the initial receptor density is observed, but with CCR1, no recycling is observed [22]. While the CCR3 receptors that do not recycle have been shown to traffic to the lysosomal compartment where they are degraded, the fate of CCR1 remains to be established. Therefore the apparent redundancy of a chemokine binding to more than one receptor may not be as redundant as meets the eye.

An additional layer of complexity has been found for the chemokine CXCL12, where six splice variants have been identified (Figure 1.2b [25]. The main difference is the extended C-termini of the δ and γ isoforms. The γ isoform has an extremely large number of basic residues resulting in a significantly increased affinity for GAGs [26, 27]. Beyond this observation little is known about the biological relevance of these iosforms, and the vast majority of data concerns CXCL12α. Similarly, the vast majority of data for CCL3 is for the LD78α allele and not the high affinity ligand, CCL3L1 described above.

1.3 Ligand Structure

1.3.1 Tertiary and Quaternary Structures

As described above, chemokines are ∼70–125 amino acid proteins that usually contain two disulfide bonds, with the exception of a few that have a single or three disulfides (e.g., XCL1 and CCL21, respectively). The presence of the disulfides enables primary sequences of low homology to adopt similar tertiary folds that

would likely be stable in the absence of the covalent structural constraints. CX3CL1 and CXCL16 are unique among the chemokines in that they contain a chemokine domain fused to a long extracellular mucin-like stalk, a single transmembrane helix and a short cytoplasmic domain; thus in contrast to most chemokines that are soluble secreted proteins, these chemokines are tethered to cell surfaces or can be proteolytically cleaved into a soluble form [2, 28]. Sequence similarity of the chemokine domain can vary from 20% to 80–90%. Nevertheless, chemokines adopt a remarkably conserved tertiary structure consisting of a disordered N-terminal region that is always important for signaling, a disordered "N-loop" ending in a 3_{10} helix, a three-stranded antiparallel beta-sheet and a C-terminal alpha-helix that packs against the sheet [29] (Figure 1.3a). Some chemokines like CCL27, JE and XCL1 have domains that extend beyond the C-terminal α-helix, and like the N-termini, these domains are disordered. In mouse JE the extension is 49 amino acids longer than the human homolog and it is glycosylated in mammalian cells. However, the functional roles of these extended regions are unclear. For example, an 8.5-kDa truncated form of JE missing most of the extra C-terminal residues is completely functional in migration assays of mouse and human monocytes *in vitro* [30]. However, whether it is fully functional *in vivo* remains to be determined.

While the tertiary structures are similar among all chemokines characterized to date, and some are monomeric in solution, many chemokines oligomerize, forming

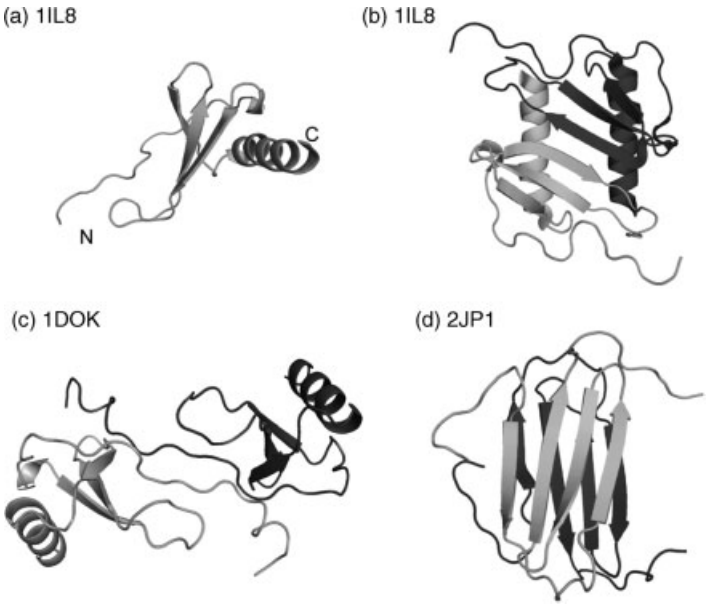

Figure 1.3 Ribbon diagrams of chemokines. (a) Monomer structure of CXCL8. (b) Dimer structure of CXCL8. (c) Dimer structure of CCL2. (d) Noncanonical structure of XCL1 stabilized by low salt and high temperature. PDB IDs are indicated next to each figure. The figures were generated in PyMol (DeLano Scientific).

dimers, tetramers and higher order oligomers. As described below, emerging data suggests that these different oligomeric forms are functionally significant and thus could add a great deal of biological diversity to an otherwise common structural fold. The formation of the oligomeric structures is not an all or nothing situation, however, and the propensity for oligomerization varies significantly from those that form stable multimers in solution under physiological conditions, to those that have weaker tendencies to oligomerize and can be readily shifted between oligomeric states by solution conditions (chemokine concentration, pH, salt, buffer), or by interactions with other molecular entities such as glycosaminoglycans (GAGs). For example, CCL7 is a monomeric ligand of CCR2, whereas another CCR2 ligand, CCL2, dimerizes in solution but can be shifted into a monomeric or tetrameric form by adjusting solution conditions or by interaction with GAGs [31, 32]. Likewise, CXCL12 exists in a monomer–dimer equilibrium and can be shifted towards the dimer by GAGs, phosphate ions and sulfate ions [33]. CXCL4 is a stable tetramer in solution [34], while CCL5, CCL3 and CCL4 form higher order oligomers under relatively normal physiological conditions [35], but can be destabilized into dimers by low pH and high salt (e.g., CCL5) concentrations [36]. Indeed, when one reads papers that report the oligomerization states of chemokines it is important to note the solution conditions, as many chemokine structures have been solved at low pH to disfavor oligomerization. By contrast, the observation of tetramers in crystal structures may be facilitated by favorable packing interactions in the crystal. However, independent of these biophysical studies, there is significant *in vitro* and *in vivo* biochemical evidence for the function of chemokine oligomers, and it is clear that one such role involves interactions with GAGs (see below).

Two common dimer motifs are generally associated with CC and CXC chemokines. CXC dimers, formed by the prototypical chemokine CXCL8, interact through amino acids in the first strand of the beta-sheet to form a dimer with an overall six-strand beta-sheet platform topped by two alpha-helices (Figure 1.3b) [29]. By contrast, most CC chemokines that dimerize (e.g., CCL2, CCL8) do so through residues near the N-termini forming a much more elongated structure than the CXC dimers (Figure 1.3c) [37]. However, at least one CC chemokine CCL20 has been reported to form a CXC-like dimer, calling into question the strict assignment of CC and CXC dimer motifs to ligands from the respective CC and CXC families [38]. CX3CL1 was solved as a monomer by NMR [39], although it showed a tendency to dimerize (Mizoue and Handel, unpublished data) and crystallized as a tetramer with the main dimeric substructure reminiscent of the CC-dimer motif [40]. XCL1 is the most unique chemokine and forms two entirely different structures that interconvert rapidly (100 ms); one structure is a canonical monomeric chemokine fold, which is stabilized by low temperature and high salt conditions (10 °C, 200 mM NaCl). The other structure is favored by low salt and high temperature conditions (0 M NaCl, 40 °C), and is a four-stranded antiparallel beta-sheet that self-associates as a novel head to tail dimer (Figure 1.3d) [41]. Interestingly, only the canonical chemokine fold acts as a receptor agonist and binds weakly to GAGs, while the novel dimer binds strongly to GAGs, but does not effectively activate the receptor XCR1.

CXCL4, the first chemokine structure to be solved, forms a stable tetramer in solution. Interestingly, a positively charged ring of lysine and arginine side chains encircles the PF-4 tetramer sphere, presenting a continuous binding site for heparin [34]. Recently, it was shown that the presence of unfractionated heparin, stabilizes these tetramers effectively into strings of tetramers and, notably, these are forms that are recognized by heparin-induced thrombocytopenia antibodies [42]. Although CCL2 forms predominantly dimers in solution, the addition of heparin octasaccharides causes it to form tetramers. High protein concentration and the presence of phosphate shift the equilibrium toward larger multimers as well (Handel, unpublished data). Interestingly, despite solution studies that show a bias toward dimers, crystallization of CCL2 trapped a tetrameric form similar to the CXCL4 tetramer. Like CXCL4, the CCL2 tetramer has an elongated ring of basic residues which have been shown to be important for heparin binding (Figure 1.4a) [32]. Furthermore, both of these structures contain CXC and CC-like dimers as substructures, and like the CCL20 dimer, suggest that CC and CXC dimers can be formed from chemokines from different families. CXCL10 was solved as a typical monomer in solution [43] but crystallized in three different tetramer forms; one resembles the CXCL4 and CCL2 tetramers while the other two are unique extended 12-stranded beta sheet structures, all with CXC dimers as substructures [44] (Figure 1.4a, c). Mouse IP-10 also crystallized as yet a fourth type of tetramer with heparin binding sites localized to the interfaces of each of the dimers, suggesting stabilization of the tetramer by heparin as observed for CCL2 [45]. The tetramer in the crystal structure of CX3CL1 is also entirely unique and mediated by water molecules

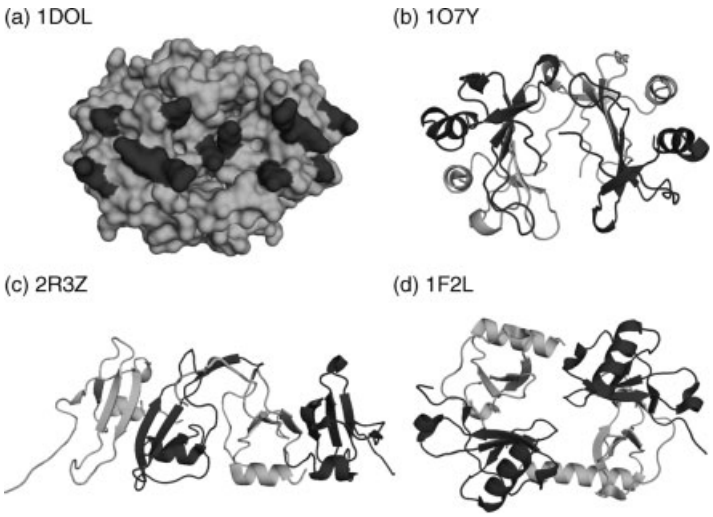

Figure 1.4 Structures of chemokine tetramers. (a) Tetramer structure of human CCL2 with GAG-binding eptiopes highlighted in dark gray. (b) Tetramer structure of human CXCL10, M form. (c) Tetramer structure of mouse CXCL10. (d) Tetramer structure of human CX3CL1. PDB IDs are indicated next to each figure. The figures were generated in PyMol (DeLano Scientific).

thus explaining the tendency to remain monomeric in solution, at least in the absence of GAGs (Figure 1.4d) [40]. In principle, different oligomeric forms like the four CXCL10 tetramers could represent structures capable of discriminating between different types of GAGs, but further study is necessary to determine if this is the case.

1.3.2 Functional Role of Tertiary and Quaternary Structures

As alluded to above, several studies suggest a functional role for chemokine oligomers, however monomeric forms of chemokines are sufficient for binding receptors to induce cell migration and cellular activation *in vitro*. Rajarathnam and coworkers demonstrated this finding by chemically synthesizing a variant of CXCL8 in which the amide nitrogen of leucine-25 was methylated to selectivity block formation of hydrogen bonds between monomers and thereby prevent dimerization [46]. Similarly a synthetic N-methylated variant of CCL5 was made for the same purpose [47]. In a different approach, mutants of CCL2 and CCL4 containing Pro to Ala substitutions in the N-terminal region were engineered to prohibit dimerization [48, 49]. In all cases, the variants were significantly or completely impaired in their ability to dimerize yet they showed full wild-type affinity and activity when tested in *in vitro* receptor binding assays and functional assays of receptor activation including *trans*-filter cell migration. From these data, it was concluded that the monomeric forms of chemokines are sufficient to activate the receptor for these biological responses.

Nevertheless, the functional importance of oligomerization was revealed with subsequent *in vivo* studies using an intra-peritoneal recruitment assay and the monomeric variants of CCL2, CCL5 and CCL4. In contrast to their ability to recruit cells *in vitro*, these mutants were incapable of causing cell migration into the peritoneal cavity *in vivo* [47]. While the mechanism is not entirely clear, biophysical and biochemical studies have associated oligomerization with binding to glycosaminoglycans, which are also required for *in vivo* but not *in vitro* cell migration [32, 50] (see below). These results also demonstrate the conflicting results that one can observe between *in vitro* and *in vivo* studies because of the complex nature of *in vivo* cell migration which involves interactions of migrating cells with other cells (e.g., endothelial cells), interactions between adhesion proteins on opposing cell surfaces, transcytosis of chemokines across endothelial cells, and other phenomena that are poorly recapitulated with simple *in vitro trans*-filter migration assays.

Although less well studied, chemokine oligomerization also appears to be important for cellular activation and related signaling processes. For example, while CCL5 monomers are capable of inducing cell migration, only wild-type CCL5 which forms large oligomers, but not an E66S mutant which is dimeric, was capable of activating the protein tyrosine kinase pathway in T cells leading to cell activation and associated events [51]. Similarly, the E66S mutant failed to induce T cell apoptosis [52]. As discussed below, obligate monomeric variants have been shown to have anti-inflammatory properties.

1.3.3
Hetero-Oligomerization

Chemokines also form hetero-oligomeric complexes. CCL3/CCL4 heterodimers were first shown to be secreted by human monocytes and peripheral blood lymphocytes by immunoprecipitation and immunoblot [53]. Subsequently, CXCL4/CXCL8, CXCL4/CCL5, CCL2/CCL8 and CCL21/CXCL13 were also shown to heterodimerize by co-immunoprecipitation and/or NMR [54–58]. Functional consequences have been associated with the ability of the requisite heterodimerizing chemokines to cause synergistic or additional responses not seen with either chemokine alone. For example, the presence of angiogenic CXCL8 with anti-angiogenic CXCL4, which form CXC heterodimers, increases the anti-proliferative activity of PF-4. Similarly, CCL5 and CXCL4, which form CC heterodimers, synergistically enhanced proinflammatory interactions, such as monocyte recruitment. Furthermore, peptides that inhibit heterodimerization not only attenuated monocyte recruitment, but were able to reduce the progression of diet-induced atherosclerosis in mice, a disease with a significant monocyte/inflammatory cell component [58]. However, the CCL5/CXCL4 study is the only example where there is strong direct physical evidence that the associated dimers are actually responsible for the observed functional effects rather than just the mere presence of the two chemokines in solution that happen to be able to associate. If further studies provide evidence that heterodimers are truly important for signaling, interfering with this interaction could prove to be a viable therapeutic strategy.

1.4
Receptor Structure

Chemokine receptors belong to the Class A family of G protein-coupled receptors (GPCRs), whose prototypic member is rhodopsin, a GPCR involved in light perception. Like other GPCRs, chemokine receptors are characterized by seven transmembrane helices connected by extracellular and intracellular loops of varying lengths, as well as extracellular N-terminal and intracellular C-terminal domains which show the most sequence diversity. A conserved structural feature of chemokine receptors includes the presence of a disulfide bond between the extracellular side of transmembrane segment 3 (TMS 3) and extracellular loop 2 (ECL 2); it is thought to have a role in structure/folding as well as functionally coupling changes in the conformation of ECL 2 upon ligand binding with TMS 3, a helix known to be critical to receptor activation in bovine rhodopsin and the β2 adrenergic receptor [59–61]. In chemokine receptors, there is also a disulfide between the N-terminus and ECL 3 (Figure 1.5a). Since the receptor N-terminus is involved in chemokine binding (see below) and since TMS 6 and 7 are connected by ECL 3 and are thought to be important for the activation switch, this disulfide may also couple ligand binding on the extracellular side to activation on the intracellular side through these helices. Other important motifs include the DRY motif at the base of TMS 3 just prior to ICL 2

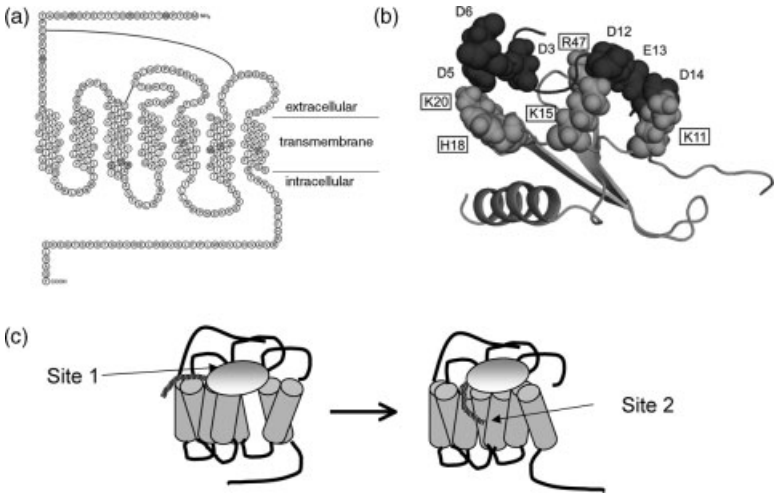

Figure 1.5 (a) Snake-like diagram of CCR1 as an example of the topology and post-translational modifications observed in chemokine receptors. The two conserved disulfides are connected by dark lines. Potential N-linked glycosylations sites and tyrosine sulfation sites in the N-terminal domain are shown as shaded circles labeled N and Y, respectively. The DRY box, required for G protein coupling is highlighted on the intracellular side of transmembrane 3. Prolines and the Tryptophan rotamer toggle, thought to be important for receptor activation, are shaded in helices 5, 6 and 7. Many chemokine receptors have cysteines in their C-terminal domain which are modified by palmitoylation. CCR1 does not get palmitoylated but CCR5 has such sites for example. (b) Monomer structure of CXCL8 (light gray) complexed with a modified peptide derived from the N-terminus of the receptor CXCR1 (PDB ID 1ILQ). The peptide binds across the surface in an extended fashion, binding in a cleft formed by a loop and beta-hairpin. Some of the basic residues on the ligand (in boxes) and acidic residues on the peptide (not boxed) are labeled and may confer specificity although most intermolecular contacts are hydrophobic. Figures were generated in PyMol (DeLano Scientific). (c) Cartoon illustration of the two site model of receptor activation. In this model, the main body of the chemokine (round oval) docks with the receptor N-terminus and extracellular domains (dark lines). The N-terminus of the ligand (stippled line) than interacts with the receptor helical bundle to induce the requisite conformational changes required for receptor activation.

which is required for G protein coupling, several conserved prolines that are thought to be important for conformational changes by introducing weak pivot points in helices, and a Trp that is thought to act as a "rotamer toggle" activation switch by rotamer interconversion [59, 61]. Other patterns include sequence motifs in the C-termini which are involved in receptor internalization often through binding of beta-arrestins and in intracellular trafficking patterns that dictate whether the receptor recycles to the cell surface or gets targeted for degradation. Chemokine receptors also frequently contain palmitoylation sites on cysteines in their C-termini, which tether the flexible C-termini to the membrane. The extracellular N-terminal domains which are relatively short and thought to be fairly flexible, at least in the absence of bound ligand, frequently contain putative N-linked glycosylation sites, as well as tryosine

sulfation motifs which are characterized by tyrosines flanked by acidic residues. While the functional roles of tyrosine sulfation and glycosylation have not been fully elucidated, tyrosine sulfation has been shown to modulate the affinity of certain receptors for their ligands [62]. Figure 1.5a shows a snake-like diagram of the chemokine receptor CCR1, illustrating some of the motifs described above.

No structures of chemokine receptors have been determined. However, on the basis of mutagenesis studies, it is clear that the N-termini of chemokine receptors are involved in ligand binding. Capitalizing on this observation, several studies have been done in which peptides from the N-termini of chemokine receptors have been synthesized and utilized in binding and structural studies with ligands [63–65]. These studies generally suggest that the receptor N-terminus binds to chemokines in a relatively extended conformation, with acidic residues from the receptor interacting with basic residues on the ligand (Figure 1.5b). Receptor peptides that are tryosine sulfated typically bind with higher (micromolar) affinity compared to nonsulfated peptides, confirming the importance of this post-translational modification. While some studies suggest that the peptides bind preferentially to the monomeric form of chemokines, consistent with the concept that monomeric forms are sufficient for activating the receptor, in the case of SDF-1/CXCL12 and a sulfated peptide from CXCR4, a 2:2 complex was favored [63]. However, one must keep in mind that these studies are taken out of context of the full receptor and the results may be influenced by the lack of other relevant interactions. In an attempt to recapitulate additional interactions between ligands and receptors, chimeric soluble proteins with the receptor N-termini and extracellular loops were made by attaching these elements to a soluble scaffold (the B1 domain of protein G) [66]. Importantly, the binding affinities of the soluble receptor mimics were highly correlated with the apparent affinities of the native receptor, demonstrating that the chimeras could capture some of the relevant interactions. However, it is obvious that to truly understand how chemokines bind and activate their receptors, that structures with full length receptors will be needed. Fortunately, progress in the determination of GPCR structures has exploded since 2007, and thus one can hope that chemokine receptors will yield to structure determination as well [67]. Until then, the two-site model of receptor activation continues to be the prevailing model (Figure 1.5c).

The two-site model is similar to that proposed for the chemoattractant protein C5a and suggests that the main body of the ligand first interacts with extracellular domain (s) of the receptor. Based on the NMR studies described above, the N-terminus of chemokine receptors would then feature prominently in interactions with the main body of the ligand, although other ECLs are also known to contribute. This interaction then positions the chemokine such that the flexible N-terminal region is able to interact with a second site, possibly in the receptor helical bundle similar to the binding site of small molecule ligands in the β2 adrenergic receptor. These interactions together induce the requisite conformational changes in the receptor, thereby triggering signal transduction. In contrast to the chemokine systems where the flexible N-terminal region is known to play the role of the signaling trigger, it is the flexible C-terminal domain in the case of C5a as well as another chemoattractant called chemerin [68].

1.4.1
Modifications to the N-Termini of Chemokines and their Effect on Receptor Activation

There is substantial evidence for the triggering role of the N-termini of chemokines, in support of the two-site model (Figure 1.5c). The importance of the N-termini of chemokines in receptor activation was first demonstrated with bacterially expressed [69] or chemical synthesized mutants of CXCL8 [70]. Both approaches demonstrated that the three-amino-acid "ELR" motif preceding the CXC sequence in the N-termini of CXCL8 was required for optimal binding and signaling. The ELR motif is found on all neutrophil attracting CXC chemokines, but is absent on members such as CXCL4 and CXCL10 that lack this activity. Interestingly, when the ELR motif was introduced into CXCL4, it was able to attract neutrophils [71]. By contrast, removal of the first five amino acid residues from CXCL8, which retained R6 of the ELR motif, produced a protein that was able to bind to its receptor with reasonably high affinity but could not induce signaling [72]. Furthermore this analog could antagonize the actions of CXCL8 highlighting the importance of the N-terminus in activation through site 2, while not affecting site 1. Other modifications to the N-termini of several chemokines further strengthened this argument, and their antagonist properties are discussed below, since they retain biding to site 1 but do not trigger signaling through site 2.

In additional to artificial mutations, natural modifications of the N-terminal domains have been described for many chemokines [73]. Several classes of enzymes have been shown to be responsible for N-terminal truncations including matrix metalloproteases (MMPs), DPIV/CD26, leukocyte elastase and cathepsins to name a few. These enzymatically cleaved forms usually are completely or partially inactivated with respect to signaling relative to the wild type, but sometimes they show increased activity and often they retain high affinity binding. Sometimes there is an effective switch in receptor specificity. For instance, (3–68)-CCL5, a cleavage product of CCL5 produced by CD26, has impaired signaling activity on monocytes through loss of affinity for CCR1, the principal receptor on circulating monocytes, and antagonizes monocyte chemotaxis. However its affinity for CCR5 is enhanced, and it is more potent in inhibiting HIV infectivity [74]. When modified CCL5 variants which have anti-inflammatory properties are administered to mice, their processing in the circulation can be directly demonstrated using surface enhanced laser desorption ionization (SELDI) – a protein chip technology coupled to a MALDI spectrometer. Using this technique, the GAG-binding mutant, ^{44}AANA^{47}-CCL5, was shown to be rapidly oxidized, cleaved to the 3–68 form and then further truncated to produce the 4–68 form [75].

As mentioned above, there are also numerous reports of N-terminal truncated variants that have increased activity relative to their wild-type counterparts: truncation of CXCL1 and CXCL3 resulted in chemokines with enhanced abilities to induce calcium signaling and neutrophil chemotaxis [76]. Furthermore, (5–73)-CXCL2 is ten times more potent than the full length chemokine [77]. Perhaps the most impressive gain of function is for the low affinity CCR1 ligands, CCL15 and CCL23 which have an extended N-terminus of 16–20 amino acids and a third disulfide bridge; processing of

their extended N-terminal by elastase, cathepsin-G or chymase, or synovial fluid, results in up to a 1000-fold increase of affinity for CCR1, rendering them more potent than CCL5 and CCL3 [78].

Conversely many chemokines are rendered inactive by N-terminal processing. For example, MMP cleavage of CCL2 to produce (5–76)-CCL2 [79] and DPPIV/CD26 cleavage of CCL11 to (3–74)-CC11 [80] abrogates activity, and the physiological relevance of the latter result was confirmed by enhanced eosinophil mobilization induced by the administration of CCL11 into DPPIV-deficient mice.

Chemokine processing is not limited to the N-terminus. In addition to CD26 and MMP N-terminally truncated versions of CXCL9, CXCL10 and CXCL11, truncation is also observed at the C-terminus. The signaling properties of (1–77)-CXCL10 and (1–73)-CXCL10 are very similar, whereas processing of the C-terminus of CXCL9 diminishes signaling [81]. In addition, heparin binding of CXCL11 is significantly reduced in the 1–58 cleaved form compared to the full-length 1–73 protein.

1.5
Glycosaminoglycan Binding Sites

Several years ago the immobilization of CXCL8 on endothelial surfaces first demonstrated the importance of chemokine/glycosaminoglycan (GAG) interactions [82]. Subsequently selectivity in this interaction was demonstrated using electrophoresis of chemokines in the presence of different heparin fragments [83] and a solid phase heparin binding assay [84]. Interestingly, GAGs have been shown to both potentiate and inhibit chemokine activities. The potentiation of chemokine activity by GAGs was reported *in vitro* using CHO cells that are deficient in cell surface GAGs where it was demonstrated that while biological activities such as receptor binding and activation is independent of the presence of GAGs, the expression of cell surface GAGs helped to sequestrate the chemokines [85]. Using chemokine mutants with abrogated GAG binding capacity, the chemokine/GAG interaction has been shown to be essential for the ability of chemokines to recruit cells *in vivo*, while this interaction is not required in the commonly used *in vitro trans*-filter chemotaxis assay [47]. Other examples of biological relevance include the fact that CCL5 secreted from the alpha granules of platelets is found in large GAG associated complexes, and the GAG association is required for the anti-HIV inhibitory properties of this chemokine [86]. CCL5 has also been shown to enhance infection of HIV-1 in macrophages [87], as well as other viral infections *in vitro*, albeit at micromolar concentrations, which is attributed in part to the oligomerization property of this chemokine [88, 89]. As discussed below, this property is mediated by the binding of CCL5 to GAGs.

GAG binding sites are generally identified by alanine scanning mutagenesis studies of the chemokine of interest. However, biochemical methods can be used to predict the regions that are involved, which can be subsequently confirmed by restricted mutagenesis. An example of such a method involves the use of protein bound to heparin beads, which is subsequently covalently stabilized by chemical

crosslinking. Following proteolytic digestion, peptides that remain bound to heparin, presumably GAG-binding epitopes, are identified by N-terminal sequencing [90]. In a second related approach, samples of chemokine/heparin complexes or chemokine alone are submitted to tryptic digestion and mass spectrometry identification; peptide fragments that are present only in the heparin containing samples correspond to fragments protected by heparin and therefore GAG-binding sites.

The relative of affinities of chemokines or chemokine mutants for GAGs has been measured by several different methods. These include heparin sepharose chromatogaphy [91–93], electophoresis in the presence of different GAG species [83], solution binding assays with radiolabeled heparin [94], binding of radiolabelled chemokines to heparin immobilized on beads [84, 93] and an ELISA-type assay of chemokine binding to heparin immobilized on specialized plates (Epranex plates, Plasso Technologies Ltd.) [95, 96]. The most common procedure is the analysis of the NaCl concentration required to elute the chemokine from heparin sepharose columns. In these analyses, certain mutants are no longer able to bind to the column, as was observed for MIP-1α [92] and MIP-1β, where the binding was abrogated in the presence of physiological concentrations of NaCl [91]. However, in the case of chemokines that bind much more strongly to heparin, such as CXCL8, CXCL12 and CCL5, mutation of the heparin binding sites does not totally abolish the capacity to bind to heparin, indicating either that other specific sites exist, or that there is a certain amount of nonspecific electrostatic interaction. For example, analyses of the CCL5 triple ^{44}RKNR^{47} alanine mutant indicate the former. The residual 20% binding capacity that this mutant retained for heparin, had the same affinity for heparin as wild-type CCL5, but lost selectivity for the four GAG families, heparin, heparin sulfate, dermatan sulfate and chondroitin sulfate [93]. Recently, a binding assay using Epranex plates has been frequently used. This assay was used to compare the GAG binding affinities of citrullinated CXCL8 to those of the wild type [96]. It was also used to demonstrate that eotaxin selectively binds to heparin, but not to heparan sulfate or a range of other GAGs [95].

Receptor binding regions and GAG binding regions delineated by mutagenesis studies for several chemokines initially led to the idea that receptor and GAG binding domains were spatially separated in that receptor binding was governed by the N-terminus and GAG binding by the C-terminal helix. For example, the importance of the C-terminal helix in GAG binding had been shown for CXCL4 [97]. However, it appears that this generalization is not true for many chemokines, now that the GAG binding sites have been delineated for more chemokines. In fact, the 20s loop contains residues important both for receptor binding and GAG binding in several chemokines such as CXCL8, CXCL10, CXCL12 and CCL2 [32, 98–100].

The GAG binding sites have now been mapped for several chemokines. The residues on chemokines that interact with GAGs identified to date are represented in Figure 1.6. Heparin binding regions are often defined by a cluster of basic residues, forming either a BBXB or a BBXXB motif (where B is a basic amino acid). These residues have been mutated to Ala in the CCR5 ligands CCL3 [92, 101], CCL4 [91] and CCL5 [93], showing that they constitute the principal heparin binding site(s). While no effect on receptor binding was observed for the R46A mutant of MIP-1β

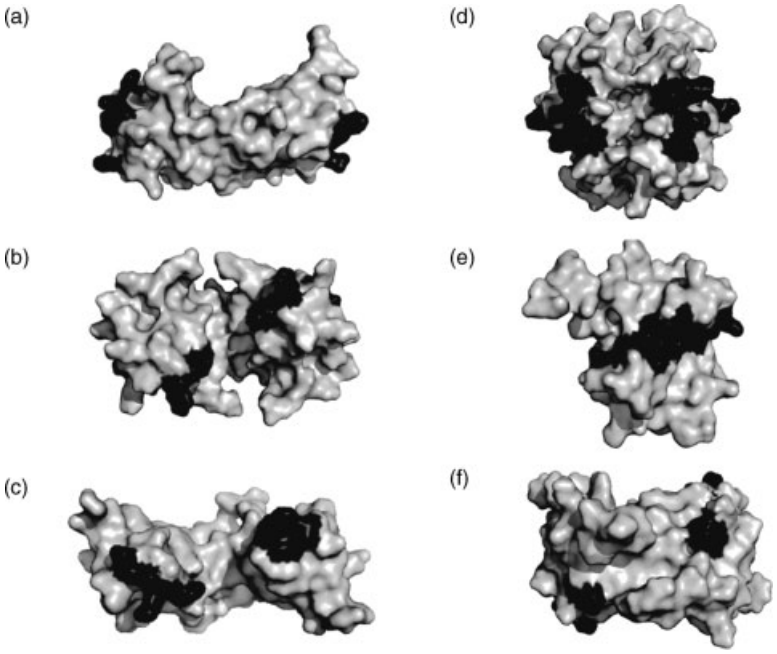

Figure 1.6 Representative dimer structures of CC chemokines (a–c), CXC chemokines **(**d, e**)**, and the C chemokine **(**f**)** with GAG binding epitopes identified by mutagenesis, highlighted in dark gray. (a) CCL2 with GAG epitopes R18, K19, R24, K48, K58, H66. (b) CCL3 with GAG epitopes R18, K45, R46, K48. (c) CCL5 with GAG epitopes R44, K45, R47. (d) CXCL8 with GAG epitopes K20, R60, K64, K67, R68. (e) CXCL12 with GAG epitopes K24, H25, K27, R41, K43. (f) XCL1, low salt high temperature form, with GAG epitopes K23, R43. The figures were generated in PyMol (DeLano Scientific).

to its receptor CCR5, the mutation R46A in MIP-1α abolished binding to CCR1. Our results with CCL5 have shown a similar phenomenon. The mutations of the residues R44, K45 and R47 individually to Ala had no significant effect on all the parameters tested – binding to heparin, CCR1, CCR5 or on *in vitro* chemotaxis. However, the concomitant mutation of the basic residues to Ala in the ^{44}RKNR^{47} motif had a profound effect on heparin binding and a significant effect on binding to CCR1, while wild-type activity was retained on CCR5. However, while GAG binding was not completely abrogated, the mutations were sufficient to eliminate *in vivo* cell migration [102]. CCL5 has a second cluster of basic residues immediately preceding the C-terminal helix, ^{55}KKWVR^{59} but mutation of these residues either individually or as a triple mutation had no effect in all of the above assays. However, using the elegant assay discussed above to involving covalent linking of the protein to a heparin bead, followed by enzymic digestion of the complex and finally N-terminal sequencing of the peptides bound to the heparin, the sequence ^{55}KKWVR^{59} was found when using an increased ratio of chemokine to heparin [90]. Interestingly this region was subsequently found to play a role in binding to tissue in the kidney [103].

The C-terminal helix of CXCL8 presents four basic residues which interact with GAGs: K64 and R68 play the major role, but R60 and K67 also contribute [98]. K20, in the 20s loop which has been shown to be involved in receptor binding, is also involved in GAG interactions. However, in the CXCL8 protein the main receptor interface is spatially separated from the C-terminal helix which is the principal GAG binding region. CXCL12 has introduced an entirely new region that is implicated in GAG binding [100]. In this chemokine, GAG binding is mediated by a BBXB motif, but which is located in the 20s loop, since it consists of the residues ^{24}KHLK^{27}. However these basic amino acids play no role in CXCR4 activation. Interestingly, while no GAG biding is observed by surface Plasmon resonance at concentrations $\leq$200 nM, micromolar concentrations show GAG binding equivalent to the wild type [100]. However the loss in GAG binding is sufficient to abrogate the ability to recruit leukocytes into the peritoneal cavity. The GAG binding residues on MCP-1 were initially described as being located towards the C-terminus, formed by two residues: K58, which is located immediately before the C-terminal helix, and H66, which is found at the end of the helix. The other basic residues in this region, K56, K69 and K75 are not involved in GAG binding [94]. An extensive alanine scanning mutagenesis study of this chemokine delineated residues on the 20s loop, R18 and K19, as being the predominant amino acids for GAG binding, with a lesser contribution provided by R24 [32]. The 20s region is also involved in GAG binding for the chemokine CXCL10. Residues Arg20, Arg22, Ile24, and Lys26, as well as Lys46 and Lys47 were found to constitute the main GAG binding domain, with the mutation of Arg22 resulting in the largest reduction of heparin binding affinity [99].

The C-terminal truncation influences the GAG binding properties of several chemokines. C-terminal cleavage of CXCL11(1–73) to CXCL11(1–58) by MMP-8 or MMP-9 significantly diminishes heparin binding affinity [104]. For the case of CXCL12a, C-terminal cleavage also decreases its heparin binding capacity, as well as its ability to attract B-lymphocytes and to stimulate pre-B cell proliferation [105].

1.6
Chemokine Analogs– Research Tools and Potential Therapeutics?

As discussed above, alterations in the N-termini of certain chemokines have resulted in profound changes in their activity, and there are many examples of modifications of the N-terminus to produce antagonists. N-terminally truncated CCL5, MCP-1 and MCP-3 proteins have antagonized the effect of their parent ligands *in vitro* [106]. Interestingly, removal of the first eight residues from the N-terminus of CCL5 changes its specificity, since the truncated protein is able to bind to CCR2, to which the full-length protein does not bind. The removal of seven residues from the N-terminus of MCP-1 (7ND-MCP-1) forms an antagonist protein that was initially proposed to act by preventing the formation of the active MCP-1 dimer, analogous to dominant negative mutations [107]. Interestingly these proteins have not been extensively studied *in vivo*, and there is only one report of the activity of a truncated chemokine *in vivo*, where the administration of the (9–68)-MCP-1 protein into mice

that spontaneously develop arthritic symptoms was very effective in reducing the inflammatory symptoms, when administered in a therapeutic protocol [108]. However, 7ND-MCP-1 has been extensively used in the form of gene therapy where the cDNA has been administered and has shown to be effective in reducing symptoms in several disease models, for example experimental autoimmune myocarditis (EAM), an animal model of human myocarditis [109] and cancer [110].

The extension of the N-terminus of certain chemokines can also have a profound effect on the biological properties. In the case of CCL5, when the recombinant human protein is produced in *Escherichia coli*, despite being correctly folded as assessed by NMR spectroscopy [36], the chemokine had no activity [111]. It was established that the retention of the initiating methionine was responsible for this effect, and that the protein was a potent antagonist – an entirely serendipitous finding. This analog, Met-CCL5, retains high affinity binding for human CCR1 [111] and CCR5 [112] but only moderate affinity for CCR3 [22, 102, 113, 114], and we have recently shown that it only binds to CCR1 and CCR5 in the mouse system [115]. Met-CCL5 has been tested in numerous rodent inflammatory models which have served to highlight the efficacy of chemokine receptor antagonism in preventing inflammation. Thus Met-CCL5 has been reported to reduce inflammation in models of arthritis [116], nephritis [117], organ transplant [118], colitis [119] and asthma [120]. Since this analog has high affinity for mCCR1 and mCCR5, chemokine receptors expressed on leukocytes implicated in many inflammatory disorders such as T lymphocytes and monocytes, these results indicate that these two receptors are good therapeutic targets. It is however interesting to note that Met-CCL5 is also effective in reducing airways inflammation in the mouse ovalbumin sensitization model, despite the fact that it has no affinity for mCCR3. Since CCR3 is the predominant CC chemokine receptor expressed on eosinophils and this cell type is widely thought to be the main pathogenic culprit in asthma, this receptor was widely believed to be a prime target for this disease. However the use of this analog demonstrates that antagonism of receptors other than CCR3 is effective in reducing airway inflammation.

Our initial studies characterizing Met-CCL5 were carried out on the promonocytic cell line, THP-1, where we found it to be devoid of activity. However, on eosinophils from certain patients, weak partial agonist activity was observed [102]. Through a semisynthetic approach aimed at producing a derivative that was devoid of this partial agonist activity, we identified an analog, AOP-CCL5 (aminooxy pentane CCL5) that had higher affinity for CCL5 receptors than Met-CCL5 [102, 112]. Again the N-terminally extended CCL5 protein was inactive on monocytes, and antagonized the effects of CCL5, CCL3 and CCL4 on this cell type. This analog has since taught us new mechanistic pathways that play a role in disease. AOP-CCL5 was found to be a particularly potent inhibitor of HIV-1 infection, being significantly more potent than CCL5 itself [112]. The inhibition of HIV-1 infection by chemokines could be mediated by two mechanisms – either simply by steric hindrance, or by inducing downregulation of the receptor from the cell surface. Although AOP-CCL5 was initially thought to be an antagonist, subsequent studies demonstrated that it also retained partial agonist activity on CCR1 and CCR3, but was a full agonist of CCR5 [102, 121]. In fact in certain activities, it was even more potent than CCL5

itself [23, 121]. Thus its potent HIV-1 inhibitory properties were attributed to its greater effectiveness in downregulating CCR5 and furthermore in preventing the recycling of the receptor to the cell surface [23, 121]. However AOP-CCL5 did not target the receptor to the lysosomal compartment where it is degraded, as has been shown to occur for other chemokine receptors such as CCR3 [24, 121]. It has subsequently been demonstrated that CCR5 does in fact recycle to the cell surface after AOP-CCL5 treatment, but through a mechanism that remains to be elucidated [122]. Thus modification of the receptor endocytotic pathways is an effective mechanism of receptor antagonism.

Two programs capitalized on this unusual property of AOP-CCL5 and developed variants that were considerably more potent. An optimization program led to a variant, PSC-CCL5 that was 50-fold more potent [123] and showed efficacy as a microbicide by topical application in preventing HIV infection in macaques [124]. However this variant had the disadvantage of being costly to produce since it was made by total chemical synthesis. Therefore a second approach was adopted which was to search for a variant of CCL5 that had the properties of PSC-CCL5, but which consisted entirely of natural amino acids using phage display. Three analogs were identified that exhibited *in vitro* potency against HIV-1 comparable to that of PSC-CCL5 [125]. The first induced prolonged intracellular sequestration of CCR5, the second had no detectable G protein-linked signaling activity nor receptor sequestration and the third induced significant levels of CCR5 internalization without detectable G protein-linked signaling activity. These analogs therefore represent promising candidates for further development as topical HIV prevention strategies. Using phage display to identify chemokine receptor antagonists appears to be a powerful tool – recently an antagonist of CX3CR1 that appears to be totally devoid of agonist activity has been identified by this approach [126].

The essential roles of GAG binding for the biological activity of chemokines *in vivo*, and oligomerisation for certain chemokines, are discussed above. Therefore it is not surprising that interference with these properties could result in anti-inflammatory activities. Modulation of GAG binding has been used with two very different approaches. The first approach used chemokine analogs that had mutations removing basic residues to abrogate GAG binding, while the second introduced basic residues to enhance GAG binding. Not only was the variant ^{44}AANA^{47} CCL5 unable to induce cellular recruitment into the peritoneal cavity, but its administration prior to CCL5 or thioglycollate inhibited the cellular recruitment [47]. This anti-inflammatory property translated into potent inhibition of disease symptoms in the mouse model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) [127] and in atherosclerosis in mice [128]. The inhibitory mechanism appears to be its ability to disaggregate CCL5 oligomers and form heterodimers with the wild-type protein which are devoid of activity *in vivo*. Similar anti-inflammatory properties were described for a mutant of CCL7 which was designed to eliminate heparin binding [129]. The opposite approach has been taken for other inflammatory chemokines where the chemokine has been mutated to abrogate receptor activation, and additional basic residues have been introduced to augment heparin binding with the rationale of displacing the active

chemokine from the endothelial surface [130]. Lastly, interference with oligomerization provides another anti-inflammatory strategy – as discussed above the mutation P8A in CCL2 creates an obligate monomer that is also able to inhibit peritoneal recruitment and to reduce disease symptoms in EAE in mice [131, 132] and antigen induced arthritis (AIA) in rats [133].

In conclusion we have described how understanding the structural relationships of chemokines and their receptors to their biological activity has led to the design of antagonists that have been pivotal in validating the chemokine system as targets for disease. This knowledge, particularly the structure–function relationships of chemokine ligands, will be crucial to the design of novel therapeutic strategies. We now look forward to the next leap in chemokine structural biology, that of solving and understanding the structure and interactions of the receptors.

Acknowledgments

This work was funded by the Lymphoma Research Foundation, the Department of Defense (USAMRAA W81XWH0710446) and NIH (RO1-AI37113 and R21 AI076961) awards to T.M.H., an NRSA postdoctoral fellowship F32GM083463 awarded to D.J.H. and a European Union FP6 INNOCHEM award (LSHB-CT-2005-518167) to A.E.I.P.

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2
Structural Insights for Homology Modeling of Chemokine Receptors

Leonardo Pardo, Xavier Deupi, Arnau Cordomí, Cedric Govaerts, and Mercedes Campillo

2.1
Introduction

G protein-coupled receptors (GPCRs) constitute a large and functionally diverse family of transmembrane (TM) proteins. Recent estimations assign more than 1000 members to this family in the human genome (http://www.ebi.ac.uk/integr8/ProteomeAnalysisAction.do?orgProteomeID=25). GPCRs are receptors for sensory signals of external origin (e.g., odors, pheromones, tastes) and for endogenous signals, such as neurotransmitters, (neuro)peptides, divalent cations, proteases, glycoprotein hormones, purine ligands and chemokines, among others [1].

Chemokines (*chemo*tactic cyto*kines*), discovered in the late 1980s [2], are classified into four main classes (CC, CXC, CX_3C, C) according to the number and spacing of conserved cysteines in the N-terminal domain [3, 4]. To date, the CC class is formed by 27 members, namely CCL1–CCL28 (CCL9 and CCL10 are the same), which possess two adjacent cysteines. In the CXC class both cysteines are separated by one amino acid. There are 17 known CXC chemokines (CXCL1–CXCL17), which are divided into the ELR + and ELR– subclasses, depending on whether the Glu-Leu-Arg (ELR) motif is present (ELR +) or absent (ELR −) immediately before the CXC motif. The only known CX_3C chemokine (both cysteines are separated by three amino acids) is fractalkine (CX_3L1), which is a membrane-anchored chemokine with a mucin-like domain [5]. Finally, lymphotactin-α (XCL1) and lymphotactin-β (XCL2) are chemokines that contain only one cysteine (the C class). At the present time, 19 different chemokine receptors are known, which are classified into four families depending on whether they bind chemokines from the CC, CXC, CX_3C, or C families [6]. Thus, CCR1–CCR11 receptors bind chemokines from the CC family, CXCR1–CXCR6 from the CXC family, CX_3C binds CX_3L and XCR1 binds XCL1 and XCL2 (see Table 2.1 in Ref. [7]).

Chemokine receptors belong to the rhodopsin family of GPCRs [8]. Within this family, the crystal structures have been elucidated for cow rhodopsin (Protein Data Bank accession numbers 1F88, 1HZX, 1GZM, 1L9H, and 1U19) [9–12] and squid

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

(2Z73) rhodopsin [13] bound to the full inverse agonist *cis*-retinal, the ligand-free opsin (3CAP) [14], the ligand-free opsin in its G protein-interacting conformation (3DQB) [15], the β_1-adrenergic receptor bound to the antagonist cyanopindolol (2VT4) [16], the β_2-adrenergic receptor bound to the partial inverse agonist carazolol (2RH1 and 2R4R) [17–19] and the A2A adenosine receptor in complex with the antagonist ZM241385 (3EML) [20]. All these receptors are formed by an extracellular N-terminus, seven transmembrane helices (TM1–TM7, which also terms this family of proteins as 7TM receptors) connected by alternating intracellular (i1–i3) and extracellular (e1–e3) hydrophilic loops, a disulfide bridge between e2 and TM3, and a cytoplasmic C-terminus containing an α-helix (HX8) parallel to the cell membrane (see Figure 2.1). Despite the growing amount of structural information on Class A GPCRs, the crystal structures of chemokine receptors are not yet known due to their low natural abundance and the difficulty in producing and purifying recombinant protein [21]. Therefore, we still must rely on modeling techniques to study the molecular details of the structure and mechanism of activation of these receptors [22].

In the following sections, we describe: (i) the structural insights for homology modeling of these chemokine receptors, (ii) receptor homo- or/and hetero-oligomerization; their interactions with chemokines, synthetic low molecular weight ligands and the G protein and (iii) the process of receptor activation.

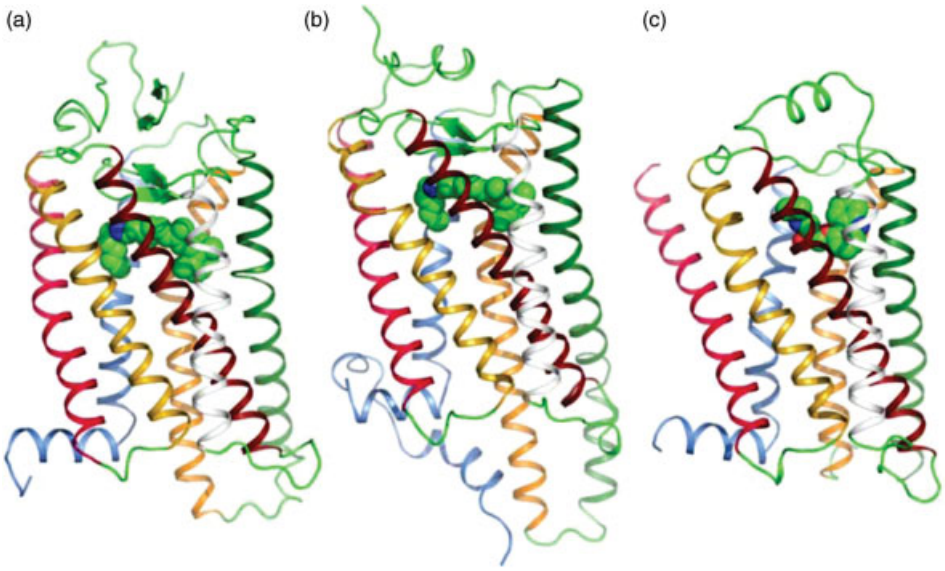

Figure 2.1 Crystal structure of (a) bovine (PDB accession number 1GZM) [12] and (b) squid (2Z73) [13] rhodopsin bound to the full inverse agonist *cis*-retinal (in spheres), and (c) the β_2-adrenergic receptor bound to the partial inverse agonist carazolol (in spheres) (2RH1) [17, 19]. Color code for these structures: the extracellular domain is in light green, the α-carbon ribbon of TMs 1 in crimson, 2 in goldenrod, 3 in dark red, 4 in gray, 5 in dark green, 6 in orange and 7 in blue; and the intracellular domain is in blue.

2.2
Chemokines

The structure of several chemokines has been solved, mainly using NMR, but also in some cases, using X-ray crystallography (see Table 2.1 in Ref. [23]). The chemokine fold (Figure 2.2) is characterized by: (i) a short nonstructured N-terminus (disordered in most NMR structures), (ii) the CC, CXC, CX_3C or C motif, (iii) a highly exposed loop of approximately ten residues, called the N-loop, (iv) a single-turn 3_{10} helix, (v) three β-strands, which form an antiparallel β-sheet and (vi) a C-terminus amphipatic α-helix [23]. Each secondary structural unit is connected by turns known as the 30s, 40s and 50s loops, which reflects the numbering of residues in the protein. The structure is stabilized by: (i) a disulfide bridge between the first Cys of the CC, CXC or CX_3C motif and a Cys on the 30s loop, and (ii) a disulfide bridge linking the second Cys and a Cys in the third β-strand near the beginning of the 50s loop. Only the last type of disulfide bond is present in the C subfamily. Mutations of these cysteines lead to disruption of the structure and impairment of the function [24]. Structural superimposition of CC, CXC, CX_3C and C chemokines shows that their fold is conserved among families.

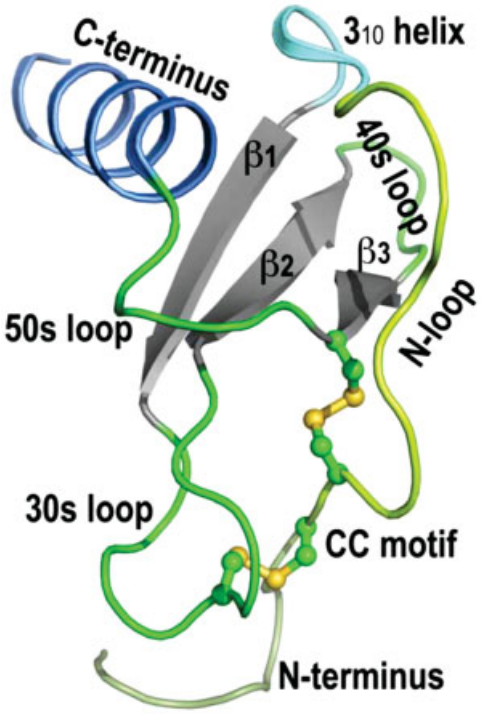

Figure 2.2 Crystal structure of CCL17 (PDB accession number 1NR4) [78]. The secondary structural units forming the conserved chemokine fold are shown.

2.3
The Transmembrane Domain of Chemokine Receptors

Statistical analysis of the residues forming the TM helices of class A GPCRs shows a large number of conserved sequence patterns [25]. Importantly, at least one amino acid is highly conserved in each TM: N in TM1 (100%), D in TM2 (94%), R in TM3 (96%), W in TM4 (96%), P in TM5 (77%), P in TM6 (100%) and P in TM7 (96%). These amino acids are easily identifiable on a multiple sequence alignment and are used as reference points to define a general numbering scheme that can be applied to all Class A GPCRs, allowing easy comparison among residues in the 7TM segments of different receptors [26]. Each residue is identified by two numbers: the first (1 through 7) corresponds to the helix in which it is located, the second indicates its position relative to the most conserved residue in the helix, arbitrarily assigned to 50. Figure 2.3 shows a multiple sequence alignment of representative members of the

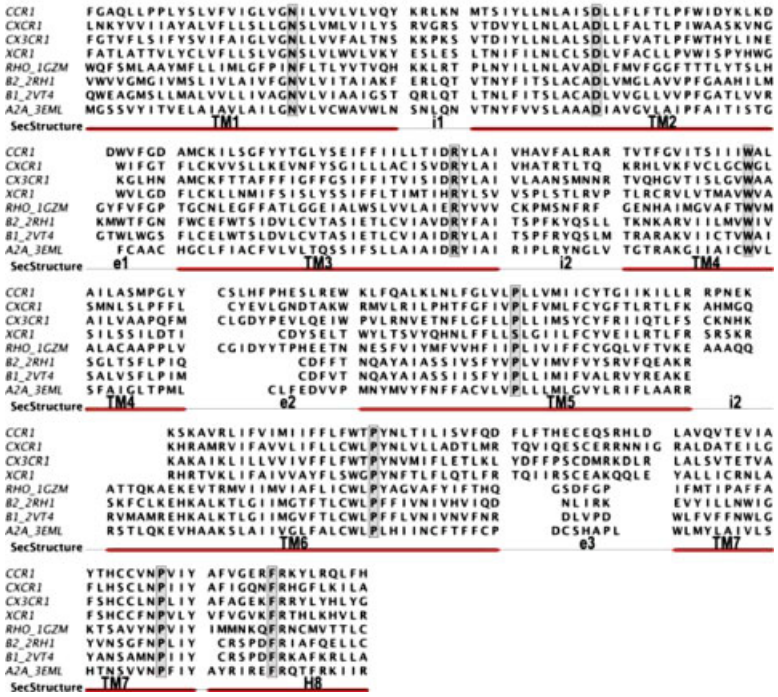

Figure 2.3 Multiple sequence alignment of representative members of the chemokine receptor family and the GPCRs with known 3-D structure. The N- and C-terminus are omitted and e2 is only shown from the disulfide-bonded cysteine to TM5. The lengths of the i2 loops of rhodopsin, β_1- and β_2-adrenergic receptors and the A2A adenosine receptor are large and they are not shown for clarity. Highly conserved amino acids in each helix used to define the general numbering scheme of Ballesteros and Weinstein [26] are boxed: N1.50 in TM1, D2.50 in TM2, R3.50 in TM3, W4.50 in TM4, P5.50 in TM5, P6.50 in TM6, P7.50 in TM7 and F8.50 in H8. The cysteines engaged in the disulfide bridge between TM3 and e2 are underlined.

chemokine receptor family and the GPCRs with known 3-D structure. Clearly, chemokine receptors contain the sequence signatures distinctive of Class A GPCRs, which suggests that the helical bundle is common to the entire family. Thus, homology-modeling techniques can be used for building 3-D models of chemokine receptors [22]. In the following sections we describe the most relevant similarities and differences between the known crystal structures and the chemokine receptors models.

2.3.1
Transmembrane Helix 1

TM1 contains the highly conserved N1.50, present in both chemokine receptors and other Class A GPCRs (Figure 2.3). The $N_{\delta 2}$-H_2 atoms of N1.50 act as hydrogen bond donors in the interactions with the carbonyl oxygens of residues at positions 1.46 and 7.46, linking TMs 1 and 7, in all known crystal structures (Figure 2.4c). In addition, there is a complex network of water molecules that mediates a number of interhelical interactions and seems conserved in Class A GPCRs (see below) [27]. Despite the similarities in the protein core, the conformation of the cytoplasmic side of TM1 varies considerably depending on whether the crystal structure of the β_1- and β_2-adrenergic receptors, rhodopsin, or adenosine A2A receptor is used (Figure 2.4a, b). Notably, the separation of TM1 from the remaining part of the bundle is the largest in adrenergic receptors, the shortest in rhodopsin, while TM1 in the adenosine receptor is located midway. Unfortunately, it is not possible to establish without ambiguity the conformation of TM1 in chemokine receptors.

2.3.2
Transmembrane Helix 2

The sequence of TM2 is highly conserved in the cytoplasmic side, particularly at positions 2.40 (N: 40%; D: 10% of the sequences), 2.42 (F: 39%; Y: 28%), 2.45 (N: 51%; S: 29%), 2.46 (L: 91%), 2.47 (A: 74%), 2.49 (A: 58%), 2.50 (D: 94%), 2.51 (L: 60%), and 2.52 (L: 60%) [25]. This conservation pattern suggests a common structural and functional role of the intracellular domain of TM2 in class A GPCRs. In contrast, the sequence is strongly divergent at the extracellular side of the helix (Figure 2.3). Rhodopsin contains two nonconserved successive Gly residues at positions 2.56 and 2.57 while the β_1- and β_2-adrenergic receptors contain Pro at position 2.59, both inducing a significant bend towards TM1 (Figure 2.4d). In contrast, Pro at the same 2.59 position in the adenosine A2A receptor induces a bend towards TM3 (Figure 2.4b). Importantly, angiotensin, opioid, and chemokine families of GPCRs possess in this region of TM2 a conserved T2.56xP2.58 motif (TxP, x being a non-conserved residue), which play a key role in receptor activation [28]. Computational simulations of the TxP motif suggest that the extracellular side of TM2 would lean towards TM3 (Figure 2.4d) [28].

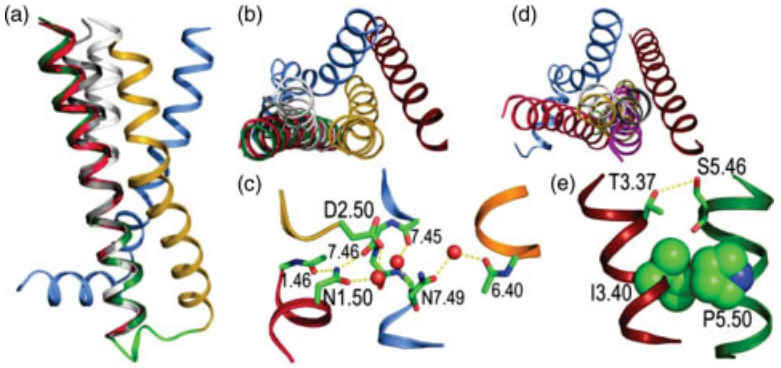

Figure 2.4 Crystal structure of the β_2-adrenergic receptor (PDB accession number 2RH1) [17, 19]. α-carbon ribbons of TMs 1 (crimson), 2 (golden red), 3 (dark red), 5 (dark green), 6 (orange), and 7 (blue) are shown. (a, b) The cytoplasmic side of TM1 of the β_1-adrenergic receptor (green; 2VT4) [16], bovine rhodopsin (white; 1GZM) [12] and A2A adenosine receptor (gray; 3EML) [20] were superimposed onto the β_2-adrenergic receptor. The separation of TM1 from the remaining part of the bundle is the largest in adrenergic receptors, the shortest in rhodopsin, while TM1 in the adenosine receptor is located midway. Panels (a) and (b) are rotated 90°. (c) Network of conserved side chains and water molecules that mediates a number of interhelical interactions, which seems conserved in Class A GPCRs (see below) [27]. (d) The cytoplasmic side of TM2 of bovine rhodopsin (white; 1GZM) [12], A2A adenosine receptor (gray; 3EML) [20] and a standard Pro-kink helix (orange) [79] were superimposed onto the β_2-adrenergic receptor. TM2 of rhodopsin has a significant bend towards TM1, while in the adenosine A2A receptor the bend is towards TM3. Computational simulations of the TxP motif of chemokines receptors suggest the extracellular side of TM2 would lean towards TM3 [28, 29]. (e) Detailed view of the interface between TMs 3 (dark red) and 5 (dark green) in the β_2-adrenergic receptor. The conformation of TM5 is stabilized by the interaction between I3.40 and the side chain of P5.50 and the carbonyl oxygen at position 5.46.

2.3.3
Transmembrane Helix 3

TM3 is also structurally conserved at the cytoplasmic end of the helix. In this region, TM3 contains the highly conserved (D/E)R(Y/W) motif, and bulky hydrophobic residues at positions 3.43 (L: 74%; I: 10%; V: 6%) and 3.46 (I: 58%; L: 15%; M: 15%; V: 8%). Thus, as for TM2, the cytoplasmic side is conserved, while the extracellular side might present family-dependent conformations. For instance, we have suggested that in chemokine receptors the bending of TM2 towards TM3, in its outer half, due to the TxP motif, is tolerated in the context of the CCR5 helical bundle as the result of the relocation of TM3 towards TM5 [29].

2.3.4
Transmembrane Helix 4

TM4 contains the highly conserved W4.50. It has been proposed that the aromatic ring of W4.50 and the hydrophobic amino acid at position 4.46 (I: 31%;

L: 14%; V: 9%) form the colesterol binding site in many Class A GPCRs [30]. No significant structural changes are observed in this helix among the known crystal structures.

2.3.5 Transmembrane Helix 5

Class A GPCRs possess a highly conserved P5.50 in TM5, present in 77% of the rhodopsin-like sequences, absent only in melanocortin, glycoprotein hormone, lysosphingolipid and cannabinoid receptors. Usually, in Pro-containing α-helices, the steric clash between the pyrrolidine ring of Pro and the carbonyl oxygen of the residue in the preceding turn induces a bend angle of approximately 20° in the helical structure [31]. Importantly, the known crystal structures have revealed a peculiar conformation of TM5 in which the Pro-kink-induced bend is decreased due to a local opening of the helix (Figure 2.4e). This unusual conformation of TM5 seems stabilized by the van der Waals interaction between the hydrophobic side chain at position 3.40 (L: 9%; V: 25%; I: 42%) and the side chain of P5.50 and the carbonyl oxygen at position 5.46 [32].

2.3.6 Transmembrane Helix 6

In all crystal structures, TM6 features a bend angle of 35°, which is much higher than the average 20° bend angle of Pro-kinked α-helices. This extreme conformation of TM6 is energetically stabilized through two structural and functional elements. First, a conserved discrete water molecule in the vicinity of P6.50. Second the ionic interaction between D/E6.30 and R3.50 of the (D/E)R(Y/W) motif in TM3 [33]. The acidic residue at position 6.30 is only conserved in 32% of the sequences (D: 7%; E: 25%). Chemokine receptors, as well as about 34% of Class A GPCRs, contain a basic residue at position 6.30. As the presence of a positively charged residue at that position is incompatible with an interaction with the R3.50 of TM3, the network of TM3–TM6 interhelical interactions in these receptors is likely different from that determined crystal structures [34].

2.3.7 Transmembrane Helix 7

The cytoplasmic end of TM7 contains the highly conserved residues N7.49 (N: 75%; D: 21%), P7.50 (96%), and Y7.53 (92%), forming the NPxxY motif. There are several key structural features within this motif (Figure 2.4c). First, the hydrogen bond between N1.50 and the backbone carbonyl oxygen at position 7.46. Second, a water molecule located between the backbone carbonyl at position 7.45 and the backbone N–H amide of N7.49. Third, an internal water molecule mediates an interhelical interaction between the side chain of N7.49 and the backbone carbonyl at position 6.40 to maintain the receptor in the inactive state [35].

In summary, chemokine receptors are expected to have a similar arrangement of TMs 4 and 7 than the known crystal structures. In contrast, the extracellular side of TM2 is likely to have a different structure due to the presence of the TxP motif at 2.58 [28]. This conformation of TM2 will probably influence the arrangement of the extracellular sides of TMs 1 and 3 [29], resulting in a modification of the binding site for chemokines. Thus, TMs 1–3 constitute an example of structural plasticity, that is, those structural differences near the binding site crevices among different receptor subfamilies responsible for the selective recognition of ligands [32]. The presence of a positively charged residue at position 6.30 in chemokine receptors is incompatible with an "ionic lock" [33] interaction with R3.50 of TM3, which suggests a different network of TM3–TM6 interhelical interactions in the inactive state of these receptors [34].

2.4
Structural and Functional Role of Internal Water Molecules

The recent crystal structures of GPCRs show the presence of water molecules in the vicinity of highly conserved residues (Figure 2.5). These water molecules mediate a number of interhelical interactions that keep the receptor in the inactive conformation and, most likely, are present in all members of the family [27]. The most important interactions are: (i) a conserved hydrogen bond network linking D2.50 and W6.48 between TMs 2 and 6, (ii) a water-mediated Pro-kink in TM6 and (iii) the water

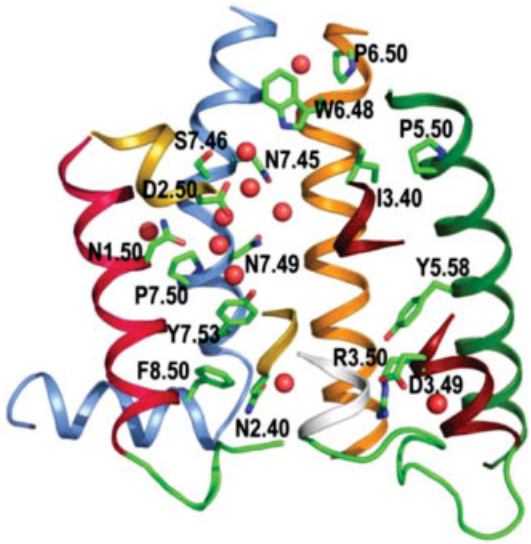

Figure 2.5 Structural and functional water molecules embedded in the TM bundle of the β_2-adrenergic receptor (PDB accession number 2RH1) [17, 19]. These water molecules are located in the vicinity of highly conserved residues [27]; thus, they are expected to be also present in chemokine receptors.

molecules in the environment of the NPxxY motif in TM7 [27]. Due to the high conservation of the residues involved in these interactions, these water-mediated networks of interactions are expected to be also present in chemokine receptors.

2.5
The Structure of the Extracellular Domain of Chemokine Receptors

The structural homology of the TM domains, among GPCRs, does not extent to the extracellular domain because of the high variability in length and amino acid composition. For instance, the extracellular domain of rhodopsin, formed by four β-strands, is highly structured, blocking the access of the extracellular ligand to the core of the receptor (Figure 2.1a). In contrast the β_1- and β_2-adrenergic receptors present a relatively exposed entrance to the core of the TM bundle, lined with an extra helical segment that facilitates the entry of diffusible ligands to the binding site (Figure 2.1c). These differences at the sequence and structure levels support the idea of divergence in this region among GPCRs. The fact that GPCRs interact with an extraordinary diversity of ligands suggests that each receptor subfamily has developed specific extracellular domains to adjust the structural characteristics of its cognate ligands. Thus, the structure of the extracellular domain of chemokine receptors remains unknown. Significant differences of chemokine receptors, as compared to other GPCRs, are the presence of an additional disulfide bridge, linking the N-terminus to e3, whose disruption leads to: (i) strong functional impairment [36], (ii) sulfation of tyrosines [37, 38] and (iii) glycosylation of asparagines [39]. Thus, the structure of the extracellular domain of chemokine receptors cannot be modeled from the known 3-D structures and remains unknown.

2.6
The Structure of the Intracellular Domain

Crystal structures of GPCRs have shown that Class A receptors contain an amphipathic Hx8 that runs parallel to the membrane. The crystal structure of squid rhodopsin has shown an additional cytoplasmic Hx9 that interacts with TM6 (Figure 2.1b). The presence in chemokine receptors of the Phe side chain at position 8.50, conserved in 68% of Class A GPCR sequences, suggests a similar Hx8 as found in the crystal structures. Finally, it has been shown that a cyteine residue at the carboxyl-terminal domain of CCR5 is palmitoylated [40].

2.7
The Binding of Chemokines to Chemokine Receptors

The structural elements involved in the binding of chemokine ligands to chemokine receptors have been mainly obtained from mutational analyses [23]. The N-loop

domain of chemokines (Figure 2.1) is the principal partner in the receptor-binding site and confers receptor specificity [41]. NMR studies using high concentrations of peptide fragments corresponding to the N-terminal sequence of the CXCR1 receptor suggest this domain as the binding partner for the N-loop of CXCL8 [42]. Mutagenesis of CCL2 identified that, in addition to the N-loop, highly conserved basic residues in 30s loop are mandatory for high affinity binding to CCR2 [43]. It has also been shown that the extracellular domain of chemokine receptors is central to ligand binding, as mutation of both the N-terminus and the extracellular loops (particularly e2) affect strongly chemokine binding [44, 45]. The sulfated tyrosines at the N-terminal region of most chemokine receptors, also contributes to the chemokine binding site [37, 38]. Mutagenesis studies have also shown that the N-terminal part of the chemokine is not important for receptor binding, but receptor activation. For instance, truncation of the two N-terminal residues of CCL5 and CCL11 changes their pharmacological profile from agonist to potent antagonist of the CCR3 receptor [46, 47]. The ELR motif preceding the first cysteine seems critical for receptor activation [48]. In summary the overall picture is that the core domain of chemokines interact with the extracellular N-terminus and, mainly, the e2 loop of the receptor for binding affinity and specificity; while their N-terminal domain interacts with the TM bundle for receptor activation (see Figure 2.5 in Ref. [7], Figure 2.7 in Ref. [49]).

2.8
The Binding of Small-Molecule Ligands to Chemokine Receptors

In the last years, many nonpeptide ligands have been reported for chemokine receptors [7, 50–53]. Most of these low molecular weight ligands contain a positively charged amine that interacts with the highly conserved E7.39 in TM7 [54]. Site-directed mutagenesis has permitted to map the additional contact regions between the ligand and the receptor [55–57]. This experimental data together with homology models of chemokine receptors are generally used to develop detailed 3-D models of the ligand–receptor complex, which provide an essential tool for rational drug design or virtual screening of chemical databases [58, 59].

2.9
Molecular Processes of Receptor Activation

The mechanism by which binding of the extracellular ligand triggers a set of conformational rearrangements of the TM segments near the G protein-binding domains remains largely unknown [60–62]. It has been proposed that the rotamer toggle switch of tryptophan at position 6.48 (W6.48) from pointing towards TM7 in the inactive state to pointing towards TM5 in the active state [63, 64] is the initial stage of the activation process, as observed in the electron microscopy density map of metarhodopsin I [65] and in solid-state NMR measurements of metarhodopsin II [66].

This conformational switch triggers the transition of N7.49 (the NPxxY motif of TM7) towards D2.50 [35]. In addition, comparison of the structure of inactive rhodopsin [12] with the recent crystal structure of the ligand-free opsin [14], which contains several distinctive features of the presumed active state, leads to the conclusion that during the process of GPCR activation the intracellular part of TM6 tilts outwards by 6–7 Å, TM5 nears TM6 and R3.50 within the (D/E)R(Y/W) motif in TM3 adopts an extended conformation pointing towards the protein core, to interact with the highly conserved Y5.58 and Y7.53 in TMs 5 and 7 [14] (Figure 2.6). As shown in the original publication of the opsin structure, these conformational changes disrupt the ionic interaction between R3.50 with negatively charged side chains at positions 3.49 and 6.30 and facilitates the interaction between K5.66 in TM5 and E6.30 in TM6. Chemokine receptors possess a positive charge at position 6.30, which precludes the interaction with R3.50, and contains a hydrophobic side chain at position 5.66. Thus, the molecular interactions locking the inactive and active conformation of TM6 in chemokine receptors are expected to be different from the opsin family of GPCRs [34]. Notably, chemokine receptors possess a short i2 loop and shorter TM6, in contrast to other D/E6.30-containing GPCRs (Figure 2.3). Thus, in chemokine receptors, the relative orientation of TMs 5 and 6 in the inactive and active state is not governed by electrostatic interactions but probably by the spatial rigidity imposed by the short loop of five residues between TMs 5 and 6.

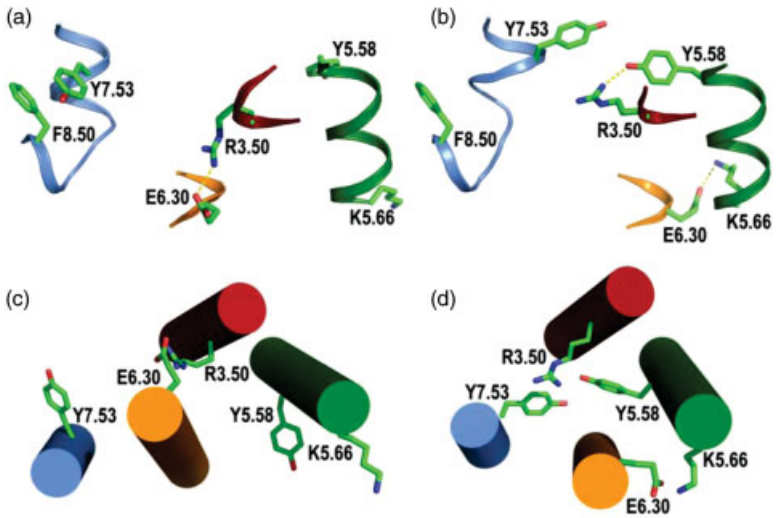

Figure 2.6 Comparison of (a, c) the crystal structure of inactive rhodopsin (1GZM) [12] with (b, d) the crystal structure of the ligand-free opsin (3CAP) [14], which contains several distinctive features of the presumed active state, in views parallel (a, b) and perpendicular (c, d) to the membrane. α-Carbon ribbons of TMs 3 (dark red), 5 (dark green), 6 (orange) and 7 (blue) are shown.

2.10
The Binding of the G Protein

The structure of the ligand-free opsin bound to a synthetic peptide derived from the C-terminus of the α-subunit of transducin has recently been obtained [15]. This structure has shown that the α5 helix of $G_{\alpha t}$ binds to a site in opsin that is opened by the movement of the cytoplasmic end of TM6 away from TM3 and towards TM5 (see above). The C-terminal domain of the G protein interacts with the extended conformation of R3.50, the short loop connecting TM7 and Hx8 and the inner side of the cytoplasmic TMs 5 and 6 (Figure 2.7). Notably, both the G protein family (positions i-2 and i-7 relative to the final amino acid) and TMs 5 (positions 5.61 and 5.65) and 6 (position 6.33) of class A GPCRs contain highly conserved hydrophobic amino acids that form key hydrophobic contacts between the receptor and the G protein. Notably, chemokine receptors also possess hydrophobic amino acids at these 5.61 (I: 75%; L: 10%; V: 5%), 5.65 (L: 90%; I: 5%) and 6.33 (A: 75%; L: 5%) positions. It, thus, seems reasonable to assume that the mode of recognition of the G protein by the chemokine receptor family resembles this structure found for opsin [15].

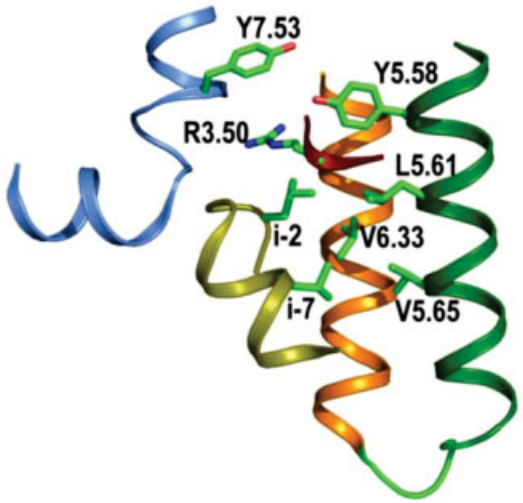

Figure 2.7 Crystal structure of the ligand-free opsin in its G protein-interacting conformation (3DQB) [15]. α-Carbon ribbons of TMs 3 (dark red), 5 (dark green), 6 (orange) and 7 (blue), Hx8 (blue) and the α5 helix of $G_{\alpha t}$ (olive) are shown. The C-terminal domain of the G protein interacts with R3.50, the short loop connecting TM7 and Hx8 and the inner side of the cytoplasmic TMs 5 and 6.

2.11
Receptor Oligomerization

GPCRs have been classically described as monomeric TM receptors that form the ligand–receptor–G protein ternary complex. This is compatible with recent observations that monomeric rhodopsin and the β_2-adrenergic receptor are capable of activating G proteins [67–69]. However, accumulating evidence indicates that many GPCRs can form dimers or higher order oligomers [70]. Several chemokine receptors have been reported to associate with another chemokine receptor, either through homo- or heterodimerization (see Table 2.1 in Ref. [71]). The distribution of the protomers in either a dimer or higher order organization is not fully understood. Disulfide crosslinking has been to map the interface between protomers [72–75]. These results are compatible with arrangements of oligomers involving TM1, TM4, or/and TM5 interfaces. However, the role of oligomerization in agonist-induced activation, inverse agonist-induced inhibition, G protein coupling and signaling, internalization and desensitization remains unknown [71].

2.12
Conclusions

Class A GPCRs are characterized by a number of highly conserved charged and polar residues located within the TM region. Mutagenesis studies indicate that most if not all of these amino acids are involved in maintaining the 3-D structure of the receptor and in the processes of receptor activation. With the exception of the TxP motif in TM2 and the charged amino acids in TMs 5 and 6 that lock the receptor in the inactive and active conformation, chemokine receptors possess in the TM domain all these key residues common to most of the GPCR members. We, thus, hypothesize an overall conserved TM architecture among the GPCR family, with particular differences in the extracellular side of TMs 1–3 related to the process of chemokine binding. The process of receptor activation and the subsequent binding of the G protein are also expected to be similar to other Class A GPCRs. The next challenge is the acquisition of direct structural data for this important family of GPCRs that will allow deciphering how chemokines activate the conserved routes of activation [76]. This knowledge can be applied to the design of new drugs with improved pharmacological properties [77].

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3
Signaling Events Involved in Chemokine-Directed T Lymphocyte Migration

Stephen G. Ward

3.1
The Role of GTPases in Chemokine-Directed Lymphocyte Migration

Cell polarization, whereby the molecular processes at the front (leading edge) and the back (uropod) of a moving cell are different, is a prerequisite for efficient migration. Hence, the uropod has the microtubule organizing center at its base and is rich in adhesion molecules [1]. It is also the site of mitochondrial redistribution during migration that ensures high ATP at this strategic position [2]. In other systems, the small GTPases Rho, Rac and cdc42, have key roles in regulating cell polarity and morphology of migrating cells through effects on the actin cytoskeleton and actomyosin contraction, usually involving crosstalk with other signaling elements such as PI3K [3–5]. These GTPases have also been implicated in actin reorganization and polarization in T cells that occurs during migratory response to chemoattractants [6]. Another GTPase Rap1, which is activated by chemokines in B cells, has also been implicated in polarization, integrin activation and motility of lymphoid cells [7–9].

Polarization and migration of T lymphocytes requires rapid Rac-driven new formation of F-actin at the leading edge [3, 10, 11]. In naive T cells, an early wave of chemokine-induced F-actin formation (<5 s) is initiated by the Rac GEF DOCK2 [6]. T lymphocytes that are DOCK2-deficient, have less chemokine-induced F-actin formation, cell polarity and *in vitro* migration [6]. This is a cell-specific defect as similar disruption of F-actin formation is not observed in DOCK2$^{-/-}$ monocytes [6]. DOCK-2 has also been implicated as being required for chemokine-promoted human T lymphocyte adhesion under shear stress mediated by $\alpha 4\beta 1$ integrin [12]. The Rac GEF Tiam1 also acts during chemokine-induced T cell migration and associates with members of the Par polarity complex that include Par3 and PKC-ζ. This complex segregates to the leading edge in polarized cells and helps to establish or stabilize the anterior-posterior axis after an initial F-actin upregulation by DOCK-2 [8, 13]. However, Tiam1 deficient mice have normal lymphoid structure and cellularity, in contrast to DOCK2- or Gαi2-deficient mice, indicating that this pathway is partially redundant with other promigratory signaling

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

modules including other Rac GEFs. In this regard, Vav is known to be involved in integrin activation and adherence of T cells [12, 14, 15]. Indeed, overexpression of Vav mutants abolished lymphocyte polarization, actin polymerization and migration in response to CXCL12, yet curiously Vav-1 deficient cells exhibit normal migration [16], possibly reflecting compensation by other Vav isoforms. Moreover, evidence also suggests that Vav localization is influenced by interactions with Tec family kinases that can in turn, be activated by CXCL12 in T cells [17, 18]. Consequently, CXCL12-induced migration, cell polarization and activation of Rac and cdc42 is impaired upon over-expression of a loss of function Itk mutant, while T cells purified from $Rlk^{-/-}Itk^{-/-}$ mice exhibited impaired migration to multiple chemokines [17, 18].

There is strong pharmacological and genetic evidence that Rho-dependent signaling is a key component of T cell migration and adhesion in response to several chemokines in mature T cells and thymocytes [19–21]. RhoA activation also appears to be necessary for integrin activation induced by Rap1 and Rac in thymocytes [21]. RhoA appears to control both LFA-1 high affinity state triggering by chemokines as well as the lateral mobility induced by chemokines [22]. In addition, formins are downstream RhoA effectors, which are actin-assembly factors. In contrast to Arp2/3, which creates branched actin networks, formins generate elongated actin filaments [23]. Lack of the lymphocyte expressed formin isoform mDia1 leads to impaired *in vitro* migration in T cells (but not in B cells), and is consistent with less chemokine-induced F-actin formation in $mDia1^{-/-}$ T cells [24, 25].

3.2
Class 1 PI3Ks and their Role in Cell Migration: An Overview

The class 1 phosphoinositide 3-kinases (PI3Ks) are composed of a regulatory subunit and a tightly associated catalytic subunit. The class 1A enzymes are represented by five regulatory subunits encoded by three genes: *PIK3r1* encodes p85α and its alternative transcripts p55α and p50α. *PIK3r2* encodes p85β and *PIK3r3* encodes p55γ. The three class 1 catalytic isoforms p110α, p110β and p110δ pair with one of these regulatory subunits which are responsible for recruitment of the complex to the plasma membrane upon receptor ligation. Class 1A isoforms are activated downstream of immune cell receptors including the TCR, BCR, costimulatory molecules and cytokine receptors that are phosphorylated by tyrosine kinases upon cognate stimulus [26–29]. A single class 1B catalytic isoform p110γ can pair with one of two regulatory subunits p84/p87 or p101 [30, 31]. This isoform is activated by G protein βγ subunits and signals downsteam of G protein-coupled receptors (GPCRs), although some GPCRs including chemokine receptors activate class IA PI3Ks, most notably p110β [32–34]. Recent evidence using cells derived from mice with conditional genetic inactivation of p110β, has revealed that p110β and p110γ can couple redundantly to the same GPCR agonists including some chemokines [33].

The major products of class I phosphoinositide 3-kinases (PI3Ks) are 3′-phosphoinositides, most notably phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] which is now recognized as a key signaling molecule. The effects of PI(3,4,5)P_3 are

counteracted by the lipid phosphatases PTEN and SHIP, which convert this lipid to PI(4,5)P_2 and PI(3,4)P_2 respectively [35]. PtdIns(3,4,5)P_3 has important biological functions that rely on interaction with effector proteins containing lipid binding domains such as pleckstrin homology domains [36, 37]. Several guanine nucleotide exchange factors, particular those with specificity for Rac, are regulated by 3′-phosphoinositides, while PI3K activity can itself be modulated by Rho GTPases [38–40]. Moreover, another well characterized downstream PI3K effector, the Ser/Thr kinase Akt, has been implicated in F-actin polymerization and myosin assembly [41–44]. Accordingly, PI3K contribute to several aspects of the migratory machinary including gradient sensing, signal amplification, actin reorganization and hence cell motility [7, 45–47]. Around 2002, several studies in neutrophils and *Dictyostelium* produced a body of evidence which indicated that PtdIns(3,4,5)P_3-dependent signals were a part of a compass mechanism, sensing and responding to extracellular gradients of chemoattractants [38, 48–50]. Several studies demonstrated that PI3K inhibitors or genetic loss of PI3Ks cause reduction in chemotactic responses of neutrophils and amoebae in a variety of *in vitro* and *in vivo* migration assays [48, 49, 51–53]. Furthermore, use of biosensors composed of fluorescent proteins fused to PH domains, which are capable of binding selectively to different phosphoinositides, revealed that PtdIns(3,4,5)P_3 becomes highly polarized to the leading edge in amoebae and neutrophil-like cell lines [38, 48–50]. Recent findings have led to a re-evaluation of the model that places PI3Ks centrally in an evolutionary conserved cell navigational mechanism. First, some experiments examining the effects of either genetic loss of PI3Ks or selective PI3K inhibitors on the chemotactic efficiency of both neutrophils and *Dictyostelium* amoebae revealed no specific deficiencies [54, 55]. Second, the PI(3,4,5)P_3 polarization to the leading edge of migrating cells was initially thought to be facilitated by the exclusion of PTEN from the leading edge and localization to the trailing edge of the migrating cell [48, 49, 56]. However, it seems that SHIP rather than PTEN provides a critical role in the polarization and motility of neutrophils [57, 58]. Finally, genetic loss of PI3Kγ or selective PI3K inhibitors caused reductions in chemokinetic cell responses (rather than ability to move toward a gradient) that could explain some of the previously reported apparent reductions in chemotactic migration [53].

3.3
Do PI3K-Dependent Signals Contribute to T Lymphocyte Migration in Response to Chemokines?

Activation of PI3K is a robust signaling event shared by most homeostatic and inflammatory chemokine receptors expressed on T lymphocytes [34, 59]. Accordingly, there has been intense interest in exploring the role of PI3K-dependent signaling in migratory responses of T cells after chemokine stimulation. Chemokine interaction with GPCRs on lymphocytes in response to either homeostatic or inflammatory chemokines has been shown to depend predominantly on Gαi proteins [60]. This led to the assumption that these chemokines receptors are

coupled to the βγ-dependent p110γ isoform. This is indeed the case, although several chemokine receptors can activate other PI3K isoforms [34, 59, 61, 62]. Early studies revealed that chemokine-stimulated migration of leukemic T-cell lines and primary T cells in *in vitro* assays of migration, is abrogated by pan-isoform PI3K inhibitors [34, 59]. Genetic and pharmacological approaches have been employed to assess the contribution of individual class I PI3K isoforms to migratory response of T lymphocytes to several chemokines. Hence, the *in vitro* migration of p110γ-deficient $CD4^+$ and $CD8^+$ T cells to CCL19, CCL21 and CXCL12 is significantly decreased compared to cells from wildtype mice [63]. Moreover, migration of freshly isolated human peripheral blood T cells is also inhibited by p110γ-targeting inhibitors, but not inhibitors directed toward the α, β or δ isoforms [62].

Surprisingly, PI3K inhibitors have little effect on T cell migration in assays that better reflect physiological conditions. Hence, T-lymphocyte arrest and adhesion to high endothelial venules in exteriorized Peyer's patches [64] or on transendothelial migration in laminar flow chambers [65] in response to either CXCR4 and/or CCR7 ligation is unaffected by PI3K inhibitors. Other lines of evidence also cast further doubt as to whether the model for PI3K/PTEN polarization in neutrophils can be applied to T lymphocytes. For example, many studies have been performed in the Jurkat leukemic T-cell line. These cells polarize and migrate normally in response to several chemokines acting on pertussis toxin-sensitive Gαi-coupled receptors despite the fact that they are deficient in both PTEN and SHIP protein expression [66, 67]. In fact, reconstitution of PTEN expression in Jurkat cells down-regulated CXCL12-stimulated cell migration suggesting a negative regulatory role for PTEN in T cells migration [68]. Introduction of a constitutively active SHIP mutant into leukemic cell lines normally deficient in SHIP, abrogates CXCL12-mediated migration [69]. This was somewhat surprising given the reported role of SHIP in neutrophil polarization [57]. However, this effect probably reflects that this construct is expressed widely throughout the plasma membrane and disrupts polarized accumulation of $PI(3,4,5)P_3$ at the leading edge. Finally, it appears that the activation status of the cell helps determine whether PI3K is required for migratory responses to chemoattractants. In this regard, *in vitro* assays have revealed migration of freshly isolated human T cells is dependent on PI3K, but after *ex vivo* maintenance and activation/differentiation the migratory response becomes PI3K-independent [62, 70].

3.4
Role of PI3K in T Lymphocyte Homing and Migration *In Vivo*

The use of genetically targeted mice in conjunction with *in vivo* models of homing of T cells to peripheral lymphoid nodes or effector T cells to sites of inflammation/antigen challenge has helped refine our understanding of the role of PI3K in T cell migration. Analysis of mice lacking DOCK2 and p110γ alone, or in combination, revealed that while DOCK-2 is the predominant molecule required for

T cell migration, p110γ can sustain a modest residual migratory response. Hence, optimum T lymphocyte migration *in vivo* is dependent on expression of both DOCK2 and p110γ [71]. Importantly, a more recent study reported no defect in the migration of $p110\gamma^{-/-}$ cells to lymph nodes *in vivo* [72]. The reason for this discrepancy with earlier studies may reflect differences in the T cell populations analyzed (e.g., bulk vs $CD8^+$ T cells). Although $p110\gamma^{-/-}$ T cells exhibit modest defects in migration *in vitro* and *in vivo*, it is notable that pan-isoform PI3K inhibitors such as wortmannin or Ly294002, effectively block *in vitro* and *in vivo* migration of naïve T cells [62, 63]. This may simply reflect off-target effects of these compounds or the involvement of other PI3K isoforms in cell migration. Certainly, recent evidence identified that p110δ is required for antigen-driven T cell localization [73] and is the dominant PI3K isoform in B cell homing [63]. Interestingly, analysis of neutrophil migration *in vivo* revealed that, in fact, while p110γ is important in early chemokine-induced emigration, p110δ replaces and maintains the delayed chemokine-induced neutrophil recruitment into inflamed tissues [74, 75]. Whether these isoforms fulfill a similar role during T cell migration *in vivo* remains to be established.

3.5
Role of PI3K in Interstitial T Lymphocyte Motility

T and B cells move vigorously within their specific microenvironments within secondary lymphoid organs following apparently random migration pathways [76–78]. This interstitial lymphocyte migration is integrin-independent, being mediated by actin flow along the confining extracellular matrix scaffold structure, shape change and squeezing [10, 79] and probably serves to increase dendritic cell screening efficiency, which may accelerate immune response initiation. Basal motility of T cells requires CCL19 and CCL21 (CCR7 agonists) that are abundant throughout the T-cell zone, together with adhesion ligands present on stromal cells [80–82]. Multiphoton and conventional epifluorescence microscopy studies have explored whether PI3K is involved in regulating basal interstitial T lymphocyte migration/motility within intact lymphoid tissue *in vivo*. Despite evidence of p110γ contributing to T cell homing to lymphoid tissue and migration [71], there was no effect on the dynamic movements of p110γ-deficient T cells or the pan-PI3K isoform inhibitor wortmannin, compared to wild-type controls inside the T cell area [80–82]. Interestingly, another group using multiphoton microscopy in conjunction with wortmannin revealed a modest reduction of T and B cell velocities compared to untreated controls. Complimentary gene-targeting strategies in which class 1A function had been ablated by deletion of the *pik3r1* (p855α, p55α, p50α null) and *pik3r2* (p85β null) gene products showed a significant decrease in velocity and a marked loss of cell polarization. However, these experiments do not distinguish whether reduced motility results from impaired class IA PI3K signaling function or from loss of adapter functions of the regulatory subunits independently of their role in activating the catalytic subunits. The reduced motility in wortmannin-treated cells supports at least some role for PI3K enzymatic subunits, but could

also be due to inhibition of other PI3K subclasses or non-PI3K targets of wortmannin [59, 62, 83].

3.6 Role of Phospholipase C and Protein Kinase C Signaling in Chemokine-Directed T Lymphocyte Migration

Activation of phospholipase C (PLC), calcium mobilization and activation of diacylglycerol (DAG)-dependent protein kinase C (PKC) isoforms are robustly induced biochemical signals associated with chemokine receptor activation and have hence, been proposed as regulators of cell adhesion and migration [84, 85]. Curiously, studies with mice deficient in PLCβ2 and β-3 appeared to suggest that the PLC pathway is not required for migratory responses in neutrophils [86]. Subsequent analysis of T lymphocyte migration in the presence of pharmacological or genetic disruption of PLCβ function revealed a key role for PLC isoforms in T cell migratory responses *in vitro* [70, 87], once again highlighting heterogeneity of signaling molecules involved in migration responses of different leukocytes. The exact role of PLC and its substrate $PI(4,5)P_2$ in cell migration is unclear, but both have been demonstrated to bind components of the actin cytoskeleton [42]. A distinct family of Ras exchange factors is regulated not only by calcium but also by membrane diacylglycerol that is generated along with $Ins(1,4,5)P_3$ during activation of phospholipase C. These are termed calcium and diacylglycerol-regulated guanine–nucleotide exchange factors (CalDAG-GEFs) [88]. One of these (CalDAG-GEF1) functions as an exchange factor for Rap. Interestingly, PLCβ-dependent T cell migration responses to chemokines was dependent on increased intracellular calcium [87], although other studies have previously reported calcium signaling does not play a critical role during lymphocyte migration [2, 59, 89]. Moreover, some studies have reported that T cell migration *in vitro* is resistant to broad-spectrum PKC inhibitors [87]. This is discrepant with evidence for involvement of conventional/novel PKC isoforms (that are dependent on calcium and/or diaclyglycerol) in migratory responses. Specifically, PKCβI and PKCδ each associate with distinct areas within the microtubules in the uropod during LFA-1-mediated locomotion of activated T cells [90]. Furthermore, in T cell models where migration to CCR4 stimulation can occur independently of PI3K, use of pharmacological tools has indicated that PKCδ is required for chemotactic responses to CCR4 ligands [19, 91]. This does not fit well the notion that PKCδ is a substrate for the 3′-phosphonositide-dependent master kinase PDK-1 [92], but it is worth noting that PKCδ is often a functional enzyme in the absence of phosphorylation in the activation loop [92]. The mechanism by which PKC isoforms regulate cell motility/migration in T lymphocytes is unclear, although it is likely to be via effects on actin reorganization/polymerization as well as changes in integrin affinity [22, 93]. Several PKC isoform knockout mice exist including those for the β, δ and ζ isoforms, but no major defect in T cell migration in these mice has so far not been reported, possibly due to redundancy in function between individual isoforms [84, 85].

3.7
Concluding Remarks and Future Directions

The diverse milieu of chemokines, adhesion ligands and stromal cell architecture in different regions within lymphoid organs and peripheral tissues as well as varying state of activation of individual cells determine the expression of antigen, costimulatory and chemoattractant receptors that likely shape the degree of PI3K involvement in T lymphocyte migration and motility and the choice of isoform (Figure 3.1). Non-agonist chemokines are capable of associating with known chemokine agonists resulting in a stronger cellular response although the molecular basis for this phenomenon has not been determined [94]. As a consequence, inflamed and other chemokine rich tissues would create an environment that renders many leukocyte types more competent to respond to migratory cues. The influence of the local environment such as partial pressure of oxygen has also been demonstrated to influence cell migration [95] while various components of the PI3K pathway (particularly the lipid phosphatase PTEN) are sensitive to local redox conditions [96]. In this regard, T cell motility has been shown to vary between the subcapsular and deep paracortical regions of the node [95]. The choice of biochemical signal elicited by individual receptors will also be further shaped according to the mode of presentation of agonist (e.g., whether it is immobilized by glycosaminoglycan binding or forms a homo- or hetero-oligomer) and/or receptor dimerization [97–99]. Certainly, chemokine receptor heterodimerisation promotes distinct signaling events from those elicited by homodimer receptors including recruitment of G proteins distinct from $G\alpha i$ [99–101].

Several therapeutic strategies have been explored to prevent leukocyte migration, including blockade of adhesion molecules, chemokine receptors and signaling events such as those mediated by p110γ [102]. The differing dependence of individual chemokine receptors on PI3K isoforms at different stages of activation, makes it difficult to design a "one fits all" drug to inhibit inflammatory recruitment of cells. Nevertheless, there is evidence that p110γ-selective inhibitors are effective in several models of chronic inflammatory disease, primarily resulting from the inhibition of neutrophil as well as $CD4^+$ T cell migration [102–104]. It is becoming clear however, that PI3K shapes T lymphocyte migration by participating in additional mechanisms that facilitate T cell movement. Hence, T cell receptor-induced p110δ activity is required for T cell localization to antigenic tissue in mice [73]. Moreover, p110δ plays an essential role in the events that lead to proteolytic shedding and reduced transcription of CD62L as well as reduced transcription of the chemokine receptor CCR7 and the S1P1 [105]. These surface proteins play an essential role in homing of $CD8^+$ cells to secondary lymphoid tissues and prevent their egress to sites of peripheral inflammation. Such roles for PI3K-dependent signaling pathways provides an additional dimension to pharmacological targeting of PI3K isoforms to control of T cell-mediated pathologies including autoimmunity and transplantation without inducing overt immune suppression.

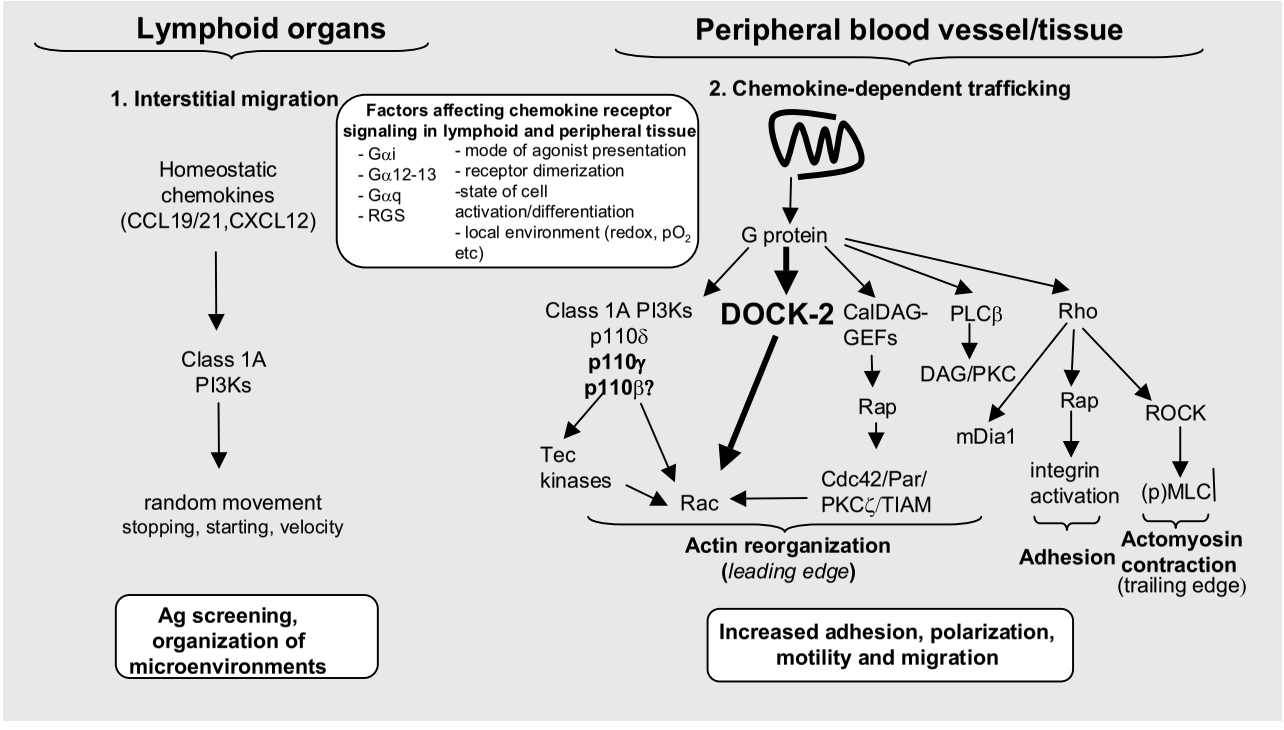

Figure 3.1 PI3K influences chemokine-stimulated T lymphocyte migration and motility. Class 1 PI3K isoforms influence T lymphocyte migration at several levels in lymphoid and peripheral tissue. Lymphoid tissue: Class 1A PI3K isoforms influence random interstitial cell migration events in lymphoid organs that underpins Ag screening and tissue architecture, although the identity of isoforms involved is unknown. Peripheral tissue: DOCK-2 is the predominant signal that leads to Rac activation and initial actin reorganization (as denoted by larger text/arrows), although there does appear to be a significant contribution provided p110γ depending on the context of cell migration. The signaling pathways linked to adhesion and formation of trailing edge are also summarized. The precise balance of signaling via p110γ (or other class 1 PI3Ks) versus DOCK-2 and other pathways leading to actin reorganization, cell polarization and adhesion will be shaped by a variety of factors, including the mode of agonist presentation (e.g. soluble vs glycosaminoglycan-bound, formation of agonist oligomers), possible receptor dimerization and environmental factors. These will likely influence the type of Gα protein subunits that become coupled to the activated receptor. The expression profile of regulator of G protein signaling (RGS) proteins (a family of GTPase activating proteins with distinct G protein selectivity [106]) varies according to the state of cell activation/differentiation and thus shapes the signaling via individual Gα subunits.

Acknowledgment

S.G.W. is the recipient of a Royal Society Industrial Fellowship.

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4
The Atypical Chemokine Receptors

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4.1
D6, an Atypical Receptor for Pro-Inflammatory CC Chemokines

Chemokines control migration by transmitting signals into cells through G protein-coupled chemokine receptors (CCR1–CCR10, CXCR1–CXCR6, XCR1, CX3CR1) [1]. However, there exists a group of proteins which bear structural similarity to these signaling chemokine receptors and bind with high affinity to a variety of different chemokines, but which appear unable to mediate leukocyte migration. Instead, these molecules (e.g., D6, CCX-CKR, CXCR7, DARC) are thought to control chemokine abundance and/or distribution to facilitate or regulate chemokine-driven leukocyte migration [2–4]. In this chapter, we explore the biochemistry and biology of these "atypical" chemokine receptors.

While searching for novel chemokine receptors for the inflammatory CC chemokine CCL3 we identified a cDNA encoding a previously unidentified putative chemokine receptor which we named D6 [5, 6]. Another group was also characterizing this molecule at this time [7]. When expressed in heterologous cell lines, D6 was able to bind with high affinity to many pro-inflammatory CC chemokines, but not to homeostatic CC chemokines or chemokines from the other subfamilies [5–7]. Recent work has extended its ligand binding profile, and it is now known that D6 interacts with at least 11 pro-inflammatory CC chemokines [5–8] (Table 4.1). Such promiscuity is not unique, and many of the inflammatory chemokine receptors show multiligand binding capability [1]. A proline residue at position 2 of the inflammatory CC chemokines appears to aid high affinity binding to D6 as evidenced by our work with proteins encoded by two nonallelic variants of human CCL3 [9]. These proteins are nearly identical, but one has a proline at position 2 while the other has a serine. The proline-containing variant CCL3L1 bound strongly to D6 while the other form did not. Moreover, a truncated variant of CCL22 lacking the first two amino acids had a much weaker affinity for D6 compared to the full-length protein [8], and we have made similar observations using a –2 form of CCL3L1 (RJBN, unpublished). Collectively, these data strongly suggest that D6 ligand binding will be influenced by CD26 and other dipeptidyl peptidases which can clip chemokines and other

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

Table 4.1 The chemokine-binding profiles of the atypical chemokine receptors.

Atypical receptor	Chemokine ligands
D6	CCL2, CCL3[a)], CCL3L1[b)], CCL4, CCL4L1[b)], CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22
CCX-CKR	CCL19, CCL21, CCL25, CXCL13[c)]
CXCR7	CXCL11, CXCL12
DARC	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL17, CXCL1, CXCL3, CXCL5, CXCL6, CXCL8, CXCL11

a) Mouse CCL3 binds with high affinity to D6, but human CCL3 interacts only weakly with this receptor.
b) CCL3L1 and CCL4L1 are non-allelic isoforms of CCL3 and CCL4 respectively which are only found in humans.
c) CXCL13 shows weak affinity for human CCX-CKR but appears unable to bind to mouse CCX-CKR.

proteins after recognition of an XP motif at the extreme N-terminus and are known to dramatically alter chemokine bioactivity [10, 11].

These early studies clearly demonstrated that D6 was a promiscuous cell surface binding protein for pro-inflammatory chemokines. However, despite showing clear structural similarity to classical chemokine receptors, D6, and the other atypical receptors described below, are characterized by their inability to direct cell migration after ligand binding, or to couple to signal transduction pathways used by other chemokine receptors. With D6, this is in part due to subtle alteration of the second intracellular loop in which the canonical DRYLAIV sequence (the DRY motif) has changed to DKYLEIV. Mutagenesis to DRYLAIV introduces weak signaling activity into human D6, while conversion of the DRY motif of human CCR5 to DKYLEIV inhibits the signaling activity of this receptor (R.J.B. Nibbs, unpublished data). Nonetheless, the strong conservation of DKYLEIV in D6 in mammals does hint that this is unlikely to simply represent a loss of function (i.e., loss of signaling capability).

4.1.1 D6 Scavenges Chemokines *In Vitro*

The lack of signaling activity led to hypotheses that D6 acts as a chemokine decoy receptor that binds to chemokines to prevent them from gaining access to signaling-competent receptors. This hypothesis has been extensively tested using transfection systems in which D6 is expressed in heterologous cell lines (e.g., HEK293 cells), and by exploring the function of endogenous D6 expressed by immortalized cell lines. What has emerged from this work is that D6 is capable of efficient scavenging of extracellular chemokines by drawing them into cells and targeting them for degradation [12, 13]. Interestingly, it achieves this by constitutively trafficking to and from the cell surface such that chemokines bound to D6 at the surface are rapidly internalized without the need for the chemokine-driven internalization signals that are required for efficient internalization of other chemokine receptors [12, 14].

Consequently, D6 is found predominantly within intracellular vesicles, with less than 5% of the total D6 expressed present at the cell surface at any one time [12, 15]. It has been suggested that this intracellular D6 represent a mobilizable pool of D6 and indeed a recent study has demonstrated limited D6 redistribution to the cell surface upon stimulation of cells with inflammatory CC chemokines [16]. This mobilization of D6 has been shown to be dependent on the small GTPase Rab11 [16], while recycling through early endosomes requires rab5 [12]. Once internalized, chemokines are rapidly dislodged by the lower pH encountered in early endosomes and then targeted to lysosomes for degradation, leaving D6 free to recycle back to the cell surface for more scavenging [12]. The mechanisms underpinning the constitutive trafficking of D6 are, in part, still unclear. While there is agreement that it enters cells via clathrin-coated pits (CCPs), the role of β-arrestins, which direct many G protein-coupled receptors to CCPs, is unclear. We have found that constitutive phosphorylation of a serine cluster in the C-terminus of D6 drives β-arrestins to the cell surface in D6-expressing cells, and controls intracellular trafficking itineraries, but we also showed that this is dispensable for chemokine scavenging [17]. Instead, in our hands, a helical region in the C-terminal tail adjacent to the seventh transmembrane helix was critical for scavenging [17]. Others reported that an acidic region at the other end of the C-terminal tail mediated constitutive interactions with β-arrestins and that these interactions were required for D6 internalization [14]. These discrepancies require further investigation. Nonetheless, the *in vitro* data have strongly indicated a role for D6 in internalizing and degrading inflammatory chemokines prompting us and others to suggest a role for D6 as a scavenger of inflammatory CC chemokines *in vivo*.

4.1.2
D6 Expression *In Vivo*

At the transcript level, D6 is expressed predominantly in barrier tissues such as the skin, gut and lung as well as in the placenta and liver [5, 6]. Within the skin, gut and lung of humans, the major cellular site of D6 expression are lymphatic endothelial cells [18], with no expression of D6 on the blood vessel endothelium. Tissue leukocytes were also seen as $D6^{+}$, and we have recently shown D6 expression by multiple subsets of leukocytes [19]. These may act as motile vehicles for D6 function [20], but perhaps surprisingly only weak expression was seen on classical inflammatory leukocytes such as neutrophils and macrophages [19]. Instead, moderate D6 expression was seen in mast cells, megakaryocytes, and haematopoietic stem and progenitor cells [19], while the major expressers were B cells and dendritic cells (DCs), the major antigen presenting cells, maybe suggesting more complexity to the range of *in vivo* D6 functions. Indeed this may go some way to explaining the phenotype displayed by the D6 null mice in the EAE model as described below. D6 is expressed at very high levels in the placenta, where it is found on the apical side of syncitiotrophoblasts and on extravillous trophoblasts [21]. Chemokine scavenging in the placenta may conceivably help prevent leukocyte infiltration of this tissue and is discussed in more detail below.

Very little is known about how the expression of D6 is restricted to specific cell lineages, and how this expression is regulated. Our recent work has revealed that D6 is regulated by the transcription factor GATA1, and is subject to reciprocal regulation in leukocytes by inflammatory and anti-inflammatory stimuli [19]. For example, TGF-β enhanced the levels of D6 expression while LPS reduced it. Thus, during an inflammatory response the levels of D6 are perhaps kept low by the presence of inflammatory mediators, such as LPS, keeping chemokine levels high to drive leukocyte recruitment to aid clearance of any invading infectious agents. Conversely, as inflammation is being resolved, the production of TGF-β increases D6 expression to enable effective removal of chemokines from the inflamed site. It is clear however that response to regulators of inflammation is dependent on the model system being used, and a recent publication has reported that pro-inflammatory cytokines can increase D6 expression on human tumor cells [22]. However, with the exception of some angiosarcomas [18] and most Kaposi's sarcomas (R.J.B. Nibbs, unpublished data), which are derived from lymphatic endothelial cells, we have been unable to detect D6 expression on any primary human tumors from a variety of different tissues (unpublished data). Clearly much more work is required to understand how D6 expression is controlled.

4.1.3
Explorations of D6 Function *In Vivo*

Studies of D6 function *in vivo* have exclusively utilized D6 "knockout" mice (Table 4.2). These mice have no apparent resting phenotype but they do have marked abnormalities in their responses to a range of inflammatory stimuli. Our work has focused on the skin, largely using a phorbol ester (TPA)-based skin inflammation model [23, 24]. D6 null mice showed an exaggerated and prolonged response to TPA compared with wildtype mice which was characterized by enhanced levels of inflammatory CC chemokines in the skin [24]. Although ultimately self-limiting, the pathology was similar in many respects to human psoriasis with an accumulation of dermal mast cells and epidermal T cells, enhanced angiogenesis, an inhibition of epidermal differentiation and excessive epidermal proliferation. This effect on inflammation was sufficiently powerful to suppress skin tumor development induced by repeated inflammatory stimulation of mutagenised back skin [23]. Moreover, transgenic over-expression of D6 in keratinocytes resulted in the suppression of cutaneous inflammation and inflammation-induced tumor development [23]. In a separate study, Martinez de la Torre and colleagues showed that D6 suppressed cutaneous inflammation induced by subcutaneous administration of complete Freund's adjuvant [25]. This work also suggested that D6 (presumably on LECs) limits inflammatory chemokine movement from inflamed peripheral tissues to local draining lymph nodes where it could conceivable drive leukocyte recruitment into the nodes. Collectively, these skin phenotypes in D6 deficient mice were consistent with a role for D6 in the resolution of the cutaneous inflammatory response through the scavenging of inflammatory CC chemokines.

Table 4.2 Phenotypes of D6 deficient mice in models of inflammation, immune response and cancer.

Model	Stimulus	Phenotype in D6 deficient mice
Cutaneous inflammation and tumorigenesis	Topical phorbol ester application (with or without mutagenesis)	Exaggerated epidermal proliferation; inhibition of epidermal differentiation; accumulation of dermal mast cells and epidermal T cells; enhanced angiogenesis; elevated chemokine abundance; increased susceptibility to cutaneous tumor development.
Cutaneous inflammation	Subcutaneous injection of complete Freund's adjuvant	Exaggerated cutaneous inflammation and elevated levels of inflammatory chemokines in skin-draining lymph nodes.
Inflammation-induced miscarriage	Injection of LPS or anti-phospholipid auto-antibodies	Enhanced levels of inflammatory CC chemokines and placental leukocyte infiltration. Increased susceptibility to miscarriage.
Allergic lung inflammation	Intraperitoneal sensitization with Ova/adjuvant then aerosolized Ova challenge, or repeated aerosolized Ova challenge only.	Enhanced levels of CCL17 and CCL22 in broncho-alveolar lavage resulting in increased abundance of T cell, DCs and eosinophils in lung. Decreased airway hyperreactivity.
Mycobacterium tuberculosis infection	Intranasal administration of *Mycobacterium tuberculosis*	Increased susceptibility to death due to systemic. Characterized by increased levels of chemokines and TNF-α IL-1β and IFN-γ levels in bronchoalveolar lavage and serum.
Autoimmune CNS disease (EAE)	Subcutaneous administration of MOG antigen in adjuvant	Reduction in spinal cord inflammation and demyelination due to defective DC migration after immunization.
Colitis	2% Dextran sodium sulfate in drinking water	Reduction in severity of clinical symptoms of colitis. No change in the levels of CC chemokine production or leukocyte recruitment compared to wild type. Elevated IL-17 production from $\gamma\delta$ T cells contributes to protection.

Inflammatory responses in the lung, another major site of D6 expression in wildtype mice, have also been explored in D6 deficient mice. Using variations of the classical ovalbumin-induced allergic lung inflammation model, Whitehead and colleagues found elevated levels of CCL17 and CCL22 in the broncho-alveolar lavage of D6 null mice compared with WT [26]. This resulted in the recruitment of large numbers of T cells, DCs and eosinophils to the lungs, and suggested that, as in skin, D6 is required for effective resolution of pulmonary inflammatory responses. However, airway reactivity, as measured by aerosolized methacholine administra-

tion, was somewhat suppressed in D6 deficient mice compared with the wild type [26]. The impact of D6 deficiency on *Myobacterium tuberculosis* lung infection has also been explored [27]. In this model, D6 deficient mice displayed exaggerated local and systemic inflammatory responses following infection, with elevated levels of chemokines and cytokines (including TNFα and IFNγ) and increased leukocyte infiltration of the lungs. However, these mice were no better at limiting bacterial growth than wildtype controls, but instead suffered greater mortality, most likely because of the increased lung inflammation, combined with kidney and liver failure driven by exaggerated systemic inflammatory responses. Antibody-mediated neutralization of chemokines in D6 deficient mice reduced mortality but impaired control over bacterial growth. Thus, in this model, D6 is able to suppress immunopathology without compromising protection.

In the placenta, it was hypothesized that D6 limits inflammatory chemokines at the fetomaternal interface to protect the fetus from the mother's leukocytes. Indeed, Martinez de la Torre and colleagues have shown that D6 null mice carrying D6 null fetuses are more susceptible than wildtype mothers carrying wildtype fetuses to inflammation-induced abortion driven by bacterial endotoxin (LPS) or antiphospholipid auto-antibodies [21]. High levels of inflammatory leukocytes and inflammatory CC chemokines were found in the placentae of D6 null mice during these treatments. Although a specific role for placental D6 was not investigated, it was clear that D6 protected animals from abortion and, again, it was loss of chemokine scavenging that was thought responsible for the D6 null phenotype.

Other studies have, at first sight, produced data that has sat less comfortably in the anti-inflammatory chemokine scavenger model. First, D6 null mice were markedly less susceptible to MOG antigen-induced experimental autoimmune encephalitis (EAE) than wildtype animals, with a marked reduction in inflammatory leukocyte accumulation in the spinal cord concomitant with lessened demyelination [28]. However, this appeared not to be due to a defect in the effector phase of the response, since adoptive transfer of encephalogenic wildtype T cells gave similar disease in wildtype or D6 deficient recipients. Instead, the authors reported a defect in T cell priming secondary to an apparent DC migratory defect from the site of immunization in the skin. This may be caused by the exaggerated inflammatory responses that develop in the skin or be due to an alteration in the way in which DCs interact with D6 deficient lymphatic endothelial cells across which DCs must pass across to gain entry to LNs. Alternatively, since we now know that DCs themselves can express D6, it is possible that the apparent block in DC movement was due to a cell autonomous defect with these cells. Clearly further experiments are required to address these issues.

The second example of what might be considered to be an unexpected D6 null phenotype came from our studies using the dextran sodium sulfate (DSS)-induced model of colitis [29]. D6 is abundantly expressed in the colon and upregulated during colitis in wildtype mice. However, we found that D6 null mice had a reduced tendency to develop the clinical symptoms of colitis compared to their wildtype counterparts, and had neither altered levels of bioavailable inflammatory CC chemokines nor

changes in leukocyte recruitment to the colon. Exaggerated production of IL17 from γδ T cells was, at least in part, responsible for the attenuated colitic response of D6 null mice, but the molecular mechanisms responsible for this are currently unknown. From these data it would appear that D6 is proinflammatory in this context, but, as in the EAE model, one has to consider the model used. In DSS colitis, the DSS damages the colonic epithelium and permits bacterial colonization of the lamina propria which drives inflammation leading to colonic damage. Thus, exaggerated inflammation early in the response, as may be expected in D6 null mice, may lead to more effective bacterial protection thus limiting the severity of the response at later timepoints and thereby suppressing clinical symptoms. Nonetheless it is clear that these and other results show that, in some contexts, chemokine scavenging can have unexpected outcomes, and that the biology of D6 is more complex than we currently think.

4.2
CCX-CKR, an Atypical Receptor for Homeostatic CC Chemokines

In 2000, the human homolog of the cow orphan receptor PPR1 was "de-orphanized" by two separate groups [30, 31]. Both claimed it was a chemokine receptor, but the reported ligand specificities were markedly different. One group, who renamed it CCR11, concluded it was a signaling-competent receptor for CCL2, 7, 8 and 13, that is, chemokines known to be active on CCR2 [31]. The other group, who called it CCX-CKR, used chemokines immobilized on stalks ("stalkokines") to provide an initial indication of binding to CCL19 and then employed heterologous and homologous radioligand binding assays to show high affinity binding to CCL19, 21 and 25, in addition to a weak interaction with CXCL13 [30]. Further investigation confirmed the conclusions of the latter study [32], and this was supported by our demonstration that the mouse homolog binds with high affinity to CCL19, 21 and 25 (although it shows no detectable interaction with CXCL13; Table 4.1) [33]. Gosling and colleagues proposed calling the molecule CCR10 (which at the time had not yet been assigned to the CCL27 and 28 receptors) [30], but its inability to couple to signal transduction pathways activated by other chemokine receptors has meant that it has remained outside the systematic nomenclature and CCX-CKR is currently the preferred name. This property has also led to its grouping with D6 as an "atypical" chemokine receptor. As with D6, CCX-CKR carries subtle variations on the canonical DRYLAIV motif in the second intracellular loop, which is present as DRYVAVT in human CCX-CKR, DRYWAIT in mice and DRYWAVT in cows. The functional consequences of these changes remains to be formally investigated, but it is tempting to speculate that they are, at least in part, responsible for the inability of CCX-CKR to couple to signal transduction pathways used by other receptors.

The function of CCX-CKR is unclear. We have found that when it is expressed in heterologous cells *in vitro* CCX-CKR can, like D6, efficiently internalize CCL19 and

target it for degradation [34]. This is in contrast to the other CCL19 receptor, CCR7, which in the same experimental system only undergoes initial CCL19 internalization before becoming desensitized. Moreover, rather like D6, the ability of CCX-CKR to internalize chemokine is enhanced by exposure to chemokine (CCL19). However, while D6 and CCX-CKR are similar in their ability to progressively scavenge large quantities of extracellular chemokine, they differ in terms of the mechanism used, with D6 entering cells via CCPs while CCX-CKR is dependent upon caveolar endocytosis. Nonetheless, these *in vitro* data indicate that CCX-CKR could be involved in scavenging homeostatic chemokines *in vivo* to regulate leukocyte migratory events mediated by CCR7 or the CCL25 receptor CCR9.

The precise cellular source of CCX-CKR expression also remains unclear primarily due to the lack of effective antibodies against this molecule. Using RT-PCR, Gosling and colleagues detected CCX-CKR mRNA in human immature DCs and activated T cells and in a variety of solid organs, such as spleen, lymph nodes, placenta, kidney and brain [30]. Using Northern blot analysis, we have found strong expression in lung, heart and gastrointestinal tract, with much weaker expression in lymphoid organs [33]. By "knocking-in" GFP into the CCX-CKR locus to use as a marker for CCX-CKR expression, Heinzel and colleagues [35] found no detectable GFP expression in leukocytes, or in any cells in spleen, heart, liver, kidney or brain, despite clear expression of CCX-CKR mRNA by some of these tissues in the previous work discussed above. However, GFP was observed in skin keratinocytes, in stromal cells around the subcapsular sinus of skin-draining lymph nodes, in putative lymphatic endothelial cells in the intestine and in thymic epithelial cells at sites thought to contain the most immature precursor cells of the adult thymus [35].

As CCL19, CCL21 and CCL25 (the ligands for CCX-CKR) are homeostatic CC chemokines with well known roles in DC, T cell and B cell trafficking, a role for CCX-CKR in regulating this process was proposed. However, CCX-CKR deficient mice appear relatively normal in terms of the structure and cellularity of their lymphoid tissue, though a statistically nonsignificant trend towards smaller lymph nodes has been reported [35]. Moreover, $CD11c^{+}MHCII^{high}$ DCs, which are believed to migrate constitutively from the skin using CCR7, were reported to be less abundant in resting skin-draining lymph nodes in CCX-CKR deficient mice than in wildtype counterparts [35], although we have not been able to verify this observation in CCX-CKR deficient mice we have generated (R.J.B. Nibbs, unpublished data). Despite strong GFP expression in the thymus of adult and embryonic CCX-CKR GFP "knock-in" mice, T cell development was not obviously affected by CCX-CKR deletion, but its over-expression in thymic epithelial cells did impair the immigration of embryonic precursors into the thymic anlage [35].

Despite the intriguing ligand binding profile of CCX-CKR, it has proven challenging to definitively determine the physiological roles of this atypical chemokine receptor *in vivo*. *In vitro* data show that it is capable of scavenging homeostatic chemokines, and it may subtly regulate homeostatic leukocyte trafficking *in vivo*, but more detailed analyses of CCX-CKR function are required, and the cellular distribution and regulation of this atypical receptor needs to be clarified.

4.3
CXCR7: A Second Receptor for CXCL11 and CXCL12 with Critical Roles in Development and Tumorigenesis

For some time there have been indications pointing to the existence of a second receptor for the primordial chemokine CXCL12. This was subsequently confirmed by the de-orphanization of RDC1 as a novel receptor for CXCL12 [36, 37]. Further competition studies revealed that RDC1 also bound CXCL11, confirming RDC1 as a chemokine receptor and earning it inclusion in the systematic nomenclature system as CXCR7 (Table 4.1). Despite this formal naming and some initial indications of the ability of CXCR7 to support leukocyte migration [36], the bulk of data now indicate that CXCR7 is likely to be a nonsignaling receptor and thus a novel member of the atypical receptor family. In keeping with the rest of the atypical chemokine receptors the DRYLAIV motif found in signaling "typical" chemokine receptors has been altered to DRYLSIT.

As befits its membership of the atypical receptor family, CXCR7 displays unanticipated expression patterns. Some data indicate expression by adult lymphocytes [36, 38], however apart from this the majority of data suggest that expression of CXCR7 is scant in adult tissues especially when measured at the protein level [39]. Curiously, however, CXCR7 is easily detectable during development as well as in the context of tumorigenesis [39, 40].

4.3.1
CXCR7 in Development

With the finding that the major sites of CXCR7 expression in nontransformed cells were within developing embryos, work was undertaken to more fully understand the developmental roles played by CXCR7. Mice with a conditional CXCR7 deletion were generated and early developmental deletion of the gene resulted in perinatal lethality at around 24 h after birth, indicating an essential role for CXCR7 in embryonic development [38]. The deletion of CXCR7 had no obvious effect on neural differentiation and hematopoiesis also appeared normal. However, careful analyses revealed that neonatal death was linked to defects in cardiac development. A consistent observation in CXCR7 deficient mice was chondrification of the aortic valves. In addition more than 90% of all CXCR7 deficient neonates had defects in the pulmonary valve. Defects caused by deletion of CXCR7 results in the heart valves being abnormally remodeled which in turn results in abnormal expression of extracellular matrix components. The cumulative effects of CXCR7 deletion on heart development ultimately resulted in the neonatal death of the mouse.

In addition to evidence for a requirement of CXCR7 in cardiac development there are also data suggesting a role in vascular development. The highly conserved nature of CXCR7 allows studies into its *in vivo* functions to be carried out in models of early vertebrate development such as those using zebrafish. Such studies have identified a role for CXCR7 in angiogenesis in addition to the organization of the vasculature during development [40]. Again, using zebrafish, it has also been shown that CXCR7

is involved in primordial germ cell migration as well as orientating cells migrating to the lateral line primordium [41, 42]. Importantly, CXCR4 is also involved in the migration of these cells. What differs between the two receptors is the part they each plays in the process. CXCR4 is expressed on the migrating cells and is therefore assumed to be supporting cell migration through traditional mechanisms of chemotaxis. The role CXCR7 plays in this process is complementary and is to ensure efficiency of the migration process by binding to, internalizing and degrading excess CXCL12, at the rear of the migrating cells, thus contributing to the maintenance of the *in vivo* gradient. CXCR7 expression is found predominantly on the somatic cells underlying the migrating cells and is thus ideally placed to continually remodel the gradient. These data are therefore striking in demonstrating that not only do the ancient receptors CXCR4 and CXCR7 play dominant roles in early development but that they do so in an interactive and complementary manner.

4.3.2 CXCR7 in Cancer

A curious aspect to the biological function of CXCR7 is that although it is expressed almost exclusively during embryo development it is also expressed in numerous tumor contexts [36, 39, 40, 43]. Indeed analysis of a range of tumor cell lines and primary mouse and human tumors has revealed extensive CXCR7 expression either in the tumor cells themselves or in the tumor associated vasculature. Strikingly, almost all human breast cancer specimens tested (97%), have been shown to be positive for CXCR7 expression [40].

The essential roles for CXCR7 in supporting tumor development have been demonstrated using both over-expressing tumor cell lines as well as CXCR7 antagonist based approaches [39, 40]. *In vivo* studies using allografted or xenografted tumor cell lines, which were either engineered to over-express CXCR7 or which endogenously expressed it, confirmed the importance of CXCR7 for tumor development. Use of antagonists in the models involving natural CXCR7 expressing tumors leant further support to the notion of a role for CXCR7 in tumorigenesis.

There also appears to be an association of CXCR7 with prostate cancer [43]. High-density tissue microarrays stained with human CXCR7 antibody revealed that tumors tend to become more aggressive in nature as the level of CXCR7 expression increases. It was further observed that CXCR7 has a role in the formation of vasculature in developing cancers as the incidence of blood vessel sprout formation dramatically increased in CXCR7 over-expressing cells. Finally, in line with previous results [39, 40], in xenograft models, tumors over-expressing CXCR7 developed *in vivo* into significantly larger tumors than nonCXCR7-expressing tumors [43]. This enhanced tumor formation could again be substantially inhibited through use of CXCR7-specific siRNA.

To conclude, it is clear that the atypical receptor CXCR7 has an essential role to play in embryonic development. It is clear however that it also plays a significant role in cancer development. The likelihood of developing disease appears to be linked with enhanced CXCR7 expression. In addition CXCR7 seems to be closely linked with the

setting up of tumor vasculature. CXCR7 is therefore a promising therapeutic target in a number of human cancers.

4.4 DARC: A Promiscuous Pro-Inflammatory Atypical Chemokine Receptor on Red Blood Cells and Endothelial Cells

The Duffy blood group antigen was identified on red blood cells (RBCs) more than 30 years ago as the cellular entry point for *Plasmodium knowlesi* and *P. vivax* [44, 45], but it was not until the mid 1990s that the gene encoding this antigen was cloned and shown to bind to chemokines [46–50], leading to its renaming as Duffy antigen/receptor for chemokines (DARC). It is now clear that DARC can bind to a broad spectrum of both pro-inflammatory CC and CXC chemokines with little or no affinity for homeostatic chemokines of either family [51] (Table 4.1). Moreover, in addition to RBCs, DARC has been found on blood vessel endothelial cells (particularly at sites of active leukocyte extravasation such as high endothelial and postcapillary venules [52, 53]), kidney epithelial cells, Purkinje cells and type II pneumocytes [54]. Regardless of the chemokine tested, ligation to DARC does not induce a calcium flux that is typically seen in classical chemokine receptor signaling [50], and no signals have been reported to be transduced through this molecule. Indeed, unlike the other atypical receptors, it appears to carry no vestige of the DRYLAIV sequence. DARC was the first chemokine receptor that showed lack of signaling capability, and was therefore the first member of the atypical chemokine receptor subfamily to be identified.

4.4.1 Function of Chemokine Binding to DARC

Chemokine binding to DARC on endothelial cells has been proposed to be involved in transcytosis, or neutralization, of chemokines, while RBC DARC is thought to regulate plasma chemokine abundance. Transcytosis would lead to the presentation of chemokines on endothelial surfaces to facilitate leukocyte extravasation, while regulation of plasma chemokines would influence the degree to which chemokine receptors on circulating leukocytes are desensitized. Thus, both proposed functions would act to fine tune inflammatory responses. Studies of transcytosis have been performed primarily by Antal Rot's laboratory [55, 56], with significant contributions from others [57], and there is now considerable evidence that DARC is capable of transporting chemokines intact across cell monolayers, possibly via the caveolar system present in endothelial cells. Indeed, DARC deficient mice show impaired neutrophil recruitment into tissues after intratracheal CXCL8 administration or intraperitoneal LPS injection, consistent with a role in aiding inflammation [57, 58]. However, others reported increased neutrophil infiltration in DARC deficient mice in response to higher doses of LPS [59], and it is still unclear how DARC deletion can lead to these apparently opposing phenotypes. One possibility is that DARC can

assume a scavenging role in some scenarios to limit chemokine bioavailability, and it is notable that mice transgenically over-expressing DARC in endothelial cells show reduced angiogenic responses to certain DARC-binding chemokines, an observation attributed to chemokine scavenging [60].

The role of RBC DARC also needs to be considered when attempting to interpret phenotypes seen in DARC deficient mice. RBC DARC has been proposed to act as a "sink" that removes chemokines from plasma, and DARC on RBCs is reported to be capable of removing high levels of chemokines produced by prostate cancer cells to allow the effective suppression of chemokine-driven angiogenesis [61]. However, DARC null mice more rapidly lose injected chemokine from their plasma compared to wildtype mice [62], and humans lacking RBC DARC show lower plasma levels of CCL2 compared to DARC positive individuals [63]. This has led to the idea that RBC DARC may also act as a chemokine "reservoir" or buffering system that maintains chemokines in the plasma at a low level, although the purpose of such a function is currently unclear. Collectively, these data could indicate that the amount of chemokine present dictates what aspect of DARC function predominates. When chemokines are abundant, DARCs scavenging/sequestering functions may be most prominent, but when they are more limited then endothelial DARC may be required for optimal chemokine presentation while RBC DARC provides appropriate buffering of plasma chemokines. Clearly further investigations are required to clarify these issues.

4.4.2 DARC Interacts with Non-Chemokine Proteins

As discussed above, DARC is exploited by some malarial species which carry aptly named Duffy-binding proteins [PvDBP (*P. vivax*), PkDBP (*P. knowlesi*)] that mediate interactions with the N-terminus of RBC DARC as part of the complex multistep RBC invasion process [64]. These DBPs show no obvious structural homology to chemokines, but it is notable that chemokine binding to DARC can inhibit parasite entry into RBCs, probably by streic hindrance [46]. Recent work has shown malaria is not the only pathogen that binds to DARC. He and colleagues have shown that the human immunodeficiency virus HIV-1 also attaches via DARC to RBCs, and this too is blocked by chemokines [65]. Importantly, HIV-1 remains viable on RBC surfaces and can be transmitted to susceptible T cells – thus, DARC may help facilitate HIV-1 infection [65, 66]. The HIV-1 proteins that mediate interaction with DARC remain to be determined but it would not be surprising if gp120 was involved. It interacts with other chemokine receptors (e.g., CCR5, CXCR4) in a manner critical for the infection of target cells and, in the same way that DARC negative individuals are resistant to P vivax infection, CCR5Δ32 homozygotes (which lack surface CCR5) are protected from HIV-1 infection. In addition to pathogen interactions, DARC has also been reported to bind to CD82 (KAI1) on tumor cells, and this is proposed to underpin the metastasis-suppressing properties of CD82 by inhibiting tumor cell proliferation and inducing senescence [67]. This requires validation, but raises the intriguing prospect that by binding non-chemokine proteins DARC can control tumor spread.

4.4.3
What Can DARC Negative People Tell Us About DARC Function?

Use of DARC as a route of entry into RBCs for some malarial species has driven the emergence of large DARC negative African populations in which a single nucleotide substitution from T to C at position 46 in the DARC promoter disrupts the binding site for the haemopoietic transcription factor GATA1 and leads to loss of DARC expression on RBCs but not endothelial cells [68]. This provides near complete protection from *P. vivax* [69] and has been proposed to contribute, through loss of chemokine scavenging, to the increased incidence of prostate cancer seen in African-American men [61]. There is also recent evidence to suggest that it controls susceptibility to asthma and atopy in Africans [70], and, because of the ability of DARC to bind HIV-1 and facilitate target cell infection, it is perhaps not surprising that DARC negative individuals infected with HIV-1 show slower progression to AIDS. Paradoxically, however, this genotype also increases susceptibility to HIV-1 infection for reasons that are currently unclear but which may be related to the role of DARC in regulating chemokine homeostasis [65, 66]. This idea makes sense since CCR5 and DARC bind overlapping subsets of chemokines, and these chemokines are known to exert complex control over target cell infection [66]. The interpretation of these various DARC-negative associated traits is further complicated by recent data implicating this genotype in the low white blood cell and neutrophil counts common in individuals of African ancestry [71, 72]. The mechanisms underpinning this aspect of DARC biology remain to be discovered, but it is clear that, even 30 years after the ground-breaking malarial studies [44], DARC negative populations continue to provide new insights into the function of this atypical chemokine receptor.

4.5
Summary

The biology and biochemistry of atypical chemokine receptors clearly distinguishes them from other chemokine receptors, and it is clear that they play critical roles in regulating and/or facilitating leukocyte migration and other chemokine-driven responses. Their identification and characterization has established new paradigms of chemokine receptor function and chemokine regulation, but much still needs to be discovered about how these molecules operate at the molecular and cellular level, where they are expressed *in vivo* and how they control immune and inflammatory responses. Perhaps more importantly, we need to investigate whether this growing knowledge base can be exploited therapeutically to aid the treatment of the broad array of human diseases in which chemokines play critical roles.

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5
Targeting Chemokine Receptor Dimers: Are there Two (or More) to Tango?

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5.1
Introduction

Chemokine receptors play a central role in the immune defense by directing the migration, activation and differentiation of the billion of leukocytes that roam our body during immune surveillance and inflammation. Chemokine receptors are integral membrane proteins belonging to the superfamily of G protein-coupled receptors (GPCRs). Differential spatiotemporal expression of chemokine receptor subtypes allows specific subpopulations of leukocytes to respond to local gradients of the cognate chemokines [1]. In addition, some elements of the chemokine system are involved in organogenesis, angiogenesis and human immunodeficiency virus (HIV) infection. Importantly, inappropriate expression of chemokines and/or their receptors is associated with various pathologies, including autoimmune and inflammatory diseases, as well as tumor growth survival and metastasis [1–3]. Not surprisingly, chemokine receptors have been recognized by both academia and pharmaceutical industry as valuable targets for therapeutical intervention in a variety of major diseases [2, 4].

5.2
Chemokines and their Receptors

Chemokines are small proteins (8–14 kDa) that share a similar tertiary folding, characterized by a flexible N-terminal tail that is connected via an N-loop to a core domain consisting of three antiparallel β-strands and a C-terminal α-helix, and stabilized by disulfide bonds between four conserved cysteine residues [5]. At least 45 chemokines have been identified to date in humans. They are subdivided into four classes (i.e., CC, CXC, CX3C, XC) on the merit of the number and sequential spacing of the first two conserved cysteine residues [1]. Chemokines have been shown to oligomerize, a property important for the formation of stable gradients *in vivo*. Indeed, oligomerization is favored by the binding of chemokines to glycosaminoglycans, which prevents their rapid diffusion, stabilizing gradients within the extracellular matrix and the presentation of chemokines at the surface of endothelial cells [6]. It has however

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

been clearly established that only chemokine monomers are able to interact with their cognate receptors [7–10]. There is therefore no link between chemokine dimerization and the dimerization of chemokine receptors. The human genome encodes 19 functionally signaling chemokine receptors which are classified according to their ability to bind a specific chemokine subclass, with ten receptors for CC chemokines (i.e., CCR1–CCR10), seven receptors for CXC chemokines (i.e., CXCR1–CXCR7), one receptor for C chemokines (i.e., XCR1), and one receptor for the CX3C chemokine (i.e., CX3CR1) [1]. Typically, most inflammatory chemokines promiscuously bind several chemokine receptor subtypes, whereas chemokines involved in immune surveillance generally interact with a single chemokine receptor subtype [11]. In addition, three decoy chemokine receptors (i.e., D6, DARC, CCX-CKR) have been identified in humans. They regulate leukocyte recruitment by removing a wide variety of predominantly inflammatory chemokines from the extracellular microenvironment through internalization, without activating intracellular signaling [12].

The majority of all human beings is latently infected by one or more herpesvirus species. Interestingly, most human herpesviruses encode at least one GPCR that will appear on the surface of human cells upon viral infection [13]. Both Epstein–Barr virus (EBV) and human herpesvirus 8 (HHV8) encode a single receptor (i.e., BILF1 and ORF74, respectively), human herpesvirus 6 and 7 (HHV6, HHV7) both encode two receptors (i.e., U12 and U51), whereas the human cytomegalovirus (HCMV) encodes four receptor proteins (i.e., US27, US28, UL33, UL78). These virally encoded GPCRs have the highest sequence similarity with chemokine receptors and are believed to be host genes that have been pirated and modified by the virus at some point in evolution [13]. Indeed, US28, U12, U51 and ORF74 are each responsive to a wide spectrum of human chemokines, whereas BILF1, US27, UL33 and UL78 are still orphan receptors. These virally encoded GPCRs are thought to contribute to immune evasion, viral latency and dissemination but have also been associated with the development and progression of herpesvirus-associated inflammatory diseases and cancer [14].

GPCRs are single polypeptide chains consisting of an extracellular N-terminal domain, seven hydrophobic transmembrane helices that are connected by alternating intracellular and extracellular hydrophylic loops, and a cytosolic C-terminal domain. Chemokine receptors belong to the large family of class A GPCRs by sharing specific sequence motifs within the transmembrane domains [15]. Chemokines bind with their core domain to the N-terminus and extracellular loops of the chemokine receptors, positioning the chemokine N-terminus to interact with the bundle of transmembrane helical domains, thereby promoting receptor activation [5]. To this end, chemokines are thought to stabilize active receptor conformations by disrupting a framework of intramolecular constraints that normally keep the receptor in an inactive state [16].

5.3
GPCRs Exist and Function as Dimers

GPCRs have always been considered to exist and function as monomeric units that consecutively interact with G proteins or β-arrestins in an 1 : 1 stoichiometry upon

binding of a single ligand. However, during the past decades, this dogma was gradually challenged with increasing evidence that GPCR proteins do in fact physically interact with each other. Negative binding cooperativity among β-adrenergic receptors provided the first indirect indication for possible physical interactions between GPCR proteins as early as 1975 or 1976 [17, 18]. Although subsequent biochemical studies supported the idea that GPCRs might be organized and function as dimeric or even oligomeric complexes [19], it was not until the late-nineties that this highly controversial concept regained attention and became more generally accepted with the unambiguous demonstration that the $GABA_B$ receptor, a class C GPCR, exists and functions as an obligate heterodimer [20–22]. Both $GABA_BR1$ and $GABA_BR2$ isoforms are nonfunctional when individually expressed in cells, with $GABA_BR1$ being retained in the endoplasmatic reticulum (ER), and $GABA_BR2$ being expressed at the cell surface, but unable to bind the ligand γ-aminobutyric acid (GABA) [23–25]. However, constitutive heterodimerization of $GABA_BR2$ with $GABA_BR1$ during biosynthesis masks the ER retention motif in the C-terminal tail of the latter, allowing efficient trafficking of a functional heterodimeric $GABA_B$ receptor to the cell surface [26, 27]. Importantly, $GABA_B$ receptor function is eliminated in knockout mice in which either the $GABA_BR1$ or $GABA_BR2$ gene was deleted, confirming the necessity of $GABA_BR1/GABA_BR2$ heterodimerization *in vivo* [28, 29]. Signaling through the $GABA_B$ receptor heterodimer requires the transactivation of the $GABA_BR2$ subunit, a mechanism involving a change in the dimerization interface between the Venus flytrap extracellular domains [30]. The sweet and umami taste receptors also function as obligate heterodimers, consisting of the class C GPCR T1R3 in complex with T1R1 or T1R2, respectively [31].

In contrast to class C GPCRs for which heterodimerization is strictly obligatory for the formation of a functional receptor, many class A GPCRs seem to appear as functional (homodimeric) receptor proteins on the cell surface when individually expressed in cells. On the other hand, heterodimerization between class A GPCRs may modulate the functional properties of the individual partners, such as ligand binding, signaling and/or trafficking, in a cell type-specific and receptor expression level-dependent manner.

Atomic force microscopy studies have shown that rhodopsin in native mouse rod outer segment disk membranes is arranged as dimers in paracrystalline arrays and raft-like membrane patches [32]. The distance and orientation of rhodopsin molecules were used to model the oligomeric assembly of rhodopsin dimers.

5.4
Detection of GPCR Dimerization

Various techniques have been routinely used to provide evidence for GPCR dimerization. However, each of these methodologies has its caveats, which should be considered for optimal experimental design and data interpretation. In fact, one definitive approach does currently not exist, and consequently multiple approaches

are usually applied in a single study to accumulate supportive data that in combination should provide enough evidence for GPCR dimerization [33, 34].

Co-immunoprecipitation (Co-IP) followed by SDS-PAGE and immunoblotting provided in 1996 the first direct biochemical evidence for physical interactions between GPCR proteins and is still widely being used [35]. Endogenous GPCR dimers can be detected in *ex vivo* samples by using specific antibodies against the GPCRs of interest for immunoprecipitation and immunoblotting [21, 36]. However, the absence of high quality antibodies against the majority of GPCRs has so far hampered the analysis of GPCR complexes in native tissues [37]. Therefore, GPCRs are routinely tagged with distinct epitopes (e.g., hemagglutinin, FLAG, c-myc), which allows Co-IP analysis using commercially available high affinity antibodies against these tags upon recombinant expression of these tagged GPCRs in cell lines (Figure 5.1a). GPCRs are integral membrane proteins and the use of detergent is required to release GPCRs from their bilayered phospholipid environment. However, this procedure can give rise to artificial aggregation of GPCRs, for example through the formation of free sulfhydryl groups [38]. In addition, if membranes are incompletely solubilized, co-expressed GPCRs can be co-immunoprecipitated without having actual physical interaction. Conversely, dimers may lose stability during the procedure, and high detergent concentrations may actually disrupt existing GPCR dimers. Crosslinking may be performed prior to solubilization, in order to stabilize the complexes during the subsequent steps. Eventhough appropriate controls may allow to deal with most technical caveats of the Co-IP procedure, additional methodologies should be applied to ensure that specific GPCR interactions are detected [34, 38].

Resonance energy transfer (RET)-based methodologies are nowadays more and more used to monitor interactions between GPCRs. In contrast to Co-IP methods, these biophysical approaches allow real-time analysis of protein–protein interactions in living cells. RET is the nonradiative transfer of energy from a light-emitting donor molecule to a suitable fluorescent acceptor molecule, causing the emission of fluorescence with a lower energetic wavelength by the latter. RET only occurs if donor and acceptor molecules are within 10 nm of each other, which is an indicative distance for protein–protein interactions (Figure 5.1b) [39]. RET efficiency is determined by the alignment and the inverse sixth power of distance between donor and acceptor dipoles. Consequently, changes in RET can reflect changes in the distance and/or orientation between donor and acceptor molecules.

To analyze GPCR interactions, donor and acceptor molecules are genetically fused to the C-terminus of GPCR proteins of interest and subsequently expressed in heterologous cells (Figure 5.1d). RET occurs when donor and acceptor molecules are brought into close enough proximity as a consequence of interactions between the GPCRs of interest. When both donor and acceptor are engineered variants of green fluorescent protein from the jellyfish *Aequorea victoria*, fluorescent resonance energy transfer (FRET) may occur upon excitation of the donor fluorophore by monochromatic light of an appropriate wavelength from an external light source. Alternatively, the RET donor can be (variants of) the enzyme luciferase (Rluc) from the sea pansy *Renilla reniformis* which generate bioluminescence by oxidation of

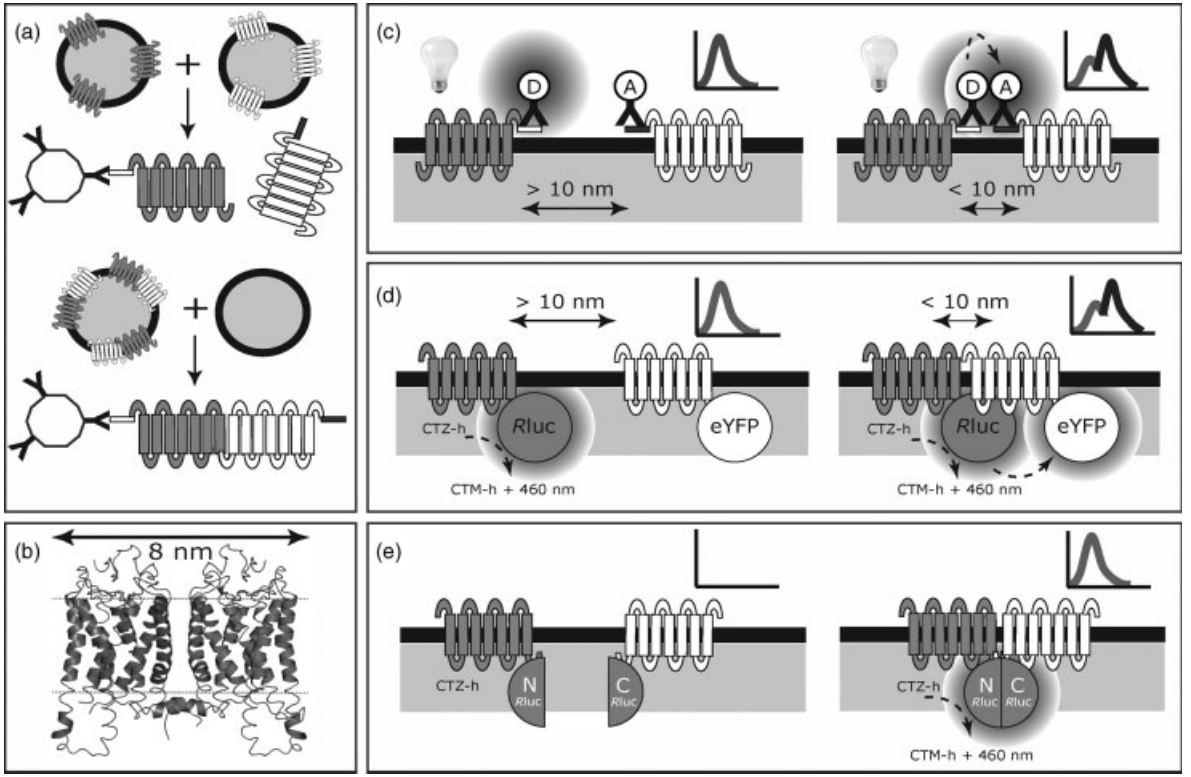

Figure 5.1 Detection of GPCR dimers. (a) Example of Co-IP setup. Cells are transfected with GPCR 1 (gray) and/or GPCR 2 (white). Prior to solubilization cells expressing GPCR 1 are mixed (1 : 1) with GPCR 2 expressing cells (upper panel), whereas cells co-expressing these receptors are mixed (1 : 1) with control cells (lower panel). GPCR 1 is isolated from cellular extracts using immobilized antibodies. GPCR 2 is only co-immunoprecipitated if it interacts physically with GPCR 1 (lower panel). (b) Dimension of a GPCR dimer. (c) TrFRET technology. Cell surface expressed GPCR 1 is labeled with Eu^{3+}-conjugated antibodies (Donor), whereas GPCR 2 is labeled with XL665-conjugated antibodies (Acceptor). Excitation of Eu^{3+} with light of 337 nm causes emission at 620 nm. If XL665-conjugated antibodies are in close proximity as a consequence of GPCR dimerization, RET occurs and light is emitted at 665 nm. (d) BRET technology. GPCR 1 and 2 are fused to Rluc (donor) and eYFP (acceptor) proteins, respectively, and co-expressed in cells. Rluc catalyzes the oxidation of coelenterazine-h (CTZ-h) to coelenteramide-h (CTM-h), which is accompanied by the emission of 460 nm light. If GPCR 2-eYFP is in close proximity (<10 nm), RET occurs resulting in the emission of 535 nm light by eYFP. (e) PFC technology. GPCR 1 and 2 are fused to the N- and C-terminal fragments of Rluc, respectively, and co-expressed in cells. The Rluc protein is functionally reconstituted when these two GPCRs heterodimerize.

a substrate, resulting in bioluminescence resonance energy transfer (BRET) to suitable acceptor fluorophores that are in close proximity (Figure 5.1d) [40]. Importantly, BRET circumvents some of the technical pitfalls of FRET that are associated with using an external light source, such as photobleaching and the simultaneous excitation of both donor and acceptor fluorophores. Monitoring BRET as a function of increasing acceptor/donor ratios results in a hyperbolic saturation curve if fusion proteins of interest are specifically interacting, whereas nonspecific interactions as a consequence of random collisions give a quasi linear increase in BRET signal [41]. The acceptor/donor ratio resulting in half maximal BRET saturation ($BRET_{50}$) is a measure for the propensity of GPCRs to form dimers [41, 42]. Conjugation of specific antibodies with donor (e.g., europium chelate/cryptate) and acceptor (e.g., allophycocyanin, Alexa Fluor 647, D2) fluophores allows the exclusive detection of GPCR dimers at the cell surface by time-resolved FRET (trFRET) (Figure 5.1c) [43, 44]. Alternatively, cell surface-expressed snap-tagged GPCRs can be covalently labeled with similar membrane-impermeant donor and acceptor fluorophores [45]. Recently, protein fragment complementation (PFC) techniques have also been applied to detect GPCR dimerization and oligomerization when used in combination with FRET/BRET measurements [46–49]. To this end, a reporter protein (e.g., green fluorescent protein or luciferase variants) is split into two nonfunctional fragments, which are genetically fused to the C-terminus of the GPCRs of interest. If these GPCR fusion proteins are in close proximity, the protein fragments will reconstitute to form a functional reporter protein (Figure 5.1e). Using such strategies, the formation of homotetramers of the dopamine D_2 receptor has been demonstrated [46], as well as the hetero-oligomerization of the cannabinoid CB_1, adenosine A_{2a} and dopamine D_2 receptors [50]. Although carefully designed RET and/or PFC-based experiments using lowest possible GPCR levels and bearing the appropriate controls may provide convincing evidence for specific GPCR interactions, one has to keep in mind that these methods actually detect close proximity between proteins and not necessarily physical interactions.

5.5 Chemokine Receptor Dimerization

Various chemokine receptor homo- and heterodimers have been identified over the last decade, and the list of dimerization partners is still expanding (Table 5.1). Initially, research has mainly focused on the traditional chemokine receptors (e.g., CCR2, CCR5, CXCR1, CXCR2, CXCR4) with known therapeutic potential in the treatment of chronic inflammatory diseases, HIV infection and cancer [1, 3]. In addition to heterodimerization between chemokine receptors, these receptors have also been reported to heterodimerize with other class A GPCRs that are co-expressed on immune cells, such as the C5AR, opioid receptors (OPRs) and the EBV-encoded GPCR BILF1 (Table 5.1).

Table 5.1 Chemokine receptor dimerization.

GPCR-1	GPCR-2	Methodology	Induced/ constitutive	Effect(s) of dimerization	References
CCR2	CCR2	CL/Co-IP	Induced		[61, 62]
CCR2	CCR2	Co-IP, BRET	Constitutive		[68, 70, 73]
CCR2	CCR5	CL/Co-IP	Induced	Synergized signaling, additional G protein coupling	[66]
CCR2	CCR5	Ab-FRET, BRET, Co-IP	Constitutive	Negative binding cooperativity (chemokines and small molecule antagonists)	[62, 68, 70]
CCR2-$V^{64}I$	CCR5	CL/Co-IP	Induced	Delayed HIV progression	[89]
CCR2	CXCR4	CL/Co-IP	Induced	Antibody-induced dimerization	[62]
CCR2	CXCR4	BRET	Constitutive	Negative binding cooperativity (chemokines and small molecule antagonists) Transinhibition of chemokine-induced Ca^{2+} mobilization and migration	[72, 73]
CCR2-$V^{64}I$	CXCR4	CL/Co-IP	Induced	Delayed HIV progression	[89]
CCR5	CCR5	CL/Co-IP	Induced	Ligand and antibody-induced dimerization decreases HIV infection	[63]
CCR5	CCR5	Co-IP, BRET, FRET/FLIM	Constitutive		[51, 63, 70, 90]
CCR5	CXCR4	Co-IP, BRET	Constitutive	Translocation to immunological synaps during T cell activation	[91]
CCR5	C5AR	BRET	Constitutive		[90]
CCR5	δ-OPR	Co-IP	Constitutive		[92]
CCR5	κ-OPR	Co-IP	Constitutive		[92]
CCR5	μ-OPR	Co-IP	Constitutive	Transinhibition of ligand-induced signaling	[87, 92]
CCR5Δ32	CCR5	Co-IP	Constitutive	HIV resistance	[93]

(*Continued*)

Table 5.1 (*Continued*)

GPCR-1	GPCR-2	Methodology	Induced/ constitutive	Effect(s) of dimerization	References
CCR5Δ32	CXCR4	Co-IP	Constitutive	HIV resistance	[93]
CXCR1	CXCR1	Co-IP, (tr)FRET, BRET, ER-t	Constitutive		[52]
CXCR1	CXCR2	Co-IP, (tr)FRET, BRET, ER-t	Consitutive	No effect on CXCL1-induced signaling	[52]
CXCR2	CXCR2	Co-IP, (tr)FRET, BRET, ER-t	Constitutive		[52, 94]
CXCR2	δ-OPR	Co-IP, (tr)FRET, BRET, FC	Constitutive	CXCR2 antagonist enhances δ-OPR function	[77]
CXCR4	CXCR4	CL/Co-IP	Induced	JAK/STAT signaling	[65]
CXCR4	CXCR4	Co-IP, BRET, FRET, PFC	Constitutive		[51, 67, 71, 73, 76]
CXCR4	CXCR7	Co-IP, PFC	Constitutive		[76, 95]
CXCR4	δ-OPR	Co-IP, FRET	Constitutive	Impaired signaling upon co-stimulation	[78]
CXCR7	CXCR7	PFC	Constitutive		[96]
BILF1	CCR6	BRET	Constitutive		[85]
BILF1	CCR7	BRET	Constitutive		[85]
BILF1	CCR9	BRET, trFRET, Co-IP	Constitutive		[85]
BILF1	CCR10	BRET, trFRET, Co-IP	Constitutive		[85]
BILF1	CXCR3	BRET, trFRET, Co-IP	Constitutive		[85]
BILF1	CXCR4	BRET, trFRET, Co-IP	Constitutive		[85]
BILF1	CXCR5	BRET	Constitutive		[85]
BILF1	CXCR7	BRET	Constitutive		[85]

Abbreviations: cross-linking (CL), anti-body FRET (Ab-FRET), FLIM (fluorescence lifetime imaging microscopy), ER-trapping (ER-t), functional complementation (FC).

5.6
Constitutive Versus Induced Chemokine Receptor Dimerization

Dimerization of chemokine receptors is readily initiated during protein synthesis and maturation in the ER as indicated by the detection of CCR5 homodimers in this organelle upon cellular fractionation [51]. Fusion of the ER retention motif sequence of the α2c-adrenoreceptor to the C-terminal tail of an N-terminally HA-tagged CXCR1 not only impaired its own cell surface detection but also that of co-expressed FLAG-tagged CXCR1 or CXCR2, revealing that CXCR1 homodimers and CXCR1/CXCR2 heterodimers are formed during biosynthesis and prior to translocation to the cell surface [52]. Constitutive dimerization between CCR5 and the frame-shift mutant CCR5Δ32 was proposed to trap the normal receptor in the ER, thus reducing its expression at the cell surface [53]. Such dominant negative effect, proposed to explain the delayed disease progression in heterozygous CCR5/CCR5Δ32 individuals [54, 55], was however not confirmed, as other groups showed that the reduced cell surface expression of the co-receptor can be fully attributed to CCR5 gene dosage, without sequestration of the wildtype receptor [56, 57].

The majority of biochemical and biophysical evidence confirmed that chemokine receptor dimers are indeed formed in a ligand-independent (i.e., constitutive) manner (Table 5.1), which is consistent with the current paradigm that GPCR homo- and heterodimerization during biosynthesis is a requirement for passing quality control checkpoints to reach the cell surface as properly folded GPCR proteins [58–60]. In contrast, some early studies suggested that chemokine receptor dimerization is strictly dependent on chemokine ligand binding, as detected by co-immunoprecipitation of crosslinked receptor dimers. In these studies, homodimerization of CCR2 [61, 62], CCR5 [63, 64] and CXCR4 [65] was only detected in the presence of their cognate chemokines, whereas the simultaneous presence of CCL2 and CCL5 was strictly required to induced CCR2/CCR5 heterodimerization [66]. The apparent absence of constitutive chemokine receptor dimers might be related to the more stringent denaturation conditions used in these studies as compared to other studies in which constitutive dimers were detected by co-immunoprecipitation [51, 67, 68]. In addition, chemokine binding to receptor dimers might significantly increase the disuccinimidyl suberate-mediated crosslinking efficiency as compared to unbound receptor dimers, possibly by bridging together receptor protomers in an optimal conformation. Consequently, more chemokine-bound dimers are recovered under these denaturating conditions leading to the potential misinterpretation that chemokine receptors are monomers under basal conditions and that dimerization is promoted upon chemokine stimulation [69]. In addition, RET-based methodologies confirmed that many if not all chemokine receptors form homo- and heterodimers in the absence of ligand stimulation (Table 5.1). Numerous studies have reported that constitutive RET signals between homo- or heterodimeric chemokine receptors were not or only minimally affected by ligands [51, 52, 67, 70], whereas others observed a clear modulation of RET signals upon the addition of ligands [63, 71–73]. Such ligand-induced increases and decreases in RET signals have often been interpreted as respectively *de novo* formation or dissociation of GPCR dimers in response to ligand

stimulation. Importantly, however, RET intensity is determined by both distance as well as relative orientation between donor and acceptor dipole moments. Hence, observed alterations in RET intensities not necessarily reflect changes in dimer numbers, but may also be related to conformational alterations in pre-existing dimer partners to which the energy donor and acceptor molecules are fused [42, 74, 75]. Indeed, CCL2 induced opposite BRET efficiencies on CXCR4-Rluc/CCR2-YFP dimer as compared to the CCR2-Rluc/CXCR4-YFP dimer, as well as changes in maximal BRET intensities without affecting the apparent dimerization propensity (i.e., $BRET_{50}$). These observations could be best explained by conformational rearrangements in the pre-existing dimer [73]. Interestingly, CXCL12 had opposite effects on constitutive firefly luciferase PFC of CXCR4 and CXCR7 homodimers, which were postulated to be the consequence of distinct ligand-induced conformational changes in these receptors [76]. The chemokine CXCL11 induced a similar increase in reconstituted firefly luciferase-mediated bioluminescence on CXCR7 homodimers as CXCL12. However, both chemokines were ineffective in modulating basal firefly luciferase PFC of CXCR4/CXCR7 heterodimers [76]. Hence, the majority of data supports that GPCRs dimerize during biosynthesis and are transported as preformed dimers to the cell surface. Consequently, the ratio between homo- and heterodimers that are formed, is determined by the respective propensity to homo- or heterodimerize in combination with the relative expression levels of the two GPCRs, and is not affected by ligand binding [52, 70, 77]. In contrast, however, the ratio between CXCR4 and δ-opioid receptors (δ-OPR) homodimers, and CXCR4/δ-OPR heterodimers on the cell surface appears to be in a dynamic equilibrium, which seemed to be sensitive to the presence of respective ligands in the microenvironment of the cells [78]. In this study, the δ-OPR-mediated disruption of FRET between CXCR4 homodimer partners was inhibited by the δ-OPR agonist [D-Pen2, D-Pen5]enkephalin (DPDPE), but not by the simultaneous addition of DPDPE and CXCL12. Interestingly, the CXCR4/δ-OPR heterodimer was found to be functionally silent. Hence, individual (homodimeric) receptor responses can be suppressed by inducing receptor heterodimerization through co-administration of both ligands.

Some antiCCR2- and CCR5-specific monoclonal antibodies (mAb), but not Fab fragments, have been reported to promote CCR2 and CCR5 homodimerization, respectively [61, 64]. Such antibody-induced CCR5 homodimerization was proposed to inhibit HIV infection *in vitro* and *in vivo* [64], while HIV-1 entry was also suggested to be inhibited *in trans* via a CCR2-dependent mechanism involving the anti-CCR2 mAb-induced formation CCR2/CCR5 and CCR2/CXCR4 heterodimers [62]. These mAbs did not compete for chemokine or HIV-1 envelope glycoprotein gp120 binding, or promote receptor signaling or downmodulation [62, 64]. Hence, the mechanism by which these mAbs would inhibit HIV entry is puzzling, as the applied crosslinking co-immunoprecipitation methods and FRET techniques to assess receptor interactions, did not allow to discriminate between *de novo* formation of receptor dimers or clustering of pre-existing dimers in higher order oligomers. In other studies, CCR5-specific mAbs have been reported to increase the BRET signal between CCR5 partners [51, 69, 79]. Monovalent forms of these anti-CCR5 mAbs were ineffective in modulating the BRET signal, suggesting that the observed effects are not the consequences of conformational

changes within dimers. Disruption of lipid rafts by methyl-β-cyclodextrin-induced cholesterol depletion prevented both receptor clustering and antibody-promoted BRET increases, without affecting constitutive BRET signals [51]. This suggests that antibodies do not induce *de novo* dimerization of receptor monomers at the cell surface, but rather promote clustering of preexisting dimers [79].

5.7
Functional Consequences of Chemokine Receptor Dimerization

Functional consequences of chemokine receptor dimerization have been investigated in recombinant cells expressing a single or several receptors, and confirmed afterwards on native leukocyte populations endogenously expressing the receptors of interest. Heterodimerization has obvious consequences on the pharmacological properties of the receptors in binding assays. This was first demonstrated for CCR5 and CCR2. Following co-expression of the two receptors, CCR5-specific ligands acquired the ability to compete for the binding of CCL2, a CCR2-specific tracer, and CCR2-specific ligands competed for the CCR5 tracer CCL4. The extent of this cross-competition correlated with the relative amount of receptors expressed in these cells [68]. This competition within CCR5/CCR2 heterodimers was demonstrated to involve an allosteric mechanism, as increased dissociation of the ligand of one receptor was observed following chemokine binding to its dimerization partner [70]. Similar observations were made for other receptor pairs, including CCR2 and CXCR4 and CCR5 and CXCR4. Moreover, for these pairs, the negative binding cooperativity was extended to some receptor antagonists, including TAK-779 (a CCR5 and CCR2 inverse agonist) and AMD-3100 (a CXCR4 antagonist). Therefore, these antagonists were demonstrated to partially inhibit the signaling promoted by chemokines acting on receptors to which the antagonists did not bind directly. The binding and functional data were confirmed on native leukocyte populations expressing naturally CCR5, CCR2 and CXCR4, namely T lymphoblasts and monocytes, demonstrating the existence of functional chemokine receptor heterodimers in these cells ([72] and unpublished data).

These data support therefore that chemokine receptor homodimers and heterodimers are able to bind a single chemokine with high affinity. This allosteric mechanism presumably involves a concerted conformational change of the two protomers following ligand binding to one of them. Whether this conformational change is dependent on the asymmetric docking of the G protein to the receptor dimer remains to be determined.

5.8
(Patho-)Physiological Consequences of Chemokine Receptor Dimerization

In particular chemokine receptor heterodimers can have distinct pharmacological properties as compared to individual receptors (i.e., monomers and/or homodimers). Eventhough GPCR dimers are being identified and functionally characterized

at a rapid pace using heterologous expression systems, very limited data is available on the presence and role of these heterodimers in native tissues. Importantly, evaluating the tissue-specific pharmacological performance of such heterodimers in response to their cognate ligands is essential to define their (patho-)physiological significance. Pin *et al.* (2007) have proposed recommendations regarding the criteria used to recognize the existence of a GPCR heterodimer [80]. Such heterodimers should be firmly demonstrated in native tissues, by the evidence for physical association in native tissue or primary cells, the existence of a specific functional property for the heterodimeric receptor and modifications of these specific functional properties in knockout animals or following RNAi-mediated gene knockdown.

5.8.1
Chemokine Receptor Heterodimerization with Chemokine Receptors

A number of chemokine receptors have been demonstrated to homodimerize. This includes CCR2, CCR5, CCR7, CXCR1, CXCR2, CXCR4 and CXCR7 (see Table 5.1 for references). As stated above, these interactions are believed to be constitutive, occurring during, or shortly after synthesis, and being unaffected by chemokines. A growing number of chemokine receptor heterodimers have also been reported: CCR2-CCR5, CCR2-CXCR4, CCR5-CXCR4, CXCR1-CXCR2, CXCR4-CXCR7 (Table 5.1). As absence of dimerization between a tested pair of chemokine receptors was not reported so far, it is quite likely that all chemokine receptors are able to homodimerize and heterodimerize according to their relative expression levels and affinity for one another. Functional consequences of heterodimerization have been reported for several of these pairs (see above).

5.8.2
Chemokine Receptor Heterodimerization with Decoy Chemokine Receptor

DARC is expressed on erythrocytes and venular endothelial cells and is upregulated during inflammation [12]. DARC is a nonsignaling chemokine receptor that binds and internalizes inflammatory CC and CXC chemokines, thereby attenuating the chemokine-mediated inflammatory response. Interestingly, DARC can also directly inhibit CCR5-mediated increases in intracellular Ca^{2+} levels and chemotaxis in response to CCL5, without affecting CCR5 internalization rates, by means of a direct physical interaction [81]. DARC/CCR5 heterodimers are constitutively formed and unaffected by chemokine stimulation. DARC and CCR5 are co-expressed on umbilical vein endothelial cells, however, the physiological consequence of DARC/CCR5 heterodimerization in relation to inflammatory and immune reactions remains to be elucidated.

5.8.3
Chemokine Receptor Heterodimerization with Virally Encoded GPCRs

Most human herpesviruses have one or more genes encoding for GPCR proteins that are produced by human host cells upon viral infection [13]. EBV is an orally

transmitted γ-herpesvirus that is associated with various carcinomas and lymphomas [82]. Upon initial infection, EBV resides lifelong in a latent form in memory B lymphocytes. Differentiation of these memory B lymphocytes into antibody-secreting cells induces the transcription of viral lytic genes, among which the EBV-encoded GPCR BILF1 [83, 84]. Interestingly, this foreign GPCR, BILF1, heterodimerizes with various chemokine receptors that are important for the directional migration of these B cells (Table 5.1) [85]. Although the functional consequence of BILF1 heterodimerization with human chemokine receptors remains to be elucidated, one might envision that BILF1 might affect *in trans* the responsiveness of these chemokine receptors to their cognate ligands, which might be beneficial for viral survival and/or dissemination and might contribute to herpesvirus-associated pathologies.

5.8.4
Chemokine Receptor Heterodimerization with Opioid Receptors

The three opioid receptors (i.e., δ-OPR, κ-OPR, μ-OPR) are widely expressed on white blood cells and modulate chemotaxis and cytokine production of these cells in response to opioids [86]. Chemokines and opioids can regulate each others' responsiveness by heterologous desensitization of their receptors, possibly through direct physical interactions as reported between CCR5 and μ-OPR [87]. The receptors CXCR4 and δ-OPR constitutively form both homodimers as well as CXCR4/δ-OPR heterodimers [78]. Stimulation of these cells with either CXCL12 or DPDPE induced pronounced CXCR4- or δ-OPR-mediated signaling, respectively. However, co-stimulation of these receptors with both agonists suppressed all signaling, without inducing phosphorylation or internalization. Since DPDPE was able to inhibit the δ-OPR-mediated disruption of CXCR4 homodimers, the authors speculated that CXCR4 and δ-OPR homo- and heterodimers exist in a dynamic equilibrium in which individual agonists induce the formation of homodimers that can activate intracellular signaling, whereas co-addition of both agonists induces CXCR4/δ-OPR heterodimerization. Interestingly, these CXCR4/δ-OPR heterodimers appear to be incapable of activating intracellular signaling, which might have interesting consequences for inflammation and the sensation of pain [78]. In contrast, however, the CXCR2 antagonist SB225002 enhanced the activity of opioid agonists that act at the δ-OPR within CXCR2/δ-OPR heterodimers [77].

5.8.5
Chemokine Receptor Heterodimers: Innovative Drug Targets?

The existence of heterodimers involving chemokine receptors paves the way for using this class of receptors as therapeutic targets in original ways. Most available small molecule antagonists were derived from screening campaigns based on binding or functional assays directed to a single (homodimeric) chemokine receptor. The opportunity of acting on a specific receptor through one or several of its dimerization partners in an allosteric manner should however be considered. As an example, acting with a small molecule allosteric inhibitor on an abundant receptor

able to heterodimerize with a variety of other, less abundant, receptors might result in a higher efficacy than antagonizing specifically one of these receptors. Alternatively, acting on a receptor heterodimer through heterodimer-selective ligands might allow targeting more specifically a cell population in which the two receptors are co-expressed, while other cells expressing only one of the receptors would remain unaffected. The feasibility of this latter approach has been demonstrated in the field of opioid receptors [88].

Acknowledgment

H.F.V. is supported by a Veni grant from the The Netherlands Organization for Scientific Research (NWO).

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Part Two
Chemokine Receptors in Disease

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

6
Chemokine Receptors in Inflammatory Diseases

Aletta D. Kraneveld, Saskia Braber, Saskia Overbeek, Petra de Kruijff, Pim Koelink, and Martine J. Smit

6.1
Introduction

Inflammation and the immune system are closely related. An immune reaction is a complex reaction to tissue injury and infection, characterized by the classic response of rubor (redness), calor (heat), tumor (swelling), dolor (pain) and functio laesa (loss of function). The immune system consists of cells and soluble factors that mediate the reaction in order to eliminate the immune stimulus and initiate the process of immunological memory. Diseases of immunity can occur due to inappropriate inflammation or when the normal immune response progresses to chronic inflammation, either because of a long-term inappropriate response to stimuli (e.g., allergies) or because the offending agent is not removed (e.g., autoimmunity).

The major events in chronic inflammatory responses are continuous activating tissue resident immune cells and ongoing infiltration of circulating immune cells after which mechanisms of innate and adaptive immunity serve to neutralize and remove the inflammatory stimulus. Chemokines are a subset of cytokines that promote immune cell trafficking and localization to sites of inflammation.

Several pharmacological strategies are used to target the pathophysiology of inflammatory/immune diseases. One involves modification of the signaling mediators of the inflammatory process. A second approach is to induce suppression of components of the immune system. Both approaches are the rational for drugs that affect the production of inflammatory mediators and cells from the immune system. The molecular events in the relevant pathways leading to immune diseases are still not fully unraveled but more understanding promises to yield a number of new drugs in the foreseeable future.

This chapter discusses the role of chemokines and their receptors in inflammatory diseases of the airways (asthma and COPD), the intestinal tract (inflammatory bowel diseases), the joints (arthritis), the blood vessels (arteriosclerosis), the central nervous system (multiple sclerosis) and the skin (psoriasis). Investigations of receptor-mediated and intracellular signal pathways in chemokine–receptor interactions

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

might help to develop more effective therapeutic approaches for chronic inflammatory diseases.

6.2 Chemokine Receptors on Inflammatory/Immune Cells

There are many different cell types in the immune system and these cells interact in a complex reaction of signaling and communication to create the overall response. The cells of the immune system derive from two types of cells in the bone marrow; myeloid stem cells and lymphoid stem cells. Myeloid cells give rise to precursor cells of the innate immune system, whereas lymphoid cells generate precursors of cells of the adaptive immune system. Chemokine receptors are found on almost all immune cells.

6.2.1 Chemokine Receptors and Innate Immune Cells

Cells of the innate immune system are the first responders of the immune system. Innate immune cells perform three important tasks:

1) Neutralization of infectious agents by secretion of cytotoxic proteins or phagocytosis.
2) Antigen presentation for activation of the adaptive immune system.
3) Secretion of numerous cytokines that further amplify the immune response.

The major cell types of the innate immune system include monocytes (blood precursor cells of antigen-presenting cells), antigen-presenting cells (macrophages, dendritic cells), granulocytes (neutrophil, eosinophils, basophils), mast cells and natural killer cells. All these cells depend on chemokines for migration and, additionally, they are an important source for chemokines.

6.2.1.1 Monocytes and Macrophages

Monocytes originate in the bone marrow from a common myeloid progenitor and are released into the peripheral blood, where they circulate for several days before entering the tissue. In response to inflammation signals, monocytes are quickly recruited from the blood into the tissue, where they differentiate into macrophages and dendritic cells, thereby inducing the immune response [1].

Monocytes are divided in two subsets based on the expression of CD14, a component of the lipopolysaccharide receptor complex, and CD16, also known as the FcγRIII immunoglobulin receptor [2]. The first subset is characterized by a high expression of CD14 ($CD14^{hi}CD16^{-}$; $CD14^{+}$ monocyte), whereas the second subset co-expresses CD16 and low levels of CD14 antigens ($CD14^{+}CD16^{+}$; $CD16^{+}$ monocyte) [3]. These subsets express different adhesion, immunoglobulin and scavenger receptors [1]. In addition, the two subsets express distinct chemokine receptors on the cell surface, thereby reacting differently in migration.

The $CD14^+$ monocytes, which are also called the classic monocytes, express high levels of the chemokine receptors CCR1, CCR2, CCR4, CCR7, CXCR1 and CXCR2. In addition, these monocytes also express CXCR4 and CX3CR1, although in lower levels than the proinflammatory $CD16^+$ monocytes, which also express CCR5. The $CD14^+$ monocytes therefore migrate into the tissue under the influence of CCL2, whereas the $CD16^+$ monocytes respond to CX3CL1 and CXCL12 [1–3].

The monocytes and macrophages are a huge source for chemokines themselves. Upon stimulation, these cells can release the following chemokines: CCL1–CCL10, CCL12, CCL15–CCL18, CCL20, CCL22–CCL24, CXCL1–CXCL3, CXCL6, CXCL8–CXCL11, CXCL13, CXCL14 and CXCL16 [4, 5].

6.2.1.2 **Dendritic Cells**

Dendritic cells (DCs) are antigen-presenting cells which function as sentinels of the immune system [4]. After antigen phagocytosis, the DCs present the antigen to helper T cells, killer T cells and B cells, thereby activating the adaptive immune system [4, 6].

There are two major subsets identified in humans; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). The two subsets differ in function and can be recruited to promote variable types of immunologic reactions. mDCs resemble monocytes and secrete IL-12 upon activation [7], whereas pDCs are morphologically similar to plasma cells and differ functionally from mDCs by their ability to produce massive amounts of type I interferons in response to viral and microbial stimuli [7, 8].

mDC precursor cells in the peripheral blood express CCR2 and CXCR4. After migration into peripheral tissues, they differentiate to become immature mDCs. The chemokine receptor repertoire changes drastically from two receptors into a whole range of chemokine receptors, including CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2 and CXCR4. After activation and changing in the mature phenotype, the cells loses the ability to express almost all the chemokine receptors except CCR7 and CXCR4 [4, 6].

Blood pDCs express a different chemokine receptor pattern, including CCR2, CCR5, CCR7, CXCR3 and CXCR4. However, only CXCR3 and CXCR4 have been demonstrated chemotactic for the pDCs [8].

DCs can be a substantial source of chemokines. Upon stimulation, immature mDCs can release CCL3–CCL5, CXCL8 and CXCL10, whereas mature mDCs release CCL17–CCL19, CCL22 and CX3CL1 to attract NK, T and B cells [4, 6, 9]. Following stimulation, pDCs mostly release CCL3, CCL4, CCL22 and CXCL8 [10].

6.2.1.3 **Neutrophils**

Neutrophils are polymorph nuclear cells that are critical for defense against bacterial and fungal infections. Neutrophils are traditionally known to express only a very limited number of chemokine receptors and the recruitment of these phagocytes to the side of infection is mainly directed by the CXC chemokine subfamily, in particular CXCR1 and CXCR2 [11–13]. However, the expression pattern of the chemokine receptors on the neutrophil is subjected to change. Hartl *et al.* (2008) described that neutrophils while infiltrating at the pulmonary and synovial site of inflammation

acquire a distinct chemokine receptor expression repertoire, enabling them to adapt to chronic inflammatory conditions. In addition to the expression of CXCR1 and CXCR2, the infiltrated neutrophils from patients with chronic inflammatory lung diseases and rheumatoid arthritis express CCR1, CCR2, CCR3, CCR5, CXCR3 and CXCR4 [12].

Neutrophils can not only bind chemokines via the chemokine receptors. Upon stimulation, neutrophils can be a source for various chemokines by releasing CCL2–CCL4, CCL19, CCL20, CXCL1–CXCL3 and CXCL8–CXCL11 [4, 5].

6.2.1.4 **Eosinophils**

Eosinophils are polymorph nuclear cells that are associated with allergy and are thought responsible for defense against parasites. These granulocytic leukocytes derive from the bone marrow under the influence of IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor [5]. Under homeostatic conditions, eosinophils leave the bone marrow and migrate to the gastrointestinal tract. However during inflammation, both maturated eosinophils and eosinophil progenitors leave the bone marrow and enter the bloodstream under the influence of chemotactic signals released by activated endothelial cells [14]. The cells leave the bloodstream near sites of allergic inflammation and migrate into the tissue toward the site of inflammation. After activation by cytokines released by Th2 cells, these cells differentiate to mature effector cells, after which they release toxic granule proteins and free radicals, thereby killing the microorganisms and parasites [5, 14].

Eosinophils can express several chemokine receptors, such as CCR1, CCR2, CCR4, CXCR2, CXCR3 and CXCR4 [4, 11, 15–18]. However, CCR3 is the chemokine receptor which is most highly expressed by eosinophils [4, 5, 11, 15–18].

The presence of eosinophils in the lung tissue and alveolar space is one of the most profound characteristics of allergic asthma. The migration of these cells out of the blood and into the lung tissue is mediated by a gradient of CCL3, CCL7 and CCL22, expressed by lung macrophages. This gradient changes near the airway epithelium, where CCL5 and CCL11 are highly expressed [4, 9]. However, CCL5 and CCL11 are not solely responsible for the eosinophil migration to the lungs. The other CCR3 ligands CCL7, CCL8 and CCL13 might also play a role in the migration [4, 5, 19]. CCL11 uniquely binds to CCR3 on the eosinophils. In addition, two other CCR3 ligands, CCL24 and CCL26, are also known to serve as specific eosinophil chemoattractant acting via CCR3. However, the expression of CCL24 and CCL26 has not been reported in asthmatics [19].

Activated eosinophils augment the immune response by releasing chemical mediators, such as prostaglandins, leukotrienes, cytokines and chemokines. More precisely, the chemokines released by eosinophils are CCL3, CCL4, CCL11 and CXCL5 [4, 5].

6.2.1.5 **Basophils**

Basophils are derived from the bone marrow and are the least abundant granulocytes. Their function is poorly understood. Normally, these cells are rarely found in tissue.

However, during allergy in for instance the lungs or the skin, their numbers increase considerably [20].

Basophils can express several chemokine receptors, such as CCR1, CCR2, CCR3, CCR4, CXCR1 and CXCR4 [4, 8, 11, 15–17, 21]. Uguccioni *et al*. described that CCR3 expressed on the basophil is mainly responsible for the chemotaxis from the blood to the site of inflammation. These basophils migrate in response to CCL5 and CCL13. Interestingly, this study also showed that CCL11, known to be a specific eosinophil chemoattractant, is chemotactic for basophils [21].

Basophils can be a source for the chemokines CCL4 and CXCL8 [4, 5].

6.2.1.6 **Mast Cells**

Mast cells arise from bone marrow-derived progenitor cells that circulate as undifferentiated $CD34^+$ mononuclear cells in the peripheral circulation. Subsequently, these undifferentiated $CD34^+$ cells migrate into tissue and mature under the influence of locally derived growth factors and cytokines.

Mast cells contain large granules that store various mediators, such as histamine. Upon stimulation, these cells release the contents of the granules, leading to a hypersensitivity reaction. Mast cells have a pivotal role in allergic diseases such as asthma [22].

Human mast cells cultured from different sources, including the human lung, cord blood progenitors and bone marrow progenitors, have distinct chemokine patterns. For instance, human lung mast cells express CCR1, CCR3, CCR4, CCR7, CXCR1, CXCR3, CXCR4 and CXCR6 [9, 21], whereas the cord blood mast cells express also CCR5, CXCR1 and CXCR2, but no CCR7, CXCR3 and CXCR6 [22, 23] and the bone marrow mast cells express CCR1, CCR4, CCR7, CXCR2, CXCR4 and CXCR6 [22]. Moreover, in addition to the previously mentioned chemokine receptors, human mast cells can also express CX3CR1 and XCR1 [4, 5].

Human mast cells can release chemokine after cross-linking of the IgE receptor. These chemokines are CCL1-5, CCL7, CCL11, CCL19, CXCL1, CXCL2, CXCL5, CXCL8 and XCL1 [4, 5, 19].

6.2.1.7 **Natural Killer Cells**

Natural killer (NK) cells are cytotoxic lymphocytes, which kill tumors and virus-infected cells. Two major NK cell subsets have been identified in humans: $CD56^{dim}$ $CD16^+$ ($CD56^{dim}$; ~90% of all NK cells) and $CD56^{bright}$ $CD16^-$ ($CD56^{bright}$; ~10% of all NK cells) [24, 25]. The two subsets have distinct functional activity, cell-surface antigen expression and chemokine receptor expression [24]. Freshly isolated, unstimulated $CD56^{dim}$ NK cells mediate natural killing and antibody-dependent cellular cytotoxicity. In a resting state, they express CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1, and their migration is mediated by CXCL12 and CX3CL1. There are no detectable levels of CC chemokine receptors on the cell surface [24].

Freshly isolated $CD56^{bright}$ cells have weak cytolytic activity and are thought to predominantly regulate other cells through cytokine production. In contrast to the $CD56^{dim}$ NK cells, resting $CD56^{bright}$ NK cells express little CXCR1, CXCR2 and

CX3CR1 but high levels of CCR5 and CCR7. These NK cells migrate in response to CCL19, CCL21, CXCL10, CXCL11 and CXCL12 [24].

In addition, other studies revealed that NK cells can also express other chemokine receptors. Pokkali *et al.* demonstrated that NK cells during a *Mycobacterium tuberculosis* infection change their chemokine pattern; $CD56^{dim}$ NK cells express higher levels of CCR5, whereas $CD56^{bright}$ cells increase the expression of CCR1, CCR2 and CCR7 [24]. Moreover, NK cells are also known to express CCR4, CCR6 and XCR1 [4, 5, 11, 15, 18].

NK cells can produce several chemokines. After stimulation, they can release CCL1, CCL3−CCL5, CCL15, CCL22, CXCL8 and XCL1-2 [4, 5, 24].

6.2.2 Chemokine Receptors and Adaptive Immune Cells

While innate immune mechanisms contribute to the first line of defense, at the same time, pathogens are taken up and presented by antigen-presenting cells to adaptive immune cells to allow the induction of an antigen-specific immune response directed against distinctive molecular targets. This process will lead either to a humoral immune response, where antigen-specific immunoglobulins produced by B lymphocytes play a central role, or to a cellular immune response, where antigen-specific $CD4^+$ T lymphocytes (Thelper1, Thelper2, Thelper 17 cells) or $CD8^+$ T cells (cytotoxic T cells) are the central players. Chemokines are crucially involved in the migration and homing of B cells and T cells.

6.2.2.1 B Lymphocytes

B cells are one of the two major types of lymphocytes. These bone marrow derived cells express a cell-surface immunoglobulin, also referred to as a B cell receptor. Upon activation by antigen, these cells differentiate into plasma cells and produce antibodies of the same type as their receptor [26].

After being released from the bone marrow, mature B cells migrate to the secondary lymphoid organs in a process called homing, which is a CXCR5/CXCL13-dependent process. All mature B cells, including recirculating follicular B cells, marginal zone (MZ) B cells, and peritoneal B1 B cells express CXCR5. The B cells migrate to the lymph node under the influence of CXCL13, a chemokine ligand released by B cell follicles [27].

After antigen activation, B cells upregulate CCR7 and subsequently relocate from the follicle to the B/T zone. After interaction with antigen-specific T cells, B cells differentiate into activated antibody-secreting plasma cells. Due to the downregulation of CCR7 and CXCR5 and an upregulation of CXCR4, the plasma cells relocate to the red pulp of the spleen and the medullary cords of lymph nodes. CXCR4 expression is also essential for plasma cell homing to the bone marrow [11, 26, 27].

However, B cells are also capable of expressing other chemokine receptors than previously mentioned. Studies have demonstrated that B cells can also express CCR1, CCR2, CCR5, CCR6, CCR8−CCR10 and CXCR3 [4, 11, 15–18, 28].

B cells can produce several chemokines. After stimulation, they can release CCL15, CCL22, CXCL14 and CXCL16 [4, 5].

6.2.2.2 T Lymphocytes

T cells are bone marrow-derived lymphocytes. There are six different types of T cells identified in humans: helper T cells (Th), cytotoxic T cells (Tc), memory T cells, regulatory T cells (Treg), natural killer T cells (NKT) and γδ T cells. All types have different roles in the adaptive immune systems and consequentially distinct chemokine receptor expression patterns.

Naive $CD4^+$ T cells leave the thymus expressing abundant amounts CCR7, migrating in response to CCL19 and CCL21, which are produced by the high endothelial venules of lymph nodes. In addition, naïve $CD4^+$ T cells also express CXCR4. However, this receptor is not essential for entering the lymph nodes [5, 19, 26, 32]. Shortly after activation, the naïve $CD4^+$ T cells differentiate into Th0 cells, expressing CCR7, CCR5 and CXCR3. Subsequently, the Th0 cells develop in Th1 or Th2 cells, depending on the environment. These helper T cells have distinct chemokine patterns; Th1 cells express CCR5 and CXCR3, whereas Th2 cells express CCR3, CCR4, CCR8 and CXCR4 [5, 19]. However, when the helper T cells or cytotoxic T cells differentiate into effector T cells, the chemokine receptor profile changes drastically, enabling these cells to migrate into the tertiary lymphoid tissues. This migration is organ specific and chemokine provide a "homing signal" for the effector cells expressing the correct chemokine receptor [5, 29].

$CD4^+$ Th0 cells can also develop in Th3 (or Tr1; adaptive $CD4^+$ regulatory T cell), $CD4^+CD25^+FoxP3^+$ regulatory T cells ($CD4^+$ Treg cells), Th17 or ThF (Follicular helper T cell) cells, which all have a distinct chemokine receptor pattern. Th3 cells can express CCR6 [30], whereas $CD4^+$ Treg cells can express CCR2, CCR4, CCR5, CCR7 and CXCR4 [31]. Th17 are capable of co-expressing CCR4 and CCR6 [30, 32] and ThF cells can express CXCR5 [33].

Memory $CD4^+$ and $CD8^+$ T cells can be divided in "effector" and "central" memory T cells. Effector memory cells are thought to home to nonlymphoid tissues, whereas the central memory T cells localize more to secondary lymphoid tissue [29]. Although originally distinguished by the expression of CCR7 by the central memory T cells, it is clear that both subsets express CCR7 [29, 34]. However, it is thought that these memory cells can express every appropriate chemokine receptor [29].

Most peripheral blood NKT cells express CCR1, CCR2, CCR5, CCR6, CXCR3, CXCR4 and CXCR6, which mediate homing to extra-lymphoid tissue or sites of inflammation. However, a few NKT cells express lymphoid tissue homing chemokine receptors CCR7 and CXCR5 [5].

γδ T cells represent a small subset of T cells that express a distinct T cell receptor (TCR) repertoire on their surface; these cells have a TCR composed of a γ and a δ chain instead of an α and a β chain. γδ T cells can express CCR1-3, CCR5, CCR9, CXCR1-3 and CXCR5 [33, 35, 36, 40].

All T cells are capable of releasing chemokines. After the appropriate stimulation, T cells can produce CCL1, CCL5, CCL15, CCL20, CCL22, CCL24, CXCL8, CX3CL1, XCL1 and XCL2 [4, 5].

Table 6.1 CXC chemokine receptor family and inflammatory cells.

Receptor	Ligands	Expression
CXCR1	CXCL6, CXCL7, CXCL8	Neutro, baso, mono/mΦ, immature mDC, NK cell, MC
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	Neutro, eosino, mono/mΦ, immature mDC, microvascular endothelial cell, T cell, NK cell, MC
CXCR3A	CXCL9, CXCL10, CXCL11	Neutro, eosino, pDC, Th1 cell, B cell, NK cell, MC
CXCR3B	CXCL4, CXCL9, CXCL10, CXCL11	Microvascular endothelial cell, neoplastic cell
CXCR4	CXCL12	Neutro, eosino, baso, mono/mΦ, immature mDC, mature mDC, pDC, naïve T cell, memory T cell, Th1 cell, Th2 cell, Th17 cell, Treg cell, B cell, NK cell, platelets
CXCR5	CXCL13	B cell, T cell
CXCR6	CXCL16	Memory T cell, Th1 cell, NK cell
CXCR7	CXCL11, CXCL12	Tumor cell

Neutro: neutrophils; eosino: eosinophils; baso: basophils; mono: monocytes; mΦ: macrophages; DC: dendritic cells; pDC: plasmoid dendritic cells; mDC: myeloid dendritic cells; Th cell: Thelper cells; Treg cells: regulatory T cells; fibro: fibroblasten; NK cells: natural killer cells; MC: mast cells [4, 5, 11, 15–18, 37–40].

6.2.3
Conclusion

In general, CXC chemokines are attractants for neutrophils and T lymphocytes and ELR-CXC chemokines are attractants for B and T lymphocytes (Table 6.1). CC chemokines induce chemotaxis of multiple subsets of white blood cells, such as monocytes, basophils, dendritic cells, macrophages, NK cells and T cells (Table 6.2). C chemokines are important for T cells traveling to the thymus; and the CX3C chemokine, fractalkine, also acting as an adhesion molecule, seems to be important for the infiltration of T cells, NK cells and monocytes (Table 6.3).

6.3
Chemokine Receptors and Inflammatory Lung Diseases

The two most prevalent chronic inflammatory lung diseases are asthma and chronic obstructive pulmonary disease (COPD). These diseases are a major and increasing global health problem. Both asthma and COPD share some clinical features, such as increased airway obstruction, mucous hypersecretion, acute exacerbations and respiratory symptoms. Asthma and COPD are identified by the presence of chronic inflammation of the airways, which is controlled by the increased expression of inflammatory proteins, including cytokines, chemokines, receptors, enzymes and

Table 6.2 CC chemokine receptor family and inflammatory cells.

Receptor	Ligands	Expression
CCR1	CCL2, CCL3, CCL3LI, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23	Neutro, eosino, baso, mono/mΦ, immature DC, memory T cell, B cell, NK cell, MC
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16	Neutro, eosino, baso, mono/mΦ, immature DC, pDC, B cell, memory T cell, Treg cell, NK cell
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL24, CCL26, CCL28	Neutro, eosino, baso, T cell, Th2 cell, MC, platelets, endothelial cell
CCR4	CCL17, CCL22	Eosino, baso, mono/mΦ, immature DC, mature DC, Th2 cell, Treg cell, NK cell, thymocyte, platelets
CCR5	CCL3, CCL3LI, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL16	Mono/mΦ, pDC, immature mDC, mature mDC, Th1 cell, Treg cell, B cell, NK cell, thymocyte
CCR6	CCL20	Immature mDC, memory T cell, Th17 cell, Treg cell, B cell
CCR7	CCL19, CCL21	Mature mDC, pDC, naïve T cell, Th1 cell, Th2 cell, Treg cell, B cell, NK cell
CCR8	CCL1	Neutro, mono/mΦ, DC, Th2 cell, Treg cell, B cell, thymocyte
CCR9	CCL25	Memory T cell, thymocyte, epithelial cell, IgA^+ plasma cell
CCR10	CCL27, CCL28	Memory T cell, B cell, fibro, epithelial cell

Neutro: neutrophils; eosino: eosinophils; baso: basophils; mono: monocytes; mΦ: macrophages; DC: dendritic cells; pDC: plasmoid dendritic cells; mDC: myeloid dendritic cells; Th cell: Thelper cells; Treg cells: regulatory T cells; fibro: fibroblasten; NK cells: natural killer cells; MC: mast cells [4, 5, 11, 15–18, 37, 39].

adhesion molecules [41, 42]. Although there is a considerable overlap in the pathogenesis between COPD and asthma, there are marked differences in the characteristics of this inflammatory process. Asthma and COPD may be distinguished by the site of inflammation, types of inflammatory cells, different mediators, different inflammatory effects and different response to treatment [42, 43]. Due to the

Table 6.3 XC and CX_3C chemokine receptor and inflammatory cells.

Receptor	Ligands	Expression
XCR1	XCL1, XCL2	T cell, NK cell, MC
CX3CR1	CX3CL1	Neutro, mono/mΦ, DC, Th1 cell, NK cell, endothelial cell

Neutro: neutrophils; mono: monocytes; mΦ: macrophages; DC: dendritic cells; Th cell: Thelper cells; NK cells: natural killer cells; MC: mast cells [4, 5, 11, 12, 15, 17, 18, 37, 39].

growing evidence that the recruitment of the inflammatory cells in asthma and COPD is largely regulated by chemokines acting as ligands for chemokine receptors, this section briefly summarizes the involvement of chemokine receptors in the two most relevant inflammatory lung disorders.

6.3.1 COPD

COPD, a term referring to two lung diseases: chronic bronchitis and emphysema, is characterized by airflow limitation that is not fully reversible, is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases [44]. COPD is primarily associated with cigarette smoke, where recurrent lung inflammation leads to a progressive decline in lung function. Most of the pathologic changes caused by inflammation are found in the small airways and in lung parenchyma [43].

COPD is a complex inflammatory disease that involves different inflammatory cell types, like macrophages, neutrophils and $CD8^+$ T lymphocytes [42, 45, 46].

6.3.2 Chemokine Receptors in COPD

In COPD the inflammatory cascade starts with exposure to cigarette smoke or other irritants, which activate the epithelial cells and the macrophages in the respiratory tract. When these cells are activated, they have the capacity to release several chemotactic factors. Chemotactic factors use specific chemokine receptors to induce inflammatory cell migration to the airways (See Figure 6.1 [42]).

First, the chemotactic factor CCL2 (MCP-1) is a CC-chemokine that mediates its effects via the CCR2 chemokine receptor. This specific receptor for CCL2 is expressed by monocytes, macrophages, T lymphocytes and epithelial cells. It is known from literature that the chemoattractant CCL2 and the receptor CCR2 are involved in the recruitment of monocytes into the airway epithelium in COPD. Under migration into tissues, monocytes differentiate into macrophages. Macrophages are important in the pathogenesis of COPD, reflected in an increased number of macrophages in the lungs of COPD patients [47–49]. Nevertheless, the CCR2 receptor might play a crucial role in COPD, since CCL2 levels are increased in the sputum, BALF and lungs of patients suffering from COPD [48].

One of the most important chemokines associated in the recruitment of inflammatory cells in COPD is the CXC chemokine CXCL8 [46]. CXCL8 binds to both CXCR1 and CXCR2 chemokine receptors, which are expressed on a broad range of leukocytes, predominantly neutrophils.

Recent studies demonstrated a significantly increased expression of CXCR1 on circulating neutrophils in COPD patients compared to healthy controls [50]. Furthermore, it is established that the expression of CXCR2 in bronchial biopsies of COPD patients is increased [51]. These two receptors have the capacity to regulate the migration of neutrophils into the pulmonary tissue during the neutrophilic inflammation in COPD. Also, activated neutrophils are undoubtedly crucial players in the

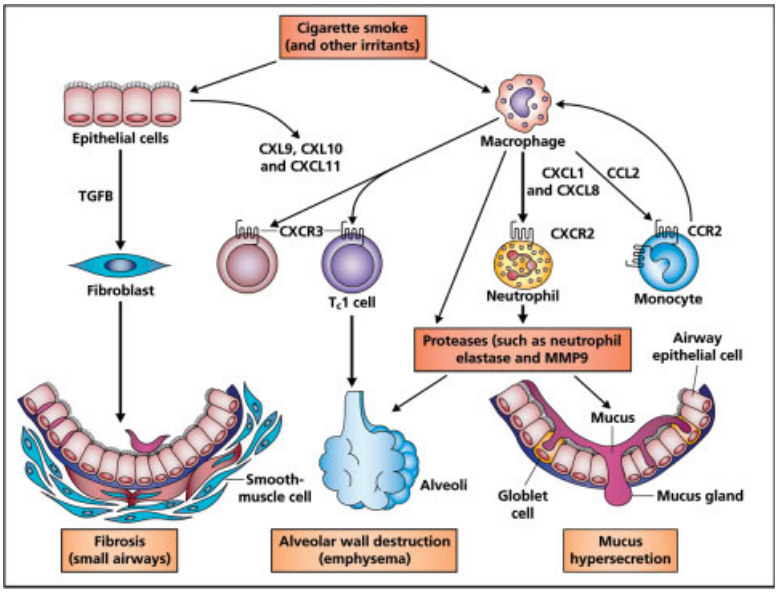

Figure 6.1 Involvement of chemokines and chemokine receptors and different cell types in the inflammation of COPD. Chemokines released from epithelial cells and macrophages in the lung recruit inflammatory cells from the circulation leading to the development of COPD. Adapted from [2].

pathogenesis of COPD. CXCL8 is secreted by several cell types, like macrophages, neutrophils and airway epithelial cells, and is a powerful chemotactic mediator for neutrophils. Among CXCL8, the CXCR2 receptor is also selectively activated by CXCL1 (GRO-α). CXCL1 is secreted by alveolar macrophages and airway epithelial cells and is a potent chemoattractant of neutrophils and monocytes.

The concentration neutrophils is increased in the sputum and bronchoalveolar lavage fluid (BALF) of COPD patients and this is related to the increased production of CXCL1 and CXCL8 [42, 46, 49, 52, 53].

It has been considered in neutrophil chemotaxis that the CXCR2 receptor responds not only to CXCL1 and CXCL8, but also to other chemokines, including CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7. CXCL5 is predominantly derived from epithelial cells and BALF cells from smokers release more CXCL5 than cells from nonsmokers [54]. CXCL7 is chemotactic for neutrophils as well as for monocytes and shows an enhanced chemotactic activity for monocytes from COPD patients which is similar to the chemotactic activity of CXCL1 [55].

In addition to macrophages and neutrophils, the T cell is also a potentially important factor in the initial inflammatory process leading to COPD. This is supported by the finding of an increased number of T cells in the airways and in lung parenchyma COPD patients, to a greater extent in $CD8^+$ T cells compared to $CD4^+$ cells [56, 57]. Lymphocytes, particularly type-1 T lymphocytes (Th1/Tc1 cells), express the chemokine receptor CXCR3. In the airways of COPD patients an increase in the number of $CXCR3^+$ T cells and an increased expression of CXCR3 was

Table 6.4 Chemokine receptors and their ligands demonstrated to be involved in COPD.

Receptor	Ligand	Target cells COPD
CXCR1	CXCL6, CXCL7,CXCL8	Neutro, mono/mΦ
CXCR2	CXCL1, CXCL2, CXCL3 CXCL5, CXCL6, CXCL7, CXCL8	Neutro, mono/mΦ
CXCR3	CXCL9, CXCL10, CXCL11	T cell
CCR2	CCL2	Mono/mΦ T cell
CCR5	CCL5	T cell

Neutro: neutrophils; mono: monocytes; mΦ: macrophages [42, 49, 59, 63, 96–102].

observed. T cells may be attracted to the lungs by IFNγ and IFNγ-induced CXCR3 receptor ligands: CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC). All three chemokines activate CXCR3 and are present at high levels in COPD airways [58, 59]. Kelsen and coworkers demonstrated that human airway epithelial cells also express the CXCR3 chemokine receptor and activation of CXCR3 by CXCL9, CXCL10 and CXCL11 may contribute to airway inflammation/remodeling in the development of COPD [60]. Furthermore, CXCR3 knockout mice showed less lung inflammation induced by cigarette smoke exposure compared with the wildtype mice [61].

Even as the CXCR3 receptor, the CCR5 receptor is also expressed on Th1 and Tc1 cells and might have a cooperative role with CXCR3 in the recruitment of these cells into the lungs [62]. The CCR5 ligand CCL5 is elevated in sputum from COPD patients, this increase is also observed in the airways and sputum of COPD patients during exacerbations [59, 63].

Due to the activity of these inflammatory cells as well as the epithelial cells by receptor–ligand interactions, the inflammatory response in COPD is further augmented leading to the induction and release of different proteases, including, matrix metalloproteinases (MMPs; e.g., MMP-9) and neutrophil elastase. This proteolytic cascade leads to the remodeling of the lung tissue by collagen and elastin degradation (emphysema) and mucus hypersecretion (chronic bronchitis). The protease–antiprotease imbalance hypothesis is thought to play a key role in the development of COPD [42, 64, 65].

Finally, epithelial cells and macrophages in the small airways also regulate the proliferation of fibroblasts by releasing transforming growth factor-β (TGFβ), resulting in fibrosis a clinical feature of COPD [66]. (See Figure 6.1 [42]). Table 6.4 summarizes the different chemokine receptors and their ligands demonstrated to be involved in COPD.

6.3.3 Asthma

Asthma is defined by three characteristic features: airway inflammation, intermittent reversible airway obstruction and airway hyperresponsiveness [67]. This results in the

clinical expression of a lower airway obstruction that usually is reversible. Asthma involves inflammation in the lower airways but without involvement of the lung parenchyma [43].

This inflammatory disease is characterized by activated T helper type 2 (Th2) lymphocytes, eosinophils and activated mast cells [68]. Several distinct asthma phenotypes exist, in particular atopic and non-atopic asthma. Atopic asthma is correlated with an elevation of total and allergen-specific IgE in the serum in contrast with the non-atopic asthmatics. In these patients there is no evidence of allergen-specific serum IgE and even total serum IgE levels are within the normal range [69].

6.3.4
Chemokine Targets in Asthma

In asthma the allergic cascade, which is crucial in the development of airway inflammation, starts with exposure to inhaled allergens and other exogenous stimuli (Figure 6.2).

These allergens bind to allergen-specific immunoglobulin E (IgE) on mast cells, crosslinking the IgE molecules and aggregating the underlying FcεRI receptors. Activation of mast cells leads to the release of several mediators, like histamine,

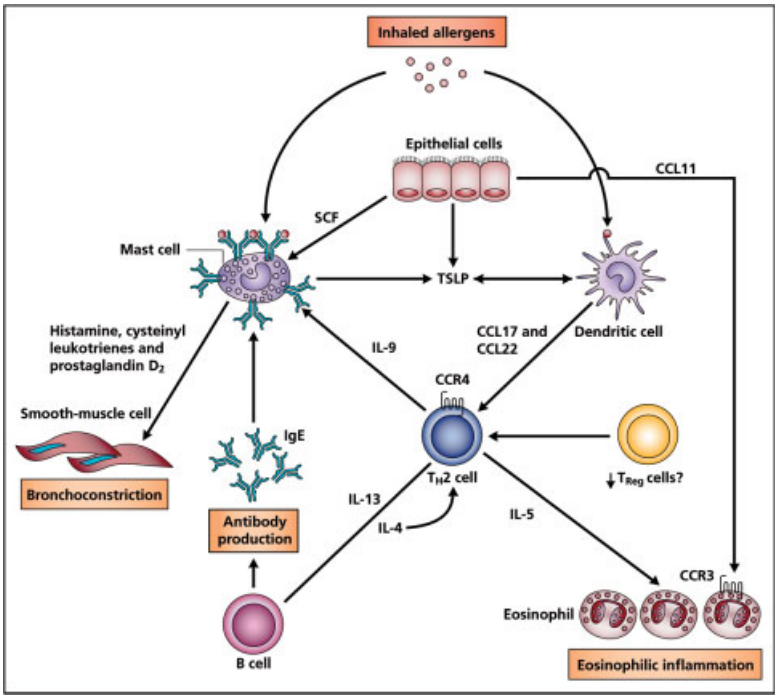

Figure 6.2 Involvement of chemokines, chemokine receptors and different cell types in the development of asthma. Adapted from [2].

leukotrienes and prostaglandins, which cause bronchoconstriction of the airway smooth muscle cells [42, 70].

In addition, mast cells also release T helper 2 (Th2)-derived cytokines, like IL-4, IL-5 and IL-13, which trigger eosinophilia, mucus hypersecretion and IgE production by B cells [71].

Besides the FcεRI receptor, human lung mast cells also express different chemokine receptors, particularly CCR1, CCR3, CXCR1, CXCR3 and CXCR4. Lung mast cell migration to the airway smooth muscle is induced through activation of these receptors by the respective ligands CCL5, CCL11, CXCL8, CXCL10 and CXCL12. This infiltration of mast cells into the airway smooth muscle has been related to airway hyperresponsiveness, found in asthma. The most abundant chemokine receptor on lung mast cells in the airway smooth muscle in asthma is the CXCR3 receptor. This is correlated with the increased expression of the CXCR3 ligand CXCL10 in bronchial biopsies of asthma patients compared to healthy controls [72–75].

Mast cells have a key role in the pathophysiology of asthma and are present in varying numbers in patients with asthma. The epithelial cells release stem cell factor (SCF), which is important for the growth, differentiation and activation of mucosal mast cells at the airway surface [76].

Epithelial cells are the first cells to encounter inhaled allergens and they interact closely with dendritic cells (DCs) by releasing thymic stromal lymphopoietin (TSLP), which is also produced by mast cells. TSLP is an interleukin (IL)-7 related cytokine. This cytokine can activate human immature DCs to promote the differentiation of naïve of T cells into Thelper 2 (Th2) cells, defined by the production of Th2 cytokines like, IL-4, IL-5, IL-9 and IL-13 [77]. All these cytokines have a specific role in the inflammatory process of asthma. IL-4 and IL13 are two cytokines which are related to the IgE production by B cells. IL-4 and IL-9 are responsible for mast cell growth. IL-5 is the key player in the eosinophilic inflammation [78–80].

Asthma is a disease in which Th2 cells play a critical role, but the mechanisms of Th2 recruitment within the lungs remain poorly defined. Th2 cells express the chemokine receptors CCR8 and CCR4 [81, 82]. After a challenge with an allergen, the CCR4 receptor and, to a lesser extent, CCR8 mark the majority of T cells infiltrating the lung of asthmatics [83]. These receptors play an important role in the recruitment of Th2 cells to the sites of inflammation in (late) asthmatic responses. It has been described that DCs can produce two ligands, which can activate the CCR4 receptor: CCL17 and CCL22. Furthermore, it is demonstrated that $CCR4^{-/-}$ and $CCR8^{-/-}$ mice develop less airway hyperresponsiveness and show a reduction of eosinophils infiltration in the airways [84, 85].

Moreover, CXCR3 and CCR5 receptors are also expressed on human lung T cells [86] and this expression increases after ovalbumin challenge in mice. Recent studies indicated that targeting these two receptors can prevent the development of asthma in a mouse model [87]. The CCR2 chemokine receptor on Th2 cells and the ligand CCL2 have also been proposed to play a role in pulmonary T cell recruitment in asthma. *In vivo* studies demonstrated that $CCR2^{-/-}$ mice are resistant to bronchial hyperreactivity after allergen challenge, compared to control mice [88]. Additionally,

Table 6.5 Chemokine receptors and their ligands demonstrated to be involved in asthma.

Receptors	Ligand	Target cells asthma
CCR1	CCL5	MC, T cell, eosino
CCR2	CCL2	T cell
CCR3	CCL5, CCL7, CCL11, CCL13	Eosino, T cell (Th2), MC
CCR4	CCL17, CCL22	T cell (Th2)
CCR5	CCL5	T cell
CCR8	CCL1	T cell (Th2)
CXCR1/CXCR2	CXCL8	MC, neutro
CXCR3	CXCL10	MC
CXCR4	CXCL12	T cell (Th2), MC

MC: mast cells; eosino: eosinophils [74, 82, 83, 87, 89, 95, 101, 103–106].

it has been suggested that the CXCR4 chemokine receptor expressed on Th2 cells is a potential therapeutic target in asthma. This because it has previously been shown that CXCL12, the CXCR4 ligand, has chemotactic properties for a variety of cells [89, 90].

Finally, there is growing evidence that besides Th2 cells, T regulatory (Treg) cells are involved in the development asthma [91, 92].

Last but not least, the eosinophil is another important participant in the pathogenesis of asthmatic inflammation and about 80% of the asthma patients show an increased number of eosinophils. Airway epithelial cells are an important source of chemokines and contribute to recruitment of eosinophils to and within the lung. Predominantly, the CCR3 ligand CCL11 (eotaxin) attracts and activates eosinophils by acting via the most prominent chemokine receptor on eosinophils: CCR3.

Ying *et al.* (1997) and coworkers demonstrated an enhanced level of eotaxin and CCR3 in asthmatic patients compared to healthy controls [93]. Other potent eosinophil chemoattractants who induce eosinophil recruitment into the airways via the CCR3 receptor are CCL5 (RANTES), CCL7 (MCP-3) and CCL13 (MCP-4) [94]. Although eosinophils are the major granulocytes in the development of COPD, a neutrophils influx is detected during acute exacerbations, in severe asthma patients and in non-eosinophilic asthma. This influx is mediated by CXCL8, which acts on the two CXCL8 chemokine receptors on neutrophils: CXCR1 and CXCR2, whereas CXCR1 is important in neutrophil activation [95, 96]. Table 6.5 summarizes the different chemokine receptors and their ligands demonstrated to be involved in asthma.

6.4
Chemokine and Inflammatory Bowel Diseases

6.4.1
Inflammatory Bowel Disease

The term inflammatory bowel disease is used to describe chronic inflammatory conditions of the gastro-intestinal (GI) tract. Inflammatory bowel disease is an

idiopathic disease characterized by swings between intestinal inflammation and remission. Patients suffer from abdominal pain and cramps, weight loss, diarrhea, cachexia, disrupted digestion, rectal bleeding and a substantial personal burden.

Inflammatory bowel disease can be subdivided into two major representatives, Crohn's disease and ulcerative colitis. Although the clinical pathological phenotype of these two disorders is similar, they can be separated by different localization of the inflammation in the GI tract and immunological and histological pattern. Roughly it can be stated that Crohn's disease is characterized by a transmural inflammation which can be found throughout the whole GI tract but mainly in the terminal ileum, ulcerative colitis is a mucosal inflammation restricted to the colon. Moreover, Crohn's disease is postulated to be a Thelper1 and Thelper17-mediated disease [107] whereas ulcerative colitis is mainly a Thelper2-mediated disorder [108].

The exact etiology of inflammatory bowel disease remains unknown but is thought to be a complex interaction of genetic, environmental (i.e., enteric microflora) and immunological factors [109, 110]. Current investigations and observations suggest that the initial event in inflammatory bowel disease is a result of a dysregulated inflammatory response rather than an aggressive inflammatory response by a defective intestinal immune system [111]. Although it is suggested that inflammatory bowel disease might be an autoimmune disease, potential enteric antigens for the exacerbation of inflammatory bowel disease are luminal bacteria, parasitic nematodes or food allergens [110, 112]. Therapy for inflammatory bowel disease is merely symptom relieving and relies highly on the use of aminosalicylates, corticosteroids and immunosuppressive drugs [113]. However, new developments in treating inflammatory bowel disease can be found in biological treatments with antibodies directed against tumor necrosis factor-alpha [114] and with pro- and prebiotics as well [115].

6.4.2
Chemokine Receptors in Inflammatory Bowel Disease

The contribution of chemokines to the pathogenesis of inflammatory bowel disease stems from a series of clinical and animal model studies (Table 6.6) [116, 117]. Several chemokines have been described in both ulcerative colitis and Crohn's disease and their expression is consistently increased during the active phase of disease. Especially, CXCL1 (Gro-α), CXCL2 (Gro-β), CXCL5 (ENA-78), CXCL6 (GCP-2) and CXCL8 (IL-8) and their receptors CXCR1 and CXCR2 are upregulated in inflammatory bowel disease as are CXCR3 and CXCR4 receptor ligands, such as interferon inducible protein 10 (CXCL10) and stromal cell-derived factor 1 (CXCL12), respectively [117–120]. In addition, CCR1, CCR2, CCR3, CCR4 and CCR5 receptor ligands are reported to be increased in inflammatory bowel disease: CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL7 (MCP-3), CCL8 (MCP-2) and regulated on activation, normal Tcells express and secrete CCL5 (RANTES) [116–118, 120]. A number of recent studies has demonstrated that antagonists targeted against chemokine or their receptors are effective in inhibiting acute and chronic inflammation in animal

Table 6.6 Chemokines and chemokine receptors and target cells demonstrated to be involved in inflammatory bowel disease.

Ligand	Receptor	Target cells IBD
CC chemokines		
CCL2		Th cell, mono, baso
CCL3		Tc cell
CCL4		Th cell
CCL5	CCR5	Th cell, mono, baso
CCL7		Mono, baso
CCL8		Neutro, baso, mono
CCL20	CCR6	Th17 cell
CCL25	CCR9	Th17 cell
?	CCR4	Treg cell
?	CCR7	Treg cell
ELR^+ CXC chemokines		
CXCL1	CXCR1/2	Neutro, baso
CXCL2	CXCR1/2	Neutro, baso
CXCL5	CXCR2	Neutro
CXCL6	CXCR1/2	Neutro, baso
CXCL8	CXCR1/2	Neutro, baso
ELR-CXC chemokines		
CXCL10	CXCR3	T cell, mono
CXCL12		T cell, B cell
CX3C chemokines		
CX3CL1	CX3CR1	Mono, DC

Tc cell: cytotoxic T cell; Th cell: T helper cell; Treg cell: regulatory T cell; neutro: neutrophils; baso: basophils; mono: monocytes; DC: dendritic cells.

models for inflammatory bowel disease. In trinitrobenzene sulfonic acid-induced colitis, the increased colonic expression of KC/mCXCL1 and CXCR2 receptor expression was associated with leukocyte recruitment, whereas increased CCL3 expression was associated with both leukocyte infiltration and the onset of ulcerative lesions [117]. In addition, CCR2 and CCR5 receptor deficient mice exhibited a reduction in colonic inflammation [121]. Notably, CCL5 (RANTES) has been shown to be crucial in the transition from acute to chronic disease in experimental model of colitis [122].

Targeting Th1 cells via CXCR3, using antibodies directed against CXCL10 or blocking the CXCR3 receptor, in different animal models demonstrated prevention of onset and cure of pre-existing colitis [120].

Th17 cells are getting more and more attention in relation to inflammatory bowel disease, especially Crohn's disease. CCR6 and its ligand CCL20 have been identified on Th17 cells in inflammatory lesions of Crohn's patients [123]. Moreover, it has also been reported that colonic epithelial cells from IBD patients show an increased expression of CCL20 [124, 125]. In addition, neutralizing CCL20 significantly ameliorated mouse colitis, which was associated with a

decreased influx of $CCR6^+$ T cells, probably of Th17 subtype, in the lamina propria [126].

CCR9, the receptor for CCL25, is abundantly expressed on intraeptithelial and lamina propria T cells. Infiltration of $CCR9^+$ T cells into the intestinal mucosa has been shown to play a role in IBD. These mucosal $CCR9^+$ T cells might be Th17 cells, since it has been demonstrated that Th17 cells express CCR9 and $CCR9^+$ T cells can produce IL17. Its targetability in IBD was recently review by Koenecke and coworkers [129]. Very recently, at the Digestive Disease Week 2009, the results of a phase II/III clinical trial using CCR9 antagonist in moderate to severe Crohn's disease patients were presented. Oral treatment with the CCR9 receptor antagonist resulted in reduced disease severity associated colonoscopic evidence of improvement [130]. Although clinical trials to are being conducted, preclinical data in animal models of IBD are lacking. Only one study reported that antibody blockade of CCR9 and CCL25 using attenuated early development of ileitis in mice but showed no therapeutic efficacy during the later stages [131]. CCR9 could be a very promising target for Crohn's disease, however more preclinical and clinical research is needed.

Regulatory T cells (Treg cells) are a critical subpopulation T cells essential for the maintenance of self-tolerance. It has been hypothesized that reduced regulatory T cell function is related to exacerbations of inflammatory bowel disease. Two reports describe the critical role of CCR4 and CCR7 in homing of Treg cells to the intestine. An inefficient accumulation of CCR4 deficient Treg cells correlated with the development of colitis [127]. And CCR7 is required for the *in vivo* function of Treg cells, since CCR7 deficient Treg cells have less capacity to provide protection in mouse colitis [128].

6.4.3
Chemokine-Mediated Neuronal Activation in Inflammatory Bowel Disease

Research has shown that chemokine receptors are not restricted to leukocytes and are also found on neurons. Chemokine receptor activity has been detected on dorsal root ganglion neurons. Moreover, human enteric nervous system neurons and glial cells have recently been demonstrated to produce chemokines such as CXCL8 and CCL4 during intestinal inflammation [132, 133]. In addition, *in vitro* studies on co-cultures of human NANC neurons and intestinal epithelial cells show that tumor necrosis factor α induced epithelium results in an upregulation of neuronal CXCL8 and CCL3 mRNA, mediated by the interleukin-1β receptor. Furthermore, pretreatment of neuronal cells with interleukin-1β resulted in chemotaxis of peripheral blood mononuclear cells which was CXCL8-dependent. Production of neuronal chemokines could therefore be responsible for the presence of leukocytes, monocytes and lymphocytes in the enteric nervous system, which could ultimately lead to the development of enteric neuropathies and central nervous system-mediated symptoms as observed in inflammatory bowel disease and irritable bowel syndrome.

Chemokines interacting with their receptors on neurons can alter excitability of NANC nerves during inflammation as well. Upregulation of CCL2 and its receptor CCR2 is described in dorsal root ganglion neurons during injury [134, 135]. CCL2/CCR2 signaling increases the excitability of NANC nerves [136]. This suggests that pain during inflammation is exacerbated by proinflammatory chemokines. Immunohistochemistry and RT PCR have revealed the expression of CCR1, CCR4, CCR5, CXCR4 and CX3CR1 receptors on populations of dorsal root ganglia that express substance P and the transient receptor potential vanilloid 1 (TRPV1) [137–139]. Of interest is that it has been reported that CX3CL1 (probably originating from epithelial cells) and $CX3CR1^+$ cells are enhanced in active Crohn's disease [140]; however a neuroimmune interaction was not investigated in these studies.

Recently, it was demonstrated that CCL3 (by interacting with the CCR1 receptor on dorsal root ganglion neurons) sensitizes TRPV1 receptor in getting more susceptible for capsaicin [138]. A similar role for the interaction CXCR4/CXCL12 on dorsal root ganglia in HIV-induced pain hypersensitivity has been reported [139–141]. CCR1 receptor co-localized with the antipain, mu opioid, receptors on dorsal root ganglia and pretreatment with CCL3, as well as CCL2, CCL5 and CXCL8, inhibited mu opioid receptor-induced calcium responses in neurons [138]. Chemokines may directly activate NANC nerves in inflammation. Interaction of CCL2 or CXCL1 with its respective receptors evoked an intracellular calcium elevations associated with the release of the neuropeptide calcitonin gene-related peptide from dorsal root ganglion neurons. Additionally, intraplantar injection of CCL2 and CXCL1 produced hyperalgesia in rats [142].

In conclusion, the mentioned studies suggest that proinflammatory chemokines can either sensitize or desensitize receptor functions on or directly activate peripheral NANC nerves. This cross-(de)sensitization between neuronal (anti)pain and chemokine receptors or direct activation of NANC nerves may contribute to the development of visceral pain observed in inflammatory bowel disease and irritable bowel syndrome.

6.5
Chemokine Receptors and Rheumatoid Arthritis

6.5.1
Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting symmetrically synovial tissue in polyarticular joints of the hands and feet. The inflammatory process is characterized by infiltration of inflammatory cells into the joints leading to swelling, proliferation of synoviocytes and painful joints. The inflammatory process may result in destruction of cartilage and bone causing disability [143]. In RA synovial tissue, the infiltrating cells such as macrophages, T cells, B cells and dendritic cells

play important role in the pathogenesis of RA. Migration of leukocytes into the synovium is a regulated multi-step process, involving interactions between leukocytes and endothelial cells, cellular adhesion molecules, as well as chemokines and chemokine receptors [144].

6.5.2 Chemokine Receptors and Their Ligands in Rheumatoid Arthritis

Chemokine expression in the inflamed synovium from human and experimental arthritis models is found to be markedly increased compared to normal tissue. For a general overview, see Figure 6.3. The neutrophil chemoattractant, CXCL8 (IL8) is abundantly present in both synovial tissue and fluid in clinically inflamed RA joints and correlates with the disease activity [145–148]. In the LPS/IL1-induced mouse model of RA, blockade of CXCL8 using an antibody prevented neutrophil infiltration reduced tissue inflammation [149]. First clinical trails using anti-CXCL8 antibodies did not result in clinical improvement in RA patients. It is premature to conclude that CXCL8 is not an appropriate therapeutic target in RA, because treatment with anti-CXCL8 resulted in increased levels of CXCL8 (probably antibody–antigen complexes) and poor clearance of the complexes [150].

CCL2 (MCP-1), the ligand for CCR2, is also highly expressed in RA synovial tissue [151]. CCR2 is found on T cells, DC, basophils and NK cells. Cells that be attracted by CCL2 and $CCR2^+$ monocytes/macrophages are found in inflamed joints [152]. Treatment with a CCL2 antibody in a rat model of collagen-induced arthritis resulted in a reduced paw swelling associated with lower numbers of macrophages in the joints [153] This finding was confirmed in a mouse model of RA using the antagonist MCP-1 (9–76) [154]. However, in the clinic monoclonal antibodies directed against CCL2 as well as CCR2 antagonist MK0812 did not show any beneficial effects in RA patients [155, 156]. Recently, the role of CCR2 in RA became ambiguous, since it was demonstrated that the absence of CCR2 worsens experimental arthritis in mice mimicking severe human RA [157]. Taking together, CCR2 is not likely to be a promising target for RA.

CCL5 (RANTES), the ligand for CCR1, CCR3 and CCR5, is an important chemoattractant for T cells and monocytes. Increased expression of CCL5, CCR1 and CCR5 is found of fibroblast-like synoviocytes, monocytes and T cells in the synovium of RA patients [158, 159]. In addition, in epidemiological studies, loss of function mutation of CCR5 has been associated with protection from juvenile RA and RA [160, 161]. Blocking CCL5 or administration of Met-RANTES (a CCR1/CCR5 antagonist) and nonpeptide CCR5 antagonist in rodent and monkey arthritis models resulted in a markedly reduced inflammatory response [162–164]. Short-term treatment of patients with active RA with the CCR1 antagonist CP-481715 showed a trend towards clinical improvement when compared to control patients [165]. This was associated with a marked decrease in the number of synovial macrophages. A phase II clinical study with CP-481715 did not demonstrate clinical efficacy after six weeks of treatment [166]. More and longer clinical trials need to be carried out to validate CCR1 as a potential therapeutic target.

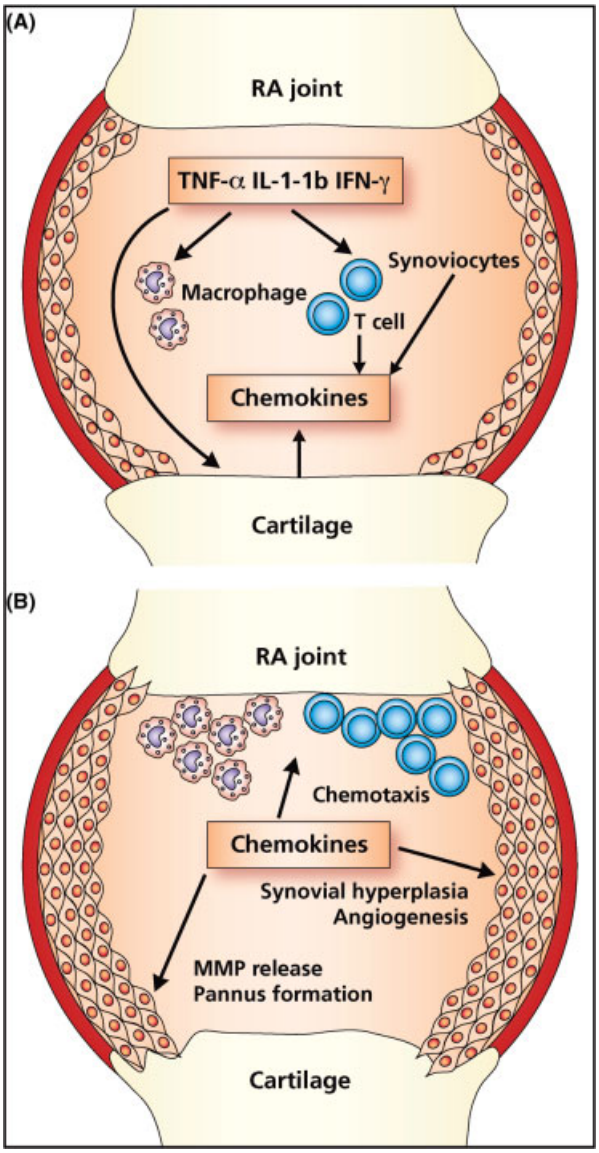

Figure 6.3 The role of chemokines in the joints of RA patients. In the synovium macrophages, T cells, synoviocytes and chondrocytes produce various chemokines, such as CXCL8, CCL2 and CCL5. Cytokines such as IL1β, TNFα and INFγ stimulate these cells in the synovium. Chemokines in the inflamed joint recruit leukocytes into the joint. In addition, chemokines have also other biological activities. Chemokines stimulate fibroblast-like synoviocytes and chondrocytes to release inflammatory mediators, such as chemokines and matrix metalloproteases (MMP). This leads to cartilage degradation and pannus formation. In addition, chemokines enhance cell proliferation and angiogenesis. Chemokines can induce in addition an autocrine and paracrine stimulation of leukocytes, fibroblasts and chondrocytes, leading to joint destruction. Adapted from [167].

6.6
Chemokine Receptors and Atherosclerosis

6.6.1
Atherosclerosis

Atherosclerosis is an inflammatory disease that is characterized by lesions in the large arteries containing lipids, immune infiltrates (particularly monocytes, macrophages, T cells), connective tissue elements and debris [168–171]. This can lead to myocardial infarction in the heart and/or to ischemic stroke in the arteries. The atherosclerotic process is initiated when plasma levels of the cholesterol-rich very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) rise. Risk factors are high-saturated fat diet, smoking, diabetes, hypertension and obesity. Once LDL concentrations rise, LDL diffuses from the blood into the innermost layer of the artery where it undergoes oxidative modification (see Figure 6.4). Oxidized LDL in turn, leads to the release of bioactive phospholipids that activate endothelial cells to express, for example, vascular cell adhesion molecule 1 (VCAM1). Both monocytes and T cells can adhere to VCAM1-expressing endothelial cells. Upon a chemokine gradient, these immune cells migrate into the arterial intima (innermost layer, see Figure 6.4). In the intima, monocytes differentiate into macrophages in response to the local overexpressed macrophage colony-stimulating factor (M-CSF). During this process, innate immune receptors are upregulated, including scavanger receptors (ScRs). ScRs mediate the uptake of oxidated LDL by macrophages and causes LDL cholesterol accumulation and consequently the transformation of macrophages into foam cells

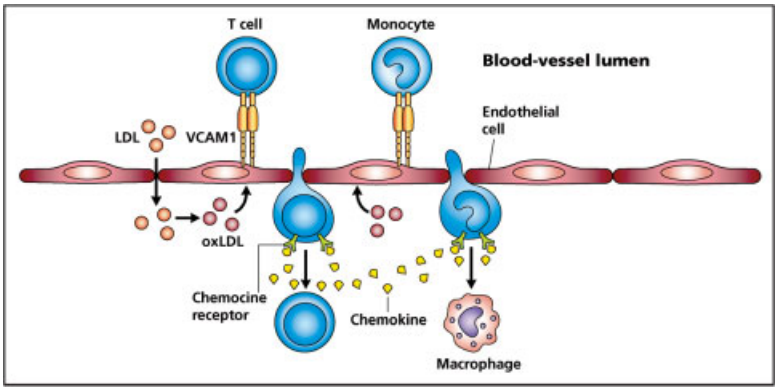

Figure 6.4 The role of chemokines in the recruitment of immune cells in atheroscelortic plaques. Low-density lipoprotein (LDL) diffuses from the blood into the innermost layer of the artery, where LDL particles can associate with proteoglycans of the extracellular matrix. The LDL of the extracellular pool is modified by enzymes and oxygen radicals to form molecules such as oxidized LDL (oxLDL). Biologically active lipids are released and induce endothelial cells to express leukocyte adhesion molecules, such as vascular cell-adhesion molecule 1 (VCAM1). Monocytes and T cells bind to VCAM-1 expressing endothelial cells through very late antigen4 (VLA4) and respond to locally produced chemokines by migrating into the arterial tissue. Adapted from [169].

(characterization of early-stage atherosclerosis). Furthermore, foam cells and activated macrophages drive lesion progression by secreting growth factors for smooth muscle cells and secreting pro-inflammatory mediators like reactive oxygen species, pro-inflammatory cytokines and of proteases. Infiltrated T cells are activated by encountering antigens, such as oxidized LDL and heat-shock proteins, bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells. In the lesion, T cell activation leads most prevalently to a T helper-1 response. Consequently, interferon-γ (IFN-γ) and tumor necrosis factor (TNF) are produced that activate macrophages. Thus, crosstalk between T cells and monocytes/macrophages induces an amplification loop of the inflammatory response, resulting in the promotion of lesion formation. As the lesion becomes more bulky, the arterial lumen narrows and this can lead to ischemic symptoms. Furthermore, depending of the stability of the lesion, the plaque may rupture and causes thrombosis, leading to acute cardiovascular events that result in myocardial infarction and stroke [168–174].

6.6.2
Chemokine and their Receptors in Atherosclerosis

Extensive research has been performed in mice to determine the factors involved in the pathogenesis of atherosclerosis (see Table 6.7). Two knock-out mouse strains were used: apolipoprein E ($ApoE^{-/-}$) mice that develop spontaneous atherosclerosis that progresses to myocardial infarction and stroke and LDL receptor ($Ldlr^{-/-}$) mice that develop hypercholesterolemia and lesion development upon fat feeding [175]. Crossbreeding of this mice with mice that carry deletions in genes of the immune system indicate an essential role of chemokines and their receptors in the early phase of atherosclerosis (for excellent reviews, see [176, 177]).

Monocyte chemoattractant protein-1 (CCL2) and its receptor (CCR2) play an important role in developing atherosclerosis as shown in both knockout mice [178, 179] and human material [180, 181]. CCR2 is expressed on monocytes and T cells, as deletion of either CCL2 or CCR2 decreases the infiltration of monyctes and T cells into the intima and subsequently the initiation of atherosclerosis. In addition to CCR2, monocytes and T cells also express CCR1 and CCR5 [182]. CCL5, the chemokine that binds both CCR1 and CCR5, is highly expressed in atherosclerotic lesions [183]. CCR5 deficiency in $Ldlr^{-/-}$ mice showed a decrease in inflammation and improved plaque stability. However, in the same mouse model, CCR1 deficiency enhanced inflammation and atherosclerotic lesion development [184, 185]. Thus, in view of developing therapeutics, a selective blocker of CCR5 is required rather than a CCR1 antagonist. Indeed, the nonpeptidergic CCR5 (and CXCR3) antagonist TAK-779, attenuates atherosclerotic lesion formation by blocking T cell migration into lesions, as shown in both DBA/1 and $Ldlr^{-/-}$ mice [186, 187]. Not only the chemokine receptors are implicated as drug targets, efforts are also being made to target the chemokine CCL5. Administration of the CCL5 antagonist Met-RANTES in $Ldlr^{-/-}$ mice reduces the progression of atherosclerosis [188]. Furthermore, a study showed that the ability of CCL5 to recruit monocytes is enhanced when a chemokine heterodimer is formed with CXCL4 [189]. Attenuation of heterodimerization of CCL5 with CXCL4 by the peptide antagonist CKEY2 or its mouse ortholog MKEY

Table 6.7 Chemokine receptors and their ligands involved in atherosclerosis.

Receptor	Ligands	Relevance
CCR2	CCL2	Presence of CCL2 in human atherosclerotic lesions $ApoE^{-/-}CCR2^{-/-}$ (less development of disease) $Ldlr^{-/-}CCL2^{-/-}$ (less development of disease)
CCR1 CCR5	CCL5	Presence of CCL5 in atherosclerotic lesions $ApoE^{-/-}CCR5^{-/-}$ (no difference compared to $ApoE^{-/-}$) $Ldlr^{-/-}CCR5^{-/-}$ (more stable plaque) $Ldlr^{-/-}CCR1^{-/-}$ (increased inflammation) Met-RANTES in $Ldlr^{-/-}$ (reduced progression) CKEY2 attenuates CCL5-CXCL4 formation and mono recruitment
CXCR2	CXCL8	Presence of CXCR2 in human atherosclerotic lesions (mΦ rich) CXCR2 overexpression: abundant in atherosclerotic lesions $Ldlr^{-/-}CXCR2^{-/-}$ (reduced progression advanced disease) CXCL8 produced by mono/mΦ/foam cells role for CXCR2 in advanced disease
CXCR3	CXCL9, CXCL10, CXCL11	Presence of CXCR3 in human disease CXCR3 antagonist (attenuation of lesion formation) $ApoE^{-/-}$ $CXCR3^{-/-}$ (less development of disease)
CXCR4	CXCL12	AMD3465 in $ApoE^{-/-}$ $Ldlr^{-/-}$ mice (increased neutro in blood) Increased plaque instability
CX3CR1	CX3CL1	Presence of CX3CL1 in human disease $ApoE^{-/-}Cx3cr1^{-/-}$ (less development of disease)

Mono: monocytes; mΦ: macrophages; neutro: neutrophils [178–181, 183–185, 188, 190, 192–196, 198–200, 203–205].

causes reduction of monocyte chemotaxis *in vitro* and atherosclerotic lesion reduction *in vivo*, respectively [190].

In human atherosclerotic lesions also expression of the chemokine CX3CL1 (fractalkine) is elevated [191, 192]. CX3CL1 causes recruitment of monocytes and T cells via its interaction with CX3CR1. In accordance, CX3CR1 deficient $ApoE^{-/-}$ mice are less prone to atherosclerosis [193, 194]. CX3CL1 is an unique chemokine (like CXCL16), because it is expressed in both a soluble and transmembrane form. Expressed on the plasma membrane of endothelial cells, CX3CL1 acts as an adhesion molecule. In its soluble form, it acts like other chemokines, as a chemoattractant [176, 177]. Thus, by these means CX3CL1 can contribute to the development of atherosclerosis.

CXCR3 is expressed on T cells and is activated by the chemokines CXCL9, CXCL10 and CXCL11. These chemokines are highly expressed in human atherosclerotic lesions [195], leading to recruitment of T cells into the intima. In CXCR3 deficient $ApoE^{-/-}$ mice the lesion formation was reduced, which was associated with an upregulation of anti-inflammatory molecules IL-10, IL-18BP and increased numbers of regulatory T-lympocytes that suppress activation of the immune system [196].

Furthermore, the CXCR3 specific antagonist NBI-74330 [197] showed attenuation of formation of atherosclerotic lesion in $Ldlr^{-/-}$ mice [198].

Oxidized LDL is known to induce the production of CXCL8 by monocytes [199]. Various studies have shown that macrophages and foam cells in atherosclerotic lesions produce CXCL8 [200, 201]. This chemokine binds to CXCR1 and CXCR2 [15]. CXCR2 was detected in the macrophage rich areas of human carotid endarterectomy atherosclerotic lesions [202]. In CXCR2 deficient $Ldlr^{-/-}$ mice the progression of advanced atherosclerosis was reduced, associated with reduced recruitment of macrophages [202]. Thus, CXCL8 plays a direct role in directing of macrophages in atherosclerotic lesions. Studies with deficient KC/mCXCl1 $Ldlr^{-/-}$ mice, a chemokine that also binds CXCR2, showed a less pronounced attenuation compared to CXCR2 deficient mice. This suggests that other CXCR2 ligands may compensate for the loss of KC/mCXCl1. Furthermore, the same study showed that KC/mCXCl1 and CXCR2 do not play an essential role in onset of early atherosclerotic lesion formation, but rather in advanced stages associated with macrophage accumulation [203].

Although neutrophils are not the key mediators in atherosclerosis, they appear to participate in atherosclerotic lesion development and are detected at sites of plaque disruption [204]. Neutrophils express among others the chemokine receptor CXCR4 [182]. The function of CXCR4 and its ligand CXCL12 in atherosclerosis was recently described by Zernecke *et al.* [205]. Under healthy conditions, CXCL12 is constitutively expressed in the bone marrow, causing homing of neutrophils into the bone marrow and subsequent clearance. Blockade of CXCR4 by the small-molecule antagonist AMD3465 in both $ApoE^{-/-}$ and $Ldlr^{-/-}$ mice resulted in expansion of bone marrow neutrophils and their egress into the blood leading and attenuating of the homing of neutrophils back to the bone marrow. Thus, the number of neutrophils is increased in the blood stream. Neutrophils express also CXCR2 and upon expression of CXCL1 and CXCL8 in the atherosclerotic plaque, neutrophils are recruited into the plaque. In the plaque they cause secretion of inflammatory mediators, thereby promoting growth of the plaque and inducing plaque instability. Thus, interference of the CXCL12/CXCR4 axis promotes lesion formation by disruption of neutrophil homeostasis [204, 205].

The human cytomegalovirus (HCMV) infects among others vascular endothelial and smooth muscle cells [170]. Upon infection, vascular smooth muscle cells express the HCMV-encoded chemokine receptor US28. Consequently, increased migration of these infected smooth muscle cells towards CCL2 and CCL5 (expressed in the intima) have been reported [206], causing increased lesion formation. Thus, US28 and its ligands CCL2 and CCL5 can promote the development of atherosclerosis (for more information, see Chapter 9).

6.7
Chemokine Receptors in Multiple Sclerosis

Chemokines and chemokine receptors appear to play an important role in the central nervous system (CNS). For more details, see Chapter 7.

6.7.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. The infiltration of mainly T lymphocytes and macrophages in the brain causes the destruction of the myelin sheaths of neurons and the apoptosis of oligodendrocytes (myelin-forming cells). Subsequently, axonal injury occurs, resulting in impairment of the signal conduction of the affected neurons, leading to both physical and cognitive disability [207–210]. Lesions in multiple sclerosis are mainly found in the optic nerves, brain stem and in the white matter of both periventricular regions and the spinal cord [207–211]. The cause of multiple sclerosis is still unknown; genetic risk factors, environmental factors and auto-immune inflammatory mechanisms are under investigation for their role in the pathogenesis of this disease [207, 209, 211, 212].

Under basal physiological conditions, the central nervous system (CNS) limits inflammation and autoimmunity by its unique structural and functional features, including the presence of the blood-brain barrier [211, 213]. It is proposed that the crucial step in the development of multiple sclerosis is the activation (e.g., by infection, superantigen stimulation, reactive metabolites, metabolic stress [214]) of circulating autoreactive T-lymphocytes (both $CD4^+$ and $CD8^+$). These activated lymphocytes interact with endothelial surface integrins, subsequently leading to breakdown of the blood–brain barrier and influx into the CNS. In addition, also matrix metalloproteinases (particular MMP-9) can disrupt the blood–brain barrier [207–209, 212, 214]. However, other studies report that the extensive apoptosis of oligodendrocytes, induced by viruses (e.g., human endogenous retrovirus type W; HERV-W) or increased levels of glutamate, are the essential step in developing multiple sclerosis. The formed myelin debris trigger the inflammatory system, leading to recruitment of T cells and macrophages into the CNS and subsequently (also) disruption of the blood–brain barrier [210]. Degradation of the blood–brain barrier allows an increased infiltration of inflammatory cells (like macrophages) in the CNS upon a chemokine gradient. Active lesions consist of monocytes/macrophages (containing myelin debris), T cells and less frequently B cells [211]. In the CNS, T lymphocytes recognize myelin as foreign. Consequently, T cells produce IL-2, IFN-γ and TNF-α to activate macrophages and local microglia cells (intrinsic CNS macrophages [213]) leading to demyelination [207, 208, 213]. Remyelination by oligodendrocytes occurs in the early phase of multiple sclerosis, but once the disease progresses, demyelination cannot be repaired. Consequently, the axons and neurons are irreversibly damaged [208, 214] causing the clinical symptoms of multiple sclerosis.

6.7.2 Chemokines and their Receptors in Multiple Sclerosis

Both patient material and *in vivo* studies using the rodent experimental autoimmune encephalomyelitis (EAE) animal model have shown that chemokines and chemokine

receptors are involved in the pathogenesis of multiple sclerosis [212, 215] (see Table 6.8). Among others, CCL2 is detected in human multiple sclerosis lesions [216, 217]. Production of this chemokine by astrocytes, microglia, endothelial cells and macrophages [218, 219] causes the recruitment of monocytes and T cells via its binding to the CCR2 expressed on these cells. CCL2 deficient mice [220] and CCR2 knockout mice [221] were both resistant to inducing EAE and developing of clinical symptoms of the disease. In addition, mice treated with either INCB3344 (small nonpeptidergic CCR2 antagonist) or P8A-MCP-1 (point mutant of CCL2) showed a significant decrease in the clinical score, indicating a role for CCR2 in MS [222, 223].

At different stages of lesion development the chemokines CCL3, CCL4 and CCL5 are detected in human brain [218, 223–225]. These chemokines bind to both CCR1 and CCR5 [182], thereby causing the infiltration of monocytes and T cells into the CNS. CCR1 knockout mice show an attenuated form of EAE [226] and administration of the CCR1 antagonist BX 471 in rat reduces EAE [227]. In addition, administration of anti-CCL3 antibodies in mice prevented the onset of EAE and infiltration of mononuclear cells into the CNS [228]. However, the CCR1 and CCR5 antagonist Met-RANTES was not able to block the leukocyte trafficking in chronic-relapsing EAE [229], whereas the RANTES variant ^{44}AANA^{47}-RANTES was indeed able to inhibit EAE development *in vivo* [215].

Table 6.8 Chemokine receptors and their ligands involved in multiple sclerosis.

Receptor	Ligands	Relevance
CCR2	CCL2	Presence of CCL2 in human MS lesions
		$CCR2^{-/-}$ mice: no EAE symptoms
		$CCL2^{-/-}$ mice: resistant to induce EAE
		P8A-MCP-1 inhibited clinical symptoms in EAE mice
		INCB3344 inhibited clinical symptoms in EAE mice
CCR1	CCL3	Presence of CCL3, CCL4 and CCL5 in human MS lesions
CCR5	CCL4	$CCR1^{-/-}$ mice had attenuated form of EAE
	CCL5	CCR1 antagonist BX 471 reduced EAE in rat
		AntiCCL3 antibody prevented onset EAE
		^{44}AANA^{47}-RANTES had inhibitory effect on EAE
		Antagonist Met-RANTES had no effect on EAE
CXCR3	CXCL9	Presence of CXCL9 and CXCL10 in human MS lesions
	CXCL10	Upregulation of CXCR3 at $CD4^+$ cells from MS patients
		$CXCR3^{-/-}$ mice were more susceptible to EAE
		$CXCL10^{-/-}$ mice were more susceptible to EAE
		AntiCXCL10 antibody induced exacerbation of EAE in rat
		AntiCXCL10 antibody decreased incidence of EAE in mice
CXCR6	CXCL16	Expression of CXCL16 in EAE mice
		AntiCXCL16 antibody: no induction of EAE in mice
CCR7	CCL 19	CCL19 and CCL21 involvement in T cell migration into CNS
	CCL 21	

[215, 216, 218, 220–223, 226–238].

Astrocytes in active demyelinating lesion express CXCL9 and CXCL10 [212, 230]. Binding of these chemokines to CXCR3 causes the influx of T cells into the CNS. Multiple sclerosis patients show an upregulation of CXCR3 on $CD4^+$ lymphocytes associated with all relapses [231]. Mice treated with antiCXCL10 antibodies showed a decreased incidence of EAE due to less accumulation of $CD4^+$ T cells [232]. However, rats treated with antiCXCL10 antibodies did not affect the induction of EAE [233]. In addition, both CXCR3 knockout and CXCL10 deficient mice were more susceptible to EAE [234, 235]. More studies are required to define the role of CXCL10 and CXCR3 in MS.

In addition to the above-mentioned receptors, T cells also express CXCR6. CXCL16, the ligand for CXCR6, has therefore chemoattractant activity for activated T cells [182, 236]. Administration of anti-CXCL16 in mice causes decreased incidence of acute EAE associated with reduced infiltration of mononuclear cells into the CNS [236]. Furthermore, T cells express CCR7 and therefore also the chemokines CCL19 and CCL21 play a role in recruiting T cells into the CNS in multiple sclerosis or EAE [238].

6.8 Chemokine Receptors and Psoriasis

6.8.1 Psoriasis

Psoriasis is an inflammatory skin disease characterized by red, scaly, raised plaques. Usually, the psoriasis lesions are several centimeters in diameter and separated by normal-appearing skin [239, 240]. Psoriasis involves a chronic cutaneous pathologic process, driven by interactions between infiltrating leukocytes (T cells, dendritic cells, macrophages, neutrophils), cytokines, chemokines and keratinocytes, the cells from the epidermis. The disease is initiated or exacerbated by infections, physical and/or emotional stress, antigenic stimuli and various medications (e.g., lithium, β-blockers [241, 243]). Psoriatic plaques can revert back to symptomless skin spontaneously or after treatment with selective immune-targeted agents [239, 240, 242–244].

Upon a stimulus (e.g., an extrinsic pathogen-associated signal or an intrinsic signal), the normal skin is converted to an acute psoriatic lesion [243]. This lesion is associated with thickening of the skin, primarily due to accumulation of scales caused by aberrant terminal proliferation of keratinocytes, elongation of epidermal rete (network of blood vessels) and enlargement of blood vessels in the dermis. In addition, the amount of lymphocytes, dendritic cells (DCs) and macrophages in the dermis markedly increases and neutrophils are found in the stratum corneum, the outer layer of the epidermis [239, 243, 245]. Lymphocytes in the lesion are mainly memory populations of skin-homing (cutaneous lymphocyte-associated antigen; CLA^+) $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T lymphocytes are mostly present in the dermis, whereas $CD8^+$ T lymphocytes are both present in the dermis and in the epidermis [239, 242]. Both classes of T lymphocytes become activated via the interaction with antigen-presenting cells (APCs), like immature myeloid dendritic

cells (Langerhans cells), plasmacotoid dendritic cells and mature myeloid dendritic cells. A so-called immunologic synapse is formed between T cells and dendritic cells [242, 245]. Subsequently, cytokines (e.g., TNF-α, IFN-γ), chemokines (e.g., CCL3, CCL4, CCL5, CCL17, CCL19, CCL20, CCL27, CXCL1, CXCL8, CXCL9, CXCL10) and growth factors (e.g., TGF-α, VEGF) are released by these T cells and dendritic APCs. This finally leads to a vicious cycle in which keratinocytes, endothelial cells, neutrophils, T cells and APCs become activated. Consequently, a chronic psoriatic plaque is formed characterized by the presence of $CD8^+$ cells and neutrophils in the epidermis, thickening of the epidermis by increased keratinocyte proliferation and an angiogenic tissue response [243].

6.8.2 Chemokines and their Receptors in Psoriasis

Chemokines play a key role in recruiting cells into the psoriatic plaque, like T cells that initiate and maintain psoriasis [244] (Table 6.9). Upon stimulation of IFN-γ (produced by T cells) keratinocytes in plaques synthesize CXCL9 and CXCL10. These chemokines bind to CXCR3 (expressed on T cells), thus causing the migration of T cells into the plaque [245, 246]. As described in Chapter 13, small CXCR3 antagonists have been considered as therapeutics in skin inflammation, including psoriasis. However, AMG487 failed in clinical II studies in psoriasis patients [247]. Expression of CCL17, CCL22 and CCL27 causes also increased infiltration of skin-homing CLA^+ memory T cells via, respectively, their interaction with the highly expressed CCR4 and CCR10 on these cells [246, 248]. Furthermore, CCR6 and its ligand CCL20 are upregulated in psoriatic skin lesions [249]. CCR6 is expressed on both T cells and dendritic cells, thus expression of CCL20 results in recruitment of both cell types into the psoriatic

Table 6.9 Chemokine receptors and their ligands involved in psoriasis.

Receptor	Ligands	Relevance
CCR2	CCL2	CCL2 produced by psoriatic keratinocytes
CCR4	CCL17	Upregulation of CCL17 and CCL22 in psoriatic lesion
	CCL22	
CCR5	CCL3	High expression of CCL3 in psoriatic tissue
	CCL4	High expression of CCL5 in psoriatic tissue
	CCL5	High expression of CCL4 in psoriatic tissue
CCR6	CCL20	Upregulation of CCL20 and CCR6 in psoriatic lesion
CCR10	CCL27	Upregulation of CCL27 in psoratic lesion
		Neutralization of CCL27-CCR10 in mice: impaired T cell recruitment
CXCR2	CXCL1	High levels CXCL1 and CXCL8 found in psoriatic epidermis
	CXCL8	Expression of CXCR2 on psoriatic keratinocytes
CXCR3	CXCL9	Upregulation CXCL9 and CXCL10 in psoriatic lesion
	CXCL10	
CX3CR1	CX3CL1	Upregulation of CX3CL1 in psoratic lesion

[227, 244, 254, 261–263].

plaque [250–252]. Also the expression of CX3CL1 (fractalkine) is increased in psoriatic tissue, leading to migration of CX3CR1 positive T cells into the psoriatic lesion [253].

The chemokines CXCL1 and CXCL8 are detected in psoriatic epidermis. Via their interaction with CXCR2, expressed on neutrophils and psoriatic keratinocytes, they recruit neutrophils into the epidermis and stimulate the growth and differentiation of keratinocytes, respectively [254]. Neutrophils in turn release reactive oxygen intermediates and proteolytic enzymes that cause destruction of the human epidermis [255]. Thus, activation of CXCR2 on both neutrophils and psoriatic keratinocytes, causes epidermal changes observed in psoriasis [244, 254, 256]. For the treatment of psoriasis and other inflammatory diseases, Abgenix has developed a human antibody against CXCL8 [257]. Unfortunately, this antibody was inactive in clinical trials of psoriasis [258]. Although the antibody against CXCL8 gave no improvement, CXCR2 can still be a potential drug target in this disease, for example, via treatment with nonpeptidergic CXCR2 antagonists [259].

Keratinocytes of psoriasis patients produce not only CXCL8, CXCL9 and CXCL10 but also CCL2, CCL3, CCL4 and CCL5 [244, 260–263]. CCL2 causes recruitment of macrophages into the psoriatic plaque via the interaction with CCR2 [256, 264], whereas CCL3, CCL4 and CCL5 recruit T cells via the interaction with CCR1 and/or CCR5 [260]. *In vivo* experiments showed that elimination of macrophages causes a reduction of both T cells and dendritic cells in the skin [265]. Clinical studies in psoriasis patients with the small molecule CCR5 antagonist SCH331125 showed no clinical effect and no difference of CCR5 expression in lesion tissue, thereby concluding that CCR5 does not play a crucial role in psoriasis [260]. In addition, targeting CCR1 with the small nonpeptidergic CCR1 antagonist BX471 also failed in clinical trials for among other psoriasis [247].

Psoriasis appears to be a uniquely human disease, as in several animal models single-gene mutations or deletions failed to generate skin lesions with relevant psoriatic characteristics [240–243]. Nevertheless, immunodeficient mice transplanted with human psoriatic plaques (SCID mice) show the implication of T cells [266]. Furthermore, human symptomless skin engrafted onto AGR129 mice spontaneously develop plaques, which are used to test drug-like compounds to prevent the development of psoriasis [242, 243].

6.9 Concluding Remarks

Taken together, the above clearly indicates that chemokines and their respective chemokine receptors play an important role in inflammatory diseases. Chemokine receptors can therefore be considered as promising drug targets.

Acknowledgements

This chapter was written within the framework of the Dutch Top Institute Pharma project number T101-3.

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7
Chemokines and their Receptors in Central Nervous System Disease

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7.1
Introduction

Chemokines are chemotactic cytokines that induce directional sensing and motile responses (defined as chemotaxis) in cells expressing the corresponding chemokine receptors. Chemokines are small (approx. 10 kDa), mostly secreted proteins that were initially implicated in the regulation of blood cell trafficking during host defense and immune responses initiated by pathogens. The first molecule with chemotactic properties (IL-8) was identified in 1987 [1] and since then the chemokine family has expanded rapidly to its current size. The family now consists in humans of 53 chemokines and 23 chemokine receptors (for an overview, see http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html) [2].

7.2
Families of Chemokines

Chemokines are characterized by four highly conserved cysteine residues in their peptide sequence [3]. Accordingly, four chemokine classes have been designated, based on the position of the first two cysteine residues relative to each other in the N-terminal region of the protein. The two major chemokine groups contain residues where the two cysteines are either separated by one amino acid (CXC or α) or are adjacent to each other (CC or β) [3, 4]. The CXC group is further divided into two subgroups depending on a distinct ELR (glutamate–leucin–arginin) motif that is located in between the N-terminus and the first cysteine [5]. Two minor classes of chemokines contain either a residue with only one cysteine in the N-terminal region (C or γ chemokines), or a residue where the first two cysteines are separated by three amino acid residues (CX_3C or δ chemokines) [4].

Because a variety of acronyms has been provided by different investigators to designate newly discovered chemokines a new nomenclature has been introduced a couple of years ago [6]. Since then, chemokines and their receptors are classified

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

according to the aforementioned subgroups they belong to (CXC, CC, C, CX3C). A chemokine is thus characterized by an "L" for ligand, followed by a number that corresponds to the order of cloning and/or characterization. As an example, the chemokine SLC (secondary lymphoid-tissue chemokine) has also been known as TCA-4 (T-cell activation protein 4), SCY21 (small cytokine 21), 6CKine (chemokine with 6 cysteines) and Exodus-2 (see also [7]). In the new nomenclature SLC became CCL21 (CC subgroup, gene description: small cytokine number 21). The nomenclature for the chemokine receptors parallels the ligand nomenclature and ends with "R" for receptor, followed by a number that indicates the order to cloning and/or characterization [8]. Chemokine receptors all belong to the family of seven transmembrane domain G protein-coupled receptors that are highly conserved in evolution (for reviews, see [9, 10]).

7.3 Chemokine Pharmacology

There are by far more chemokines than corresponding receptors (53 chemokines, 23 receptors), meaning that there is a high degree of promiscuity in the chemokinergic system. Thus, a given chemokine receptor generally recognizes multiple chemokine ligands and most chemokines can bind to a variety of different chemokine receptors [11]. A few exceptions are specific chemokine–chemokine receptor pairs (e.g., CCR8-CCL1, CCR9-CCL25, CXCR5-CXCL13, CXCR6-CXCL16, CX3CR1-CX3CL1) that to date appear to be monogamous [11].

Although most chemokines recognize more than one receptor, it seems to be a general rule that CC receptors only bind CC chemokines, whereas CXC chemokines are recognized by CXC receptors. Thus there is no crossreactivity between members of the four chemokine subfamilies [8]. However, exceptions to this rule have been published [12–15].

7.4 Chemokines and Chemokine Receptors: A Complex System

It is obvious today that chemokines are far more than just cellular attractors. The biology of chemokines is enormously complex and highly regulated by different mechanisms, like oligomerization of ligands and receptors, GAG-binding, alternative splicing and structural modulation by proteases (for reviews, see [11, 16]). Thus, different levels of control allow precise regulation of cellular trafficking and function in all tissues of the body, not only restricted to inflammation but also in development and physiological homeostasis. Accordingly, the chemokine system has been implicated in various pathologies and numerous reports suggest that the chemokine system might be a valuable drug target for the treatment of yet untreatable diseases.

There are several possibilities to target the chemokine system. Chemokines are the only cytokines that signal through G protein-coupled receptors, which are a favorite

target in medicinal chemistry. Accordingly, various small-molecule chemokine receptor antagonists have been developed by the pharmaceutical companies in the past couple of years (for reviews, see [17–19]). Thus inhibiting chemokine functions with small chemokine receptor antagonists could be a promising approach. Since some of these small molecules have already entered clinical trials, whether or not this might lead to new therapeutic treatments in the next couple of years remains to be seen [18, 19].

7.4.1
Expression of Chemokines and their Receptors in the CNS

Chemokine expression in the CNS by brain-specific cells was first described almost 20 years ago (for examples, see [20, 21]. These results were obtained in an animal model for multiple sclerosis (MS), showing for the first time a disease-associated increase in chemokine expression by distinct subsets of brain cells [20, 22]. In the mean time, many *in vitro* and *in vivo* studies on functional expression of chemokines and their receptors in the CNS have been published. Chemokines are now discussed to be part of the repertoire of signaling molecules in the brain, with numerous functions in development, physiology and pathology of the CNS, not only in rodents but also in humans [7, 23–26].

7.4.2
Involvement of Chemokines in Blood–Brain Barrier Disruption Associated with Neurodegenerative Disease

Inflammatory processes are actively restricted in the CNS, a precaution to prevent potentially harmful reactions of the immune system. This, however, does not imply a lack of immune reactions in the brain [27, 28]. The blood–brain barrier (BBB) importantly contributes to the anti-inflammatory milieu of the CNS [29]. The BBB at the cellular level consists of endothelial cells, astroglia, pericytes, perivascular macrophages and a basal lamina [30]. The cerebral endothelial cells, in conjuction with the basal lamina, are co-localized with pericytes and perivascular macrophages [31]. Furthermore, astrocytes project their endfeet close to the cerebral endothelial cell layer and thus contribute to the barrier function of the cerebral endothelium. Compared to peripheral endothelia the cerebral endothelium is very tight and contains strict intercellular tight junctional structures. These tight junctions prohibit paracellular transport across the cerebral endothelium. Under physiological circumstances the BBB maintains isolation of the central nervous system and protects against external factors [32, 33]. Thus, although a certain degree of immune surveillance of the brain has been reported [34], under healthy conditions, infiltration of the brain by immune cells from the blood is low [29].

This may change during neurodegenerative disease and associated neuroinflammation, where the isolating capacity of the blood–brain barrier deteriorates and thus allows the infiltration of blood leukocytes. The increased permeability of the blood–brain barrier is the result of the disruption of tight junctional structures as well

as other cell–cell and cell–matrix junctions by matrix metalloproteinases and the formation of transendothelial channels [35]. Increased endothelial cell permeability and degradation of extracellular matrix proteins of the vascular basal lamina have been observed after various types of neurodegeneration and inflammation [36, 37]. Subsequent infiltration of immune cells involves rolling, adhesion and transendothelial migration of these cells, with chemokines contributing importantly to all of these processes that finally lead to the infiltration of leukocytes [38, 39].

7.5
The Role of the Chemokinergic System in Multiple Sclerosis and Experimental Autoimmune Encephalitis

Multiple sclerosis (MS) is an inflammatory neurological disease that is characterized by the destruction of myelin sheaths, death of oligodendrocytes and subsequent axonal damage and loss of axons. It is generally assumed that the inflammatory reaction and demyelination in MS is due to the presence of various peripheral cells that invaded the brain. The cause of MS is still unknown, and the exact sequence of events leading to the disease has not been unraveled. Various lines of evidence argue that myelin specific auto-reactive T cells are activated in peripheral lymphoid organs, migrate over the BBB and start the devastating inflammation that finally leads to the breakdown of myelin and the clinical symptoms of MS. Other data support that the view of an immune-independent start in the CNS parchenchyma (neurodegeneration, oligodendrocytic death) and a secondary attraction of peripheral cells into the brain (for an excellent review, see [40]). Anyhow, MS is characterized by the presence of numerous T cells and macrophages in the nervous tissue that most likely amplify an ongoing inflammation by secreting various proinflammatory mediators, thus the infiltration of blood-derived cells into the perivascular space and brain parenchyma is a histological hallmark of MS [41].

7.5.1
Chemokines: Attractors for Blood Lymphocytes in MS and EAE?

Experimental autoimmune encephalitis (EAE) is a T-cell driven demyelinating disease in rodents that serves as a model for MS. The first description of chemokine expression in EAE immediately prompted the question whether brain chemokines could be involved in the attraction of mononuclear lymphocytes into the brain [42]. The past decade has accordingly yielded a great number of papers dealing with the function of chemokines and chemokine receptors in the attraction and infiltration of blood lymphocytes into the brain. As mentioned above it is known today that numerous chemokines are expressed in MS and EAE and that their expression can be related to various disease states (for reviews, see [43–47]). Activated microglia and astrocytes are a major source of brain chemokines. In EAE or MS brain these cells have been shown to express various chemokines, including CXCL1, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5 CCL7, CCL8, CCL19 and CCL22 [48–52]. The

predominant cells that infiltrate the brain in MS and EAE are $CD4^+$ Th1 and Th17 cells and monocytes/macrophages that express the chemokine receptors CXCR3, CCR1, CCR2, CCR5 and CCR2, CCR3, CCR5, respectively [44, 53]. The function of chemokines and their receptors in MS and EAE has been extensively discussed in excellent recent reviews and is therefore not further highlighted here [11, 15, 54].

Despite the fact that numerous chemokines or chemokine receptors have been implicated in the pathology of MS, a "chemokinergic" therapy is still not in sight. BX-471 is a selective CCR1 antagonist and inhibits a number of CCR1-mediated effects including calcium mobilization and leukocyte migration [55]. BX-471 has been evaluated in various peripheral inflammatory disease conditions [56–58]. Although it has been found safe in Phase I clinical trials in MS, it failed to have an effect in Phase II studies [59].

The CCR2 antagonist INCB3344 is a novel, potent and selective small molecule antagonist of mouse CCR2 and inhibits the binding of CCL2 to mouse monocytes with nanomolar potency. Similarly, INCB3344 significantly reduces disease pathology in mice subjected to EAE, a model of MS, as well as to a rat model of inflammatory arthritis [60] Although INCB3344 has served as a useful proof of concept of the potential therapeutic value of CCR2 antagonists, its selectivity for rodent versus human CCR2 renders it unsuitable for clinical development; however, related molecules such as INCB3284 are being pursued [60].

7.6
The Role of Chemokines in Brain Ischemia

Very early after ischemia neutrophils infiltrate the ischemic brain area. Neutrophil infiltration is later followed by blood macrophages, natural killer cells and T cells [61].

Specific chemokines are induced during/after ischemia and their time-dependent induction seems in accordance with the temporal profile of infiltration of blood cells. It is likely that the expression of chemokines is induced by proinflammatory cytokines which are produced in the ischemic area. For example, TNF-α and IL-1β activate transcription factors such as nuclear factor $\varkappa$B [62] that regulate the transcription of chemokines indicating that these proinflammatory cytokines activate the production of chemokines. Indeed expression of CCL2 mRNA in monocytes and astrocytes is induced by cytokines like IL-1β and TNFα and TGFβ [63–65]. Furthermore, expression of CXCL10 in the brain is induced by interferon-γ [66] and presumably also by factors such as IL-1β and TNFα. Cytokines produced in the ischemic area may thus be involved in the regulation of chemokine expression during the ischemic process.

The time course of chemokine expression seems clearly associated with the time-dependent infiltration of different immune cells in the ischemic brain area. Particularly, early expression of CXC chemokines like CXCL1, CXCL2, CXCL3 and CINC [67–69] has been observed and precedes infiltration of neutrophils [70, 71]. CXCL8 is a potent neutrophil chemotactic and activating factor and most likely plays an important role in neutrophil-mediated acute inflammation [72]. In addition to

attracting neutrophils, CXCL8 also stimulates a number of processes which are relevant for ischemia such as the release of neutrophil granules, mediation of the respiratory burst and upregulation of complement receptors and integrins in neutrophils [73–76]. Although neutrophils are the first cells that infiltrate the ischemic area, their involvement in the pathological process remains to be established [77]. A rapid but more complex ischemia-induced expression pattern was observed for CXCL10. This chemokine displays strong chemoattractant activity towards lymphocytes. In a rat model of focal ischemia (MCAO) Wang and colleagues [78] found that CXCL10 is expressed at an early phase (3–6 h after ischemia) and at later stages (10–15 days after ischemia).

The early infiltration of the ischemic area by neutrophils is followed by subsequent infiltration by monocytes and, accordingly, the expression of CXC chemokines is followed by expression of CC chemokines.

Transient increases in mRNA and protein expression of the CC-chemokine CCL2 have been detected in various cerebral ischemia models in adult rats. CCL2 protein expression in ischemic brain tissue has been localized in endothelial cells and macrophage-like cells [79, 80]. Also for CCL2 a biphasic expression pattern was found showing early expression (6–48 h after MCAO) in astrocytes surrounding the penumbra and late expression (after 4–6 days) in macrophages/microglia in the infarcted tissue [81]. The early expression of CCL2 mRNA precedes the peak of emergence of monocyte infiltrates [82] suggesting involvement of CCL2 in therecruitment of monocytes. The later phase of CCL2 expression by recruited monocytes most likely serves as a signal which further potentiates monocyte accumulation and activation [71, 83]. In stroke patients increased levels of CCL2 have been detected in the cerebrospinal fluid, supporting the clinical relevance of this chemokine in stroke pathology.

Expression of the CC-chemokines CCL3 and CCL4 is prominently induced during ischemia [80, 84, 85]. Both chemokines are potential chemoattractants for macrophages and T cells invading the ischemic area. The biological activity of CCL3 and CCL4, however, is different. Thus CCL3 stimulates secretion of TNF and interleukin-1α and interleukin-6 but CCL4 does not [86]. Furthermore, CCL3 and CCL4 act on different populations of activated T cells, CCL3 specifically attracts CD-8$^+$ cells and CCL4 acts on CD-4$^+$ T cells [87].

7.6.1
Functional Involvement of Chemokines in Ischemic Neurodegeneration

Recently several peptide- and nonpeptide chemokine receptor antagonists have been developed and characterized. Various antagonists have been tested in ischemia models and support involvement of chemokines in ischemia-mediated tissue damage. The CCR5 (and CXCR3) chemokine antagonist TAK-779 reduced ischemic brain injury in an ischemia-reperfusion model after both intracerebroventricular and intravenous injection [88]. Also the broad-spectrum chemokine antagonist NR58-3.14.3, a synthetic peptide that inhibits both CC and CXC chemokines has been tested in a rat model for ischemia-reperfusion injury [89]. Intravenous

infusion of NR58-3.14.3 in rats reduced ischemic injury after several time points post middle cerebral artery occlusion. Viral macrophage inflammatory protein-II (vMIP-II), another broad-spectrum chemokine receptor antagonist, reduced infarct volume in a dose-dependent manner after intracerebroventricular administration in a rat medial cerebral artery occlusion model [90, 91]. Finally, post ischemic gene transfer of dominant negative CCL2 attenuated the infarct volume and infiltration of inflammatory cells, suggesting the potential usefulness of antiCCL2 gene therapy [92].

7.7
Chemokines in HIV-Associated Dementia

In most people infected with HIV it is not only the immune system that suffers from the viral infection, it is also the central nervous system that undergoes significant pathology that may finally lead to dementia, called HIV-associated dementia (HAD). The pathology of HAD is characterized by neuronal loss, apoptosis of astrocytes, widespread accumulation and activation of brain macrophages (endogenous and infiltrated) and the presence of multinucleated giant cells in the CNS [93].

7.7.1
Involvement of Chemokine Receptors in HIV Infection of the Brain

HIV comes in two different forms that display different tropisms for either monocyte/macrophages (R5 or M-tropic) or T cells (X4 or T-tropic). This tropism is determined by interactions of the HIV envelope glycoprotein gp120 with chemokine receptors in these cells; CCR5 in monocyte/macrophages and CXCR4 in T cells. It is well known that HIV enters the brain shortly after the infection of the body. Since T-tropic viruses emerge at late stages in HIV infected individuals it might be HIV infected monocytes/macrophages that enter the brain and bring the virus to the CNS [94, 95]. Once in brain HIV infection can further spread to resident microglia that express the HIV co-receptor CCR5. Due to their expression of CD4 and CCR5 microglia are the only cells in the CNS that can get infected by HIV [96]. Infected microglia are therefore supposed to be the major viral reservoir in brain.

The findings in 1996 that HIV uses CCR5 as a co-receptor [97–100] and that those individuals carrying the CCR5 Δ32 mutation are almost resistant against HIV infection [101–103] indicated that the inhibition of HIV co-receptors would be a promising strategy to prevent infection with HIV [104, 105].

7.7.2
Involvement of CXCR4 in gp120-Induced Neurotoxicity

Although neurons generally do not get infected by HIV there is accumulating evidence that neuronal CXCR4 expression might be instrumental for the development of HAD. It has been described that viral gp120 affects the electrophysiology

of cultured neurons. These effects were PTX sensitive and/or were blocked by antibodies or inhibitory peptides for CXCR4, indicating that gp120 may act as an agonist ligand for CXCR4 [106–110]. Due to its agonistic activity gp120 might be responsible for hyperalgesia in AIDS patients by enhancing the excitability of dorsal root ganglion neurons [111].

Several lines of evidence moreover indicate an effect of gp120 on neuronal apoptosis, thus gp120 may have direct neurotoxic effects [112–117]. Since AMD3100 treatment or CXCR4-deficiency protects from gp120-induced neuronal apoptosis *in vitro* [118, 119], it is suggested that CXCR4 mediates the toxic effects of the viral protein. This assumption is furthermore corroborated by findings that also CXCL12 induces apoptosis in neuronal cultures [118, 119].

Recent evidence suggested that gp120 might also suppress neurogenesis [120]. Interesting in this respect was the finding that only cerebrospinal fluid (CSF) from patients with HAD inhibited the proliferation of neuronal stem cells, but not CFS from AIDS patients without neurological symptoms, although it is well known that HIV is also found in CSF from patients without HAD (reviewed in [121]). Thus gp120 not only induced apoptosis in mature neurons but might also inhibit neurogenesis and thereby restrain memory formation in AIDS patients [122].

7.7.3 The Role of CX3CL1 in HAD

Several studies showed elevated levels of CX3CL1 in the CSF of patients with HAD compared to controls (healthy individuals, AIDS patients without HAD) and a function of CX3CL1 in HAD was accordingly suggested [123–125]. Since an increase of CX3CL1 in CSF was not only found in HAD patients but also under other neurodegenerative conditions, this chemokine might be a general marker for neurodegeneration [126, 127].

The prominent expression of CX3CL1 is found in healthy neurons, making CX3CL1 one of the few chemokines that is constitutively expressed in normal brain [128]. Remarkably and unique among the chemokines, neuronal CX3CL1 is expressed in healthy brain at higher levels than in any other organ of the body suggesting a function of CX3CL1 in brain physiology [128, 129].

A seminal report has attributed a role for CX3CL1 in the control of microglia neurotoxicity [130]. Since CX3CL1 is expressed in neurons and released from these cells under stress conditions [131, 132] and since microglia express the corresponding chemokine receptor CX3CR1, a role of CX3CL1 in neuron–microglia signaling has been suggested [123–125, 130–133]. Indeed, in various models of neurodegeneration and/or neuroinflammation an increase in (neurotoxic)-microglia activity was found when CX3CL1–CX3CR1 signaling was absent [130]. These *in vivo* data are corroborated by several lines of *in vitro* evidence, showing that CX3CL1 plays a role in dampening (neurotoxic)-microglia activity [134–136]. It therefore is indicated that CX3CL1 expressed in healthy neurons belongs to the so-called "Off" signals that control microglia function in the CNS [28]. Unfortunately, no *in vivo* evidence from animal models yet exists for the function of CX3CL1–CX3CR1 in HAD.

For both HIV receptors (CXCR4, CCR5) antagonists have been developed in order to prevent viral infection. AMD3100 is a specific CXCR4 antagonist and has been shown to block HIV infection of T-tropic virus [137]. AMD3100 blocks ligand binding to CXCR4 and inhibits CXCL12-induced GTP-binding, calcium flux and chemotaxis. Conversely, AMD3100 does not inhibit calcium flux induced by activation of CXCR3, CCR1, CCR2b, CCR4, CCR5 or CCR7, thus indicating the selective antagonist activity of AMD3100 [138]. Furthermore, AMD3100 inhibits HIV-1 envelop protein gp120-induced neuronal apoptosis and thus may be useful for the treatment of HAD. Also CCR5 is essentially involved in the pathogenesis of HAD. Recently developed CCR5 inhibitors may be beneficial for the treatment of HAD. TAK-779 is a non-peptide small-molecule CCR5 antagonists that prevents ligand binding to CCR5 as well as CCR5-mediated signaling. TAK-779 is a relatively selective antagonist as the compound mediates only weak inhibiton of CCR2b and does not antagonize CCR1, CCR3 or CCR4. TAK-779 inhibits R5 HIV-1 replication [139] by blocking the interaction of the viral surface glycoprotein gp120 with CCR5.

SCH-351125 (also named SCH-C) is another CCR5 antagonist with potent activity against the CCR5 HIV-1 strain [140]. GW873140 is a potent noncompetitive allosteric antagonist of CCR5 that inhibits the calcium response effects of CCR5 activation. GW873140-induced antagonism is ligand-dependent, consistent with an allosteric mechanism of action [141]. UK-427 857 is another selective CCR5 antagonist and acts as a potent antiviral agent prevent binding of the viral envelop gp120 to CCR5. Although in a preclinical study TAK-779 was shown to protect against ischemia [142], the aforementioned CCR5 antagonists have been developed to block HIV-1 entry into cells and have not yet been evaluated for clinical application in neurodegenerative diseases.

7.8
Chemokines in Neuropathic Pain

Neuropathic pain is a pathological pain condition that often occurs in response to peripheral nerve injury. A breakthrough in our understanding of neuropathic pain was the discovery that microglia activation in the spinal cord is essential for neuropathic pain development [143, 144]. Various recent lines of evidence indicate an involvement of chemokines in spinal cord microglia activation and the development of neuropathic pain. Neuronal CX3CL1 might act as a microglia-activator in response to peripheral nerve injury [145, 146]. It has been shown that spinal injection of CX3CL1 induces tactile allodynia, whereas an inhibition of CX3CL1 signaling by antibody treatment prevented the development of neuropathic pain [146–149]. It was furthermore demonstrated recently that activated spinal cord microglia release cathepsin S, a protease which is capable to cleave CX3CL1 in the membrane of neurons, which might lead to sustained neuropathic pain conditions [147]. The resulting soluble CX3CL1 sends signals back to the already activated microglia and is critical for the maintenance of tactile allodynia and microglia activation. However, data recently published in CX3CR1-deficient mice (displaying even higher

neuropathic pain levels than the control animals) contradict a role of CX3CL1 in neuropathic pain [150], showing that more work is needed to elucidate the role of CX3CL1 here.

As described for CX3CL1, CCL2 may also play a role in the control of spinal cord microglia in response to peripheral nerve lesion. CCR2-deficient animals show significantly reduced tactile allodynia in response to peripheral nerve injury [151, 152], whereas spinal injection of CCL2 is sufficient to induce neuropathic pain behavior in mice [152]. Peripheral nerve injury or compression of the dorsal root ganglion leads to rapid CCL2 expression in the cell body of these neurons [153, 154] and in the primary afferents in the dorsal horn ipsilateral to the lesion [155]. Taken together, these data raise the possibility that CCL2 in DRG neurons might serve as a signal that activates microglia in the dorsal horn after peripheral nerve injury [155]. However, CCL2 also directly affects the electrophysiology of DRG neurons, indicating that the role of this chemokine in neuropathic pain sensations might be manifold and not restricted to the activation of microglia in the spinal cord (for an excellent review, see [156]).

7.9 Conclusions

A vast amount of data has been published in the past decade that demonstrates the function of chemokines and chemokine receptors in brain disease. Several studies in which the chemokine system was targeted (knockout animals, active immunization) revealed the impact of chemokine signaling in brain disease. These results provided the foundation for the assumption that targeting the chemokine system may be promising for the treatment of neurodegenerative diseases. Importantly the chemokine system is highly "drugable," not only because chemokine receptors are 7TMs but also because the system comprises several control levels that can be used in various ways to interfere with the signaling and function of chemokines. This combination (important system in disease + relatively easy drug target) is the reason that there are already clinical trials going on with drugs that target the chemokine system, although the research on chemokines is a relatively young field. However, the complex functions of chemokines in brain disease are not completely understood yet and much work is still needed before drugs that interfere with chemokine function in brain disease will enter the clinic.

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8
Chemokines and Cancer Metastasis

Amanda Burkhardt and Albert Zlotnik

8.1
Introduction

Chemokines are a superfamily of many small peptide ligands (47 in humans) and, to date, 19 receptors [1, 2]. Although they have many functions, they are historically best known and characterized through their ability to regulate the chemotaxis and migration of many different cell types. Many of these functions are reviewed in detail elsewhere in this book, so we restrict our comments to their role in cancer. Originally, there were a number of studies that documented the presence of chemokine receptors in various cancer cell lines. The significance of these observations was not, however, fully appreciated until we demonstrated that the CXCL12/CXCR4 axis played a significant role in a mouse model of breast cancer metastasis [3]. This study transformed the field and a PubMed search with the keywords CXCR4 and cancer currently yields 1142 references. Here, we try to organize and summarize what is currently known about this field, in order to give the reader an idea of how it has developed, what is known and what may be the key important questions that will be the focus of future studies.

It is important to review the level of understanding of chemokine biology at the time of our initial study on chemokines and metastasis. Most of the ligands were already known, and chemokines could be divided into inflammatory and homeostatic. Inflammatory chemokines were those strongly expressed by cells that participate in inflammatory responses upon activation. Interestingly, the genes encoding most of these chemokines are located in two clusters in chromosomes 17q12 (CCL family) and 4q13.3 (CXCL family). The genes encoding the homeostatic chemokines, in contrast, tend to be located in isolated chromosomal locations throughout the genome. This curious observation can be explained because the inflammatory chemokines represent very active recombination/duplication sites in the genome, and their expression is often a determinant factor for a given organism to survive a microbial outbreak. Hence, these genes represent relatively recent events in evolution and this also explains their poor conservation between the mouse and human genomes. In contrast, homeostatic chemokines are very well conserved throughout

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

evolution and often are the only chemokine genes that can be recognized between distant species. This is particularly applicable to the CXCL12/CXCR4 axis, which is one of the few chemokines that can be easily recognized between zebrafish and human [4]. Importantly, for the purposes of this discussion, homeostatic chemokines have many functions during development. In fact, the CXCL12/CXCR4 knockout mice are both embryonic lethal; and examination of the arrested developing embryos reveals significant defects in bone marrow, heart and central nervous system [5]. These early studies provided compelling evidence that chemokines are involved in many more processes than the simple chemotaxis of leukocytes in the body.

Another chemokine/receptor pair whose function was elucidated in the latter part of the 1990s was CCL21/CCR7. CCL21 was originally found to be a chemokine that exhibited significant specificity, as it was found to show its highest expression in the lymph nodes. It was further mapped to the higher endothelial venules. In contrast, CCR7, its receptor [6], was expressed in a subset of T lymphocytes, that eventually was recognized to include naïve T cells. It was soon recognized that a mouse that had no T cells in the lymph nodes (called the "paucity of lymph node T cells", or PLT mouse) was defective in CCL21 expression [7]. These observations indicated that the CCL21/CCR7 axis was necessary to allow T cells entry into lymph nodes. Similarly, another axis, CXCL13/CXCR5 was found to allow entry of B cells into lymph nodes [8]. These studies indicated that chemokines not only guided cells through chemotaxis, but were in fact absolutely necessary for certain cells to enter certain organs (i.e., lymph nodes). While this originally applied to lymphocytes, the question arose whether this paradigm was applicable to other cell types.

With this background, we set out to ask whether chemokines influenced metastasis. To this end, we first predicted that in order for this paradigm to be applicable, the expression of chemokine receptors in tumor cells could not be random. That is because different cancers exhibit specific metastatic patterns. To answer this question we measured the expression of all known chemokine receptors in breast cancer cell lines and observed that chemokine receptor expression was not random; and we identified several receptors as predominant among breast cancer cells. These included CXCR4 and CCR7. We went on to show that anti-CXCR4 antibodies significantly inhibited the metastasis of MDA-MB-231 cells to the lung [3]. The implications of these observations were apparent, and our study was soon followed by many others that support the conclusion that chemokines play a pivotal role in metastasis of cancer cells.

8.2
CXCR4 and CCR7 Receptors Play Special Roles in Cancer Metastasis

As explained above, we initially found these two receptors significantly expressed in breast cancer cells. Soon many other reports documented that they were also expressed in many cancers. There are now too many reports to discuss, so I only use some examples that highlight the dominant role of these receptors. These include

studies by Sam Hwang and colleagues. They used a mouse model of metastasis using a melanoma cell line (B16). This cell line usually does not metastasize *in vivo*. All they had to do was to transfect it with CCR7, and it then metastasized to the lymph nodes [9]. Conversely, when they transfected the same cell with CXCR4, it then metastasized to the lung [10]. These observations suggested a conclusion that agrees with a substantial body of clinical data, namely that CCR7 is a pivotal receptor regulating the metastasis of tumor cells to the lymph node, while CXCR4 regulates the metastasis of tumor cells to lung, liver, bone marrow and possibly brain. This conclusion agrees with the expression patterns of the ligands of these receptors: CCL21 is mainly expressed in the lymph nodes, while CXCL12 is strongly expressed in bone marrow, lung, liver, lymph node and some sites in the central nervous system (CNS).

It is worth discussing further the latter conclusion. There are preferred anatomical sites for metastasis within the body for most cancers. These include lung, liver, bone marrow and lymph node. Other sites occur as well but with reduced frequency, like the CNS. Finally, there are organs that represent poor targets for metastatic dissemination for most cancers. These include stomach, kidney, esophagus, pancreas and colon. These sites can be sources of metastatic cells, but they usually do not represent metastatic destinations. Accordingly, they are poor producers of CXCL12, the ligand of CXCR4. These clinical observations suggest that the CXCL12/CXCR4 axis is a pivotal controlling factor in cancer metastasis in many human cancers. Accordingly, as discussed below, CXCR4 is the most common and widely expressed chemokine receptor in many human cancers.

8.3
Retrospective Clinical Data Supports a Role for Chemokines in Cancer Metastasis

Following the initial reports, other groups performed retrospective clinical studies using paraffin-embedded tumor samples from cancer patients with detailed clinical history and outcomes. There are many such studies in the literature, and I have reviewed this area in detail [11]. We can summarize these studies by concluding that the clinical data strongly support a role for chemokines in cancer metastasis. The most widely expressed chemokine receptor in most kinds of cancer is CXCR4; CCR7 is the second most commonly expressed receptor. Other receptors that are only expressed in selected cancers include: (i) CCR10 in melanoma, (ii) CX3CR1 in glioblastoma and other CNS tumors and (iii) CCR9 in a subset of melanoma patients. Interestingly, in the latter cases, there is a correlation between the expression of these chemokine receptors and the metastatic behavior of the tumor. Melanoma can metastasize to the skin, and one of the ligands of CCR10 (CCL27) is strongly and specifically expressed in the skin. Further, glioblastoma has a unique ability to invade brain tissue, and CX3CL1 (the ligand of CX3CR1) is strongly expressed in the CNS. Finally, the expression of CCR9 in melanomas predicts metastasis to the small intestine, which is known to express CCL25 [12], the ligand of CCR9. These examples of special chemokine-mediated metastatic cases lend strong support to the overall

model where some chemokines play a strong role in defining metastatic destinations in cancer.

The expression of CXCR4 in particular has been reported to correlate with a worse prognosis in a wide variety of cancers [11]. This correlates with many mouse model reports where the expression of CXCR4 not only results in more abundant metastatic lesions, but also these lesions develop much faster than in tumors that do not express CXCR4. One of the outstanding questions refers to the mechanism of action of CXCR4 in these lesions. Unfortunately, the answer to this question is not likely to be simple, and it involves many aspects that are considered below.

8.4 How Does the CXCR4/CXCL12 Axis Influence the Development of Metastatic Lesions?

The difference in tumor spread and the development of metastatic lesions in animal models in the presence or absence of anti-CXCR4 inhibitors is very dramatic. Depending on the tumor type, the tumor size difference can easily exceed tenfold. One of the most obvious possibilities is that CXCR4 directly mediates the growth of tumor cells. This can be easily tested in tumor models when the tumors are derived from cell lines. In this context, most studies have found that CXCL12 does not directly influence tumor cell growth *in vitro*. The exception is glioblastoma or other central nervous system cells [13], where there are reports that CXCL12 has a direct growth-promoting effect in these cells. Importantly, the expression of CXCR4 in glioblastoma and other CNS tumors is significantly associated with a poor prognosis [14], as it appears that more invasive tumors express CXCR4.

But in the case of most other tumor types, especially those involving epithelial cells, it is not clear that CXCR4 signaling results in growth promotion. Instead, its function may be more related to what I would call "organogenesis", which is a function already associated with CXCR4 in development. To explain this, it is first necessary to review the findings on the role of chemokines in the developmental biology of zebrafish, an extremely interesting model that allows unprecedented and detailed analysis.

8.5 CXCR4 is a Key Player in the Development of Zebrafish; Role of CXCR7

While immunologists played a pioneering role in the development of the chemokine field, neurobiologists and developmental biologists have now discovered that chemokines are important in their fields and have made significant contributions to our understanding of the biological role of these molecules *in vivo*. An excellent example is the role of chemokines in zebrafish development. Importantly, the most interesting molecules here are the CXCR4/CXCL12 axis and, as we review below, CXCR7. A pioneering observation was that CXCR4/CXCL12 is necessary for the germ stem cells that give rise to the gonads of the fish to migrate to the correct

location in the embryo during its development [15]. This phenomenon also occurs in the mouse [16].

The zebrafish offers an excellent model for the study of embryo development. It is virtually transparent during the first week of life, so it is easy to detect and follow developmental abnormalities. These characteristics offered pivotal advantages to understand the role of a new CXCL12 receptor, CXCR7, in development and (potentially) in cancer. CXCR7 is now the name of a receptor originally described as RDC1 [17]. This is a G protein-coupled receptor that was reported in 2005 to also bind CXCL12 [18]. This was a surprising observation because for a long time we assumed that the only receptor for CXCL12 was CXCR4. This observation was followed by several studies that provided more puzzling details of the interactions between these three molecules (CXCL12, CXCR4, CXCR7). Clarification of the respective roles of these molecules has come from studies in zebrafish, where all three molecules are expressed and exhibit specific roles during development [19]. The overall observation was that mutant fish lacking CXCL12 showed a more dramatic phenotype than fish lacking CXCR4. It appears that the interaction CXCL12/CXCR4 is necessary to provide the "leading edge" for the migration of precursor cells to their proper place in the developing embryo, but CXCL12/CXCR7 is required to complete the process and bring the tail end of the migrating cells to their proper place for continued development. The latter effect may be mediated by scavenging of the chemokine; at this point the exact mechanism is not known. What appears clear is that these three molecules orchestrate a finely tuned mechanism that appears to control the fine details of stem cell homing during development [20]. The phenotype of the CXCR7 knockout mouse is consistent with the view that CXCR7 "fine tunes" the function of CXCR4, since it does not show the dramatic brain phenotype observed in the knockout of the latter receptor [21].

The discussion above confirms that CXCL12, CXCR4 and now CXCR7 play important roles in development but we do not know how these aspects translate into cancer metastasis. To apply these observations to cancer, it helps to understand that metastasis shares many aspects with development of a given organ and that the developing organ needs to have a minimum organization in order to survive and grow. This is what chemokines seem to be providing and is what can collectively be called "organogenesis." However, while a role for CXCR7 in the biological effects of CXCL12/CXCR4 seems a solid conclusion, there are other observations that suggest some caution when analyzing its potential as a cancer target. First, the phenotype of the CXCR7 knockout suggests a more discrete effect of this receptor. Second, it does not signal like other chemokine receptors; for example, it fails to elicit a Ca^{2+} flux when triggered by CXCL12 [22]. There is conflicting information on the potential of CXCR7 antagonists to prevent tumor growth or metastasis. Some reports have found inhibition by CXCR7 antagonists [22], while others have found no such evidence [23]. Taken together, these observations suggest that CXCR7 evolved to modulate the function of CXCL12/CXCR4 but is unlikely to be a key player on a par with CXCR4. As the zebrafish results have shown, CXCR7 may be necessary to finely tune the (more powerful) effects of CXCR4 in guiding stem cell homing and development. Its lack of signaling induction by CXCL12 and suggested role as a CXCL12 scavenger

suggest that it is possible that this is more akin to other decoy receptors, like Duffy, whose main function is to tightly regulate the concentrations of very powerful chemokines (CXCL12). One possibility however, may be to explore what the effect of high expression of CXCR7 would be in highly metastatic tumors that strongly express CXCR4. This and related experiments would be interesting in future studies.

8.6
The CXCR4/CXCL12 Axis in Stem Cell Homing in the Bone Marrow

Another aspect worth discussing is the role of CXCL12/CXCR4 in stem cell homing. Besides the literature documenting the effects of CXCL12/CXCR4 in zebrafish development, the data on CXCR4 and cancer metastasis has led several companies to develop small molecule antagonists. One of the better known antagonists is AMD3100, originally developed by Anormed (later acquired by Genzyme). Genzyme has developed this compound for therapeutic use. Surprisingly, one of its effects *in vivo* was the rapid mobilization of CD34 + stem cells from the bone marrow into the circulation [24–26]. It appears therefore that the CXCL12/CXCR4 axis is a key signal that "keeps" the stem cells in the bone marrow. It is part of a complex mechanism that maintains the stem cells in the bone marrow and releases them sparingly in response to as yet unknown signals.

The significance of this observation to the complex effects of CXCL12/CXCR4 in cancer development has not yet been completely understood or adequately studied. Recent studies have documented the existence of "cancer stem cells" that seed and promote tumor development [27]. The general idea is that, just like there are stem cells that selectively repopulate normal organs, there are tumor stem cells that continuously seed the tumor and promote its growth. If this hypothesis proves correct, then the therapeutic approach would be to specifically target this population of cells instead of the general tumor cell population. Many of the current targets of cancer therapeutics may or may not be expressed in tumor stem cells. Whether this hypothesis proves correct or not, it is very likely that CXCL12/CXCR4 may play a role in the homing and maintenance of tumor stem cells, although at present it is not well understood how a potential therapeutic would be used. We will have to await better characterization of tumor stem cells in order to answer these questions.

8.7
Conclusions and Future Directions

There is little doubt that the CXCL12/CXCR4 axis is a pivotal signal in the metastatic dissemination of many cancers. What we do not know is how we can harness this information for the development of novel therapeutics. The most straightforward conclusion is that, in principle, inhibitors of the CXCL12/CXCR4 axis should be beneficial in the treatment of many cancers. However, some caution is warranted, because another conclusion is that the CXCL12/CXCR4 axis represents a very

powerful, evolutionary ancestral signal of extreme importance in development and in the normal function of many systems, including the immune, cardiovascular, hematopoietic and central nervous system. We do not know enough about what the consequences of continued inhibition of this axis would be in humans. This complicates the design of long-term clinical trials using CXCR4 antagonists in cancer.

However, perhaps the key to this problem would be careful selection of the cancer to be treated. In many cancers, the first line of treatment includes surgery to try to eliminate the initial source of neoplasia. In many patients, however, the cancer is not eliminated and later returns and metastasizes. In some cancers, this process is relatively predictable. That is the case for ovarian cancer, for example. For this reason, this may be a good candidate for initial clinical trials. Another reason it is a good candidate is because CXCR4 is the only chemokine receptor expressed in ovarian cancer [28].

A very welcome development is the fact that recently a CXCR4 antagonist was approved for mobilization of bone marrow stem cells. It has been shown to synergize with GCSF in the recovery of leukocyte counts following cancer therapies (radiation, chemotherapy). The availability of this approved CXCR4 inhibitor for clinical indications will likely facilitate its use off-label in cancer indications, and as a result we are likely to obtain much needed clinical data on the potential of this novel kind of drug in the treatment of cancer.

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9
Constitutively Active Viral Chemokine Receptors: Tools for Immune Subversion and Pathogenesis

David Maussang, Gerold Bongers, Sergio A. Lira, and Martine J. Smit

9.1
Introduction

Herpesviruses are large double-stranded DNA viruses with an icosahedral nucleocapsid surrounded by a tegument and the outer lipid envelope that contains multiple viral glycoproteins. The human *herpesviridae* family encompasses eight members classified into three different subfamilies: α, β and γ. These subfamilies were established according to genetic variations in the conserved gH structural protein and possess characteristic physiological properties (Table 9.1). The alpha human herpesviruses (α-HHV) subfamily consists of HHV-1, HHV-2 and HHV-3, also known as herpes simplex virus (HSV)-1, HSV-2 and varicella zoster virus, respectively. They are neurotropic and cause fever blisters, genital sores and chicken pox and shingles respectively. The β-HHV subfamily is categorized into the *Cytomegalovirus* genus (HHV-5) and the *Roseolovirus* genus (HHV-6, HHV-7). These viruses exhibit a broad cellular tropism and are involved in a wide range of diseases. The γ-HHV subfamiliy comprises the *lymphocryptovirus* HHV-4 and the *rhadinovirus* HHV-8. While the involvement of β-HHV in pathological conditions is becoming apparent and requires further investigation, the role of γ-HHV in oncogenesis is clearly established. These lymphotropic viruses are extensively linked to proliferative diseases due to their transforming potential.

Infections by HHV are usually asymptomatic. In order to survive within the infected immunocompetent host, viruses have acquired genes that allow them to evade the immune system or to be controlled by immune cells without being eliminated. However, upon immunosuppression, viruses escape immune surveillance and can be reactivated, leading to the development of serious pathologies. During evolution, the β- and γ-HHV have pirated genes from the human chemokine receptor family (Table 9.2). Although their human counterparts require ligands in order to signal and induce intracellular events, most of the viral chemokine receptors can signal in a constitutive, that is, ligand-independent, manner. Upon viral infection, these viral receptors can perturb intracellular signaling pathways and have

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

Table 9.1 Classification and diseases association of beta- and gamma-herpesviruses.

Subfamily	Genus	Common name	Species	Prevalence in populations	Associated diseases
Beta-herpesviridae	*Cytomegalovirus*	Human cytomegalovirus (HCMV)	HHV-5	30–100%	Immunocompromised fetus: hearing loss, abnormal development
					Immunosupressed graft recipient: graft rejection/loss, graft-specific diseases (e.g., pancreatitis, hepatitis), cardiovascular diseases
					AIDS patients: end-organ diseases, colitis, retinitis
					Immunocompetent hosts: cardiovascular disease (atherosclerosis), proliferative disease (colon cancer)
	Roseolovirus		HHV-6	70–100%	Exanthema subitum in children
					CNS diseases such as encephalitis, encephalopathy, multiple sclerosis
	Roseolovirus		HHV-7	Approximately 75%	Exanthema subitum in children
Gamma-herpesviridae	*Lymphocrypto-virus*	Epstein–Barr virus (EBV)	HHV-4	Over 90%	Mononucleosis in adolescents (kissing disease)

					Immunocompetent hosts: Burkitt's and Hodgkin's lymphomas, nasopharyngeal carcinoma CNS diseases such as multiple sclerosis Other proliferative diseases, for example, gastric carcinoma, breast cancer, T cell lymphoma, follicular dendritic cell tumors Immunosuppressed graft recipients: post-transplant lymphoproliferative disease AIDS patients: Burkitt's and Hodgkin's lymphomas, CNS lymphomas, leiosarcomas, oral hairy leukoplakia
	Rhadinovirus	Kaposi's sarcoma associated herpesvirus (KSHV)	HHV-8	2–50%	Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease

multiple (patho)physiological functions or be involved in the virus life cycle (Table 9.2).

9.2
Herpesviruses and Viral Diseases

9.2.1
The Betaherpesviridae Subfamily

9.2.1.1 The Cytomegalovirus Genus: HHV-5/HCMV

The β-herpesvirus HHV-5, also known as the human cytomegalovirus (HCMV) has been detected in a wide proportion of the population raging from 30% up to 100% depending on the geographical location [1]. Acute and chronic HCMV infections lead to the development of viral diseases, in particular in immunocompromised patients. A typical manifestation of acute infection occurs during gestation where infected fetuses can present severe hear loss or development abnormalities [1]. Also, immunosuppressed transplantation patients experiencing primary, that is, acute, HCMV infection are at stake with graft rejection and diseases specific to the engrafted organ. For instance, liver and pancreas transplant patients are prone to develop hepatitis and pancreatitis, respectively [1]. Chronic HCMV infection in immunosuppressed transplant patients is a cofactor for vascular diseases resulting in allograft loss. In particular, HCMV-associated cardiac allograft vasculopathy (CAV) commonly leads to cardiac allograft loss. [1]. HCMV also has a major impact in human immunodeficiency virus (HIV)-infected patients by causing end-organ diseases, particularly in the gastrointestinal tract and the eye. In most cases, HCMV infection leads to the development of colitis and retinitis.

Yet, patients apparently immunocompetent also are prone to the development of diseases similar to those observed in immunocompromised patients [2]. Moreover, HCMV infection in immunocompetent hosts is increasingly linked to the development of inflammatory conditions such as cardiovascular and proliferative diseases [1, 3, 4]. For instance, HCMV is becoming a widely accepted pathogen involved in the development of atherosclerosis [3]. Furthermore, studies highlighted the presence of HCMV gene products in several malignancies, for example, breast, colon and prostate cancer as well as glioblastoma (reviewed in [3, 4]). In addition, HCMV is not presented as an oncogenic virus but rather as a virus that possesses oncomodulatory properties [5]. Cellular transformation and genetic instability may be a prerequisite for the virus to contribute to tumor progression. HCMV preferably infects tumor cells and can alter several molecular mechanisms to influence cell cycle progression, apoptosis and migration of cells. Cancer cells may also help the virus to evade immune surveillance [6]. As such, HCMV may further increase the transformed potential of the tumor cells while avoiding its clearance by the immune system.

Table 9.2 Chemokine-related characteristics of vGPCRs and their putative functions.

Virus	vGPCR	CKR homolog	Ligand	Putative functions
HHV-4	BILF1	CXCR4	Unknown	Immune system escape: downregulates MHC Class I and PKC Homeostasis and cellular trafficking: heterodimerizes with a variety of human chemokine receptors in B cells
HHV-5	US28	CX3CR1, CCR5	CCL2, CCL3, CCL4, CCL5, CCL7, CX3CL1, vCXCL2	Atherosclerosis: mediates smooth muscle cells migration in HCMV-infected cells and transfected cells Oncogenesis: induces a proangiogenic and inflammatory phenotype in vitro and tumor formation in xenograft models
	UL33	CCR10	Unknown	Viral life: mouse and rat orthologs important for viral replication in salivary glands
	UL78	CXCR1	Unknown	Viral life: mouse and rat orthologs important for virulence and viral replication.
	US27	CXCR3	Unknown	Unknown
HHV-6	U12	CCR10	CCL2, CCL3, CCL4, CCL5	Unknown
	U51	—	CCL2, CCL5, CCL7, CCL11, CCL13, CCL19, CCL22, CX3CL1	Immunomodulation: downregulates CCL5 and FOG-2

(*Continued*)

Table 9.2 (*Continued*)

Virus	vGPCR	CKR homolog	Ligand	Putative functions
				Viral life: important for viral replication, induction of cytopathic effects and can enhance cell-cell fusion in infected cells
HHV-7	U12	CX3CR1	CCL17, CCL19, CCL21, CCL22	Unknown
	U51	CCR2	CCL17, CCL19, CCL21, CCL22	Unknown
HHV-8	ORF74	CXCR2	CCL1, 5; CXCL1, 2, 3, 4, 5, 6, 7, 8, 10, 12; vCXCL2	Oncogenesis: induces a proangiogenic and inflammatory phenotype (release of cytokines and growth factors and upregulation of adhesion molecules) Oncogenesis: transforms cells in direct and paracrine manners Oncogenesis: induces tumor formation in xenograft models and transgenic animals

9.2.1.2 The Rhesolovirus Genus: HHV-6 and HHV-7

HHV-6 and HHV-7 from the *Rhesolovirus* genus present a high distribution worldwide with infection rates of respectively 70–100% and 75% [7, 8]. Primary infection with HHV-6 results in the development of exanthema subitum and can lead to complications such as malaise, febrile seizures and in some rare cases encephalitis [7]. HHV-6 is heavily associated with diseases of the central nervous system in immunocompromised patients such as encephalitis and/or encephalopathy [7], but also with multiple sclerosis. In particular, anti-HHV-6 antibodies and HHV-6 DNA have been detected in MS cerebrospinal fluid and MS plaques, respectively [9]. So far, the link of HHV-7 and diseases other than exanthema subitum during primary infection remains highly speculative [9–11]. Additional studies are required to understand the true contribution of HHV-7 in pathological conditions.

9.2.2 The Gammaherpesviridae Subfamily

9.2.2.1 The Lymphocryptovirus Genus: HHV-4/EBV

The Epstein–Barr virus, discovered in 1964 [12], is the fourth virus in the human herpesvirus family and belongs to the genus of the lymphocryptoviruses. After control of EBV infection by the immune system, the virus remains in a latent stage in B lymphocytes and epithelial cells. During this stage, a restricted number of latent genes is expressed [13]. EBV is involved in a broad spectrum of diseases, which can differ between immunocompetent and immunocompromised hosts. Healthy individuals infected with EBV are more prone to develop malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma. However, EBV is not the only factor involved in lymphoproliferative pathogenesis because not all EBV-infected individuals develop these diseases [14]. Immunocompromised hosts are also at risk with EBV-related diseases due to viral reactivation. In particular, transplant patients undergoing immunosuppression therapies fail to have a tight regulation of EBV-infected B cells and can develop post-transplant lymphoproliferative diseases (PTLD) [15]. However, PTLD can be reversed upon restoration of the immune system. In addition, HIV-infected patients in terminal AIDS phase may present EBV-related lymphomas such as Burkitt's and Hodgkin's types and lymphomas in the central nervous system [14]. Lytic viral replication is also responsible for oral hairy leukoplakia, a nonmalignant proliferative disease common in HIV patients [16].

9.2.2.2 The Rhadinovirus Genus: HHV-8/KSHV

The eighth herpesvirus was discovered in Kaposi's sarcoma lesions and named accordingly Kaposi's sarcoma herpesvirus (KSHV) [17]. KSHV infection rates are lower than other herpesviruses, ranging from a small percentage up to 50% in different geographical areas [18]. HHV-8 DNA was initially detected in Kaposi's sarcoma lesions and later on in primary effusion lymphoma (PEL) [19] and in multicentric Castleman's disease (MCD) [20]. Furthermore, the expression of latent viral proteins was confirmed in KS, PEL and MCD tissues, confirming the pathogenic

role of KSHV in these diseases [21, 22]. Failure of immune surveillance appears to be a necessary factor for the development of KSHV-related diseases since immunosuppressed transplant patients or AIDS patients are prone to develop KSHV-related diseases [23, 24]. HHV-8 has also been suggested to be associated with other diseases such as salivary gland tumors, lymphoproliferative diseases [23] and even multiple sclerosis [9]. However, additional studies are required to support an epidemiological link with KSHV infection.

9.3
Herpesviruses Encode Constitutively Active Viral Chemokine Receptors

9.3.1
Human Cytomegalovirus Encodes Four Different Viral G Protein-Coupled Receptors

9.3.1.1 The Chemokine Receptor US28 Mediates HCMV-Induced (Patho)Physiological Effects

HCMV encodes four different viral GPCRs (vGPCRs), namely US27, US28, UL33 and UL78, of which US28 is the best characterized [25, 26]. Numerous publications have contributed to understand the importance of this vGPCR in a wide range of pathogenic processes. US28 protein is present on HCMV virions [27] and has been found dispensable for viral growth in infected HFF cells [28]. Recently, US28 was shown to activate the major immediate early promoter of HCMV suggesting to subsequently transactivate other viral genes [29]. US28 gene product is transcribed just after IE genes and before pp65 in HCMV-infected cells *in vitro* [30]. In addition to this early phase of expression, US28 mRNA and protein expressions have been determined at later time points in various cell types [30–34]. Interestingly, US28 expression was also found *in vivo* in HCMV-infected patients. For instance, US28 mRNA expression was measured in blood samples of naturally infected hosts [35], HIV patients [36] as well as immunosuppressed lung transplant patients [37]. US28 was also detected in neonates and fetuses presenting a congenital HCMV infection [38]. Finally, it was recently discovered that serum of artery coronary patients contains antibodies that recognize US28 protein, implying that US28 is or has been expressed in these patients [39].

Ligand-Dependent US28 Signaling Activities The primary function US28 is to bind a broad range of chemokines. US28 presents highest homology to CC and CX3C chemokine receptors [40], and binds different classes of CC and CX3C chemokines including CCL2, CCL3, CCL4, CCL5, CCL7 and CX3CL1 [41–43], as well as the KSHV-encoded viral chemokine vCXCL2 [44]. Initially, chemokines were shown to induce calcium signaling in US28-transfected cells (CCL2 [41], CCL5 [41, 42], CCL7 [42]), but they appear to possess different and specific signaling capacities. Chemokines also bind to US28 in the supernatant of HCMV-infected cells, resulting in their subsequent depletion. This chemokine sink function of US28 was suggested to help HCMV to escape immune surveillance [28, 32, 42, 45, 46].

Exposure of US28-transfected cells to CCL5 induces calcium signaling and ERK activation via $G\alpha_{i/o}$ and $G\alpha_{16}$ proteins, while CCL7 only uses $G\alpha_{16}$ proteins to modulate calcium flux [42]. Also, CCL2 induces calcium signaling in US28-expressing cells, but the coupling G protein remains undetermined [41]. Although CCL3 and CCL4 bind to US28 with high affinity, these chemokines have not been shown to induce any signaling so far [41, 47]. Interestingly, the modulation of calcium influx in HCMV-infected cells has also been observed after stimulation with CCL5 [42].

One of the key features of human chemokine receptors is their ability to induce chemotaxis. As such, the ability of US28 to induce cellular migration was investigated. CC chemokines CCL2 and CCL5 were shown to induce US28-mediated chemotaxis via $G\alpha_{12}$ rather than $G\alpha_{i/o}$ proteins [48, 49]. Treatment of US28-expressing smooth muscle cells (SMC) with pertussis toxin showed no inhibitory effects of CCL2 or CCL5-induced chemotaxis [48], while co-transfection of $G\alpha_{12}$ or $G\alpha_{13}$ proteins enhanced US28-mediated migration of HCMV-infected cells [49, 50]. In addition, $G\alpha_{12}$ co-transfection potentiated US28-activated focal adhesion kinase (FAK) and RhoA kinase signaling [49, 50]. Recently, it was shown that CX3CL1 preferentially induces the chemotaxis of macrophages but not of SMC. CX3CL1 antagonizes CCL5-induced SMC chemotaxis and induces FAK activation via $G\alpha_q$ proteins [50]. In SMC, US28 was induces p42/p44 MAPK activation upon stimulation with CCL5 or CX3CL1 [50]. In addition, US28 is responsible for HCMV-induced cell migration. The deletion of the US28 gene from the HCMV viral genome impairs cellular migration observed in infected cells [48] and the co-transfection of either $G\alpha_{12}$ or $G\alpha_{13}$ further increases the migration of HCMV-infected cells [49]. Taken together, these data present US28 as a molecular mediator of atherosclerosis pathogenesis and viral dissemination in HCMV-infected patients.

Ligand-Independent US28 Signaling Besides the ligand-induced activities observed in US28-expressing cells, this vGPCR possesses prominent ligand-independent activity. We initially demonstrated that expression of US28 induces the constitutive activation of the phospholipase C and the transcription factor NF-κB [51]. This constitutive signaling involves $G\alpha_q$ and $G\beta\gamma$ subunits and pre-treatment of the cells with pertussis toxin confirmed that these cellular processes are not mediated by $G\alpha_{i/o}$ proteins. Using a G protein-uncoupled mutant of US28, US28-R^{129}A, and a US28 mutant that cannot bind ligands, US28-Δ2-22, we demonstrated that G protein activation, but not chemokine binding, were important for the constitutive formation of inositol phosphates by US28 [33, 52]. US28 is also responsible for the activation of the cyclic AMP responsive element binding protein (CREB) and the nuclear factor activated in T cells (NFAT) transcription factors through p38 MAPK- and p42/p44 MAPK-dependent pathways [53]. Pre-treatment of cells with PTX indicated that NFAT transcriptional activation partly relied on a $G\alpha_i$ component [53]. Recently, the constitutive activation of $G\alpha_q$ subunits has also been shown to induce the transcription of the serum responsive element (SRE) [54]. As such, signaling studies indicate that US28 couples to a broad range of G proteins, such as $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ subunits upon ligand stimulation, whereas US28 constitutive activity is mediated mainly by $G\alpha_{q/11}$ and $G\beta\gamma$ proteins.

Upon chemokine stimulation, the human chemokine receptors expressed on the cell surface elicit activation of intracellular signaling cascades and further undergo internalization before being degraded or recycled to the cell surface [55]. The viral chemokine receptor US28 is able to undergo internalization independently from ligand stimulation, explaining why after transfection of US28 merely 20% of the protein is expressed on the cell surface [56]. While the coupling of G proteins was not important during constitutive internalization. In contrast, the C-terminus of US28 showed to be the crucial motif responsible for the ligand-independent endocytosis of the receptor [52]. CX3CL1 is the only chemokine able to downmodulate US28 constitutive activity in several signaling assays, and it was as such classified as a partial inverse agonist [51, 53, 57]. However, when US28 internalization was impaired by removing its C-terminal tail or replacing it by that of another human or viral GPCR, the mutant receptor presented a higher signaling activity than the nonmutated receptor and CX3CL1 could further potentiate its signaling [52]. As such, the constitutive internalization of US28 affects its signaling ability and it was suggested to mask the true agonist properties of its ligands.

US28 constitutive activity may also have an impact on the signaling abilities of other ligand-stimulated chemokine receptors. We showed that although CCL5 is a ligand for both US28 and CCR1, it failed to either enhance US28 constitutive activity or stimulate CCR1 signaling towards NF-κB [58]. While unstimulated cells co-transfected with CCR1 and US28 signaled in a similar manner than US28-expressing cells, CCL5 stimulation of CCR1 and US28 co-transfected cells induced an increase of NF-κB activation compared to stimulated cells expressing only US28 [58]. Interestingly, the use of the US28-Δ2-22 and US28-R^{129}A mutants indicated that CCL5 was not mediating its potentiating effect via US28 but directly via CCR1, and that the constitutive activity of US28 was required to observe NF-κB signaling, respectively. This potentiation was entirely PTX-sensitive, demonstrating that ligand stimulation induced the coupling of CCR1 to $G\alpha_{i/o}$ proteins. As such, ligand-stimulated $G\alpha_{i/o}$ protein coupling can be diverted by US28 to enhance its own constitutive signaling.

The constitutive activity of US28 was shown in several studies in a viral setting. Deletion of the US28 gene from the genome of laboratory (AD169, Toledo) and clinical (TB40/E) HCMV strains showed that this vGPCR is the main mediator of the constitutive activation of $G\alpha_q$-mediated signaling pathways in HCMV-infected human foreskin fibroblast cells [59]. Mutant viruses presenting a deletion of the N-terminus of US28 could induce inositol phosphate formation at a similar level than WT HCMV strains, indicating that chemokine binding did not play a role in the observed PLC activation [34].

In order to block US28 constitutive activity, we have developed a series of compound that can act as inverse agonists. The first nonpeptidergic compound, VUF2274, was derived from an antagonist against the chemokine receptor CCR1 [33]. Besides inhibiting US28-mediated signaling activity in transfected cells, VUF2274 could also reduce the constitutive activity of US28 observed in HCMV-infected cells [33]. Such inverse agonist compounds may be promising antiviral drugs, but further optimization and improvement of the affinity of these compounds is still required [60, 61].

Potential Roles of US28 in HCMV-Related Pathological Conditions US28 activates proliferative and inflammatory signaling pathways that may favor tumorigenesis and the development of cardiovascular diseases. In particular, we showed that US28 constitutively activates the inflammatory transcription factor NF-κB [51] that in turn induces the transcription of the promoters of the human VEGF and COX-2 genes [62, 63]. Using the COX-2 specific inhibitor Celecoxib, COX-2 was shown to be responsible for VEGF gene activation and protein secretion [62]. US28-stably transfected NIH-3T3 cells acquired the ability to induce the formation of foci when cultured *in vitro*, highlighting their transforming potential of US28 [63]. Furthermore, these cells presented a higher proliferation rate, as measured by an increased thymidine incorporation, cell cycle progression and expression of Cyclin D1 [63], a protein involved in cell cycle progression and upregulated in various forms of cancer [64]. When US28-expressing cells were injected in the flanks of nude mice, tumors developed as early as two weeks post-injection, and the tumor incidence reached 100% within three weeks after injection. The US28-induced tumors presented high vascularization, most likely due to the increased VEGF secretion and subsequent formation of new $CD31^+$ blood vessels [63]. Interestingly, mice treated with the COX-2 inhibitor Celecoxib presented a delayed tumor formation and a slower growth rate, indicating an important role for COX-2 in the US28-mediated tumor formation [65]. In addition, deletion of the US28 gene from the viral genome impaired the HCMV-induced activation of STAT3 and the promoters of COX-2 and VEGF genes [62–64]. As such, US28 expression may increase the inflammatory state of the infected cell. Since the oncomodulatory properties of HCMV are evident in transformed cells or in inflammatory stages, the expression of US28 may further contribute to HCMV oncomodulation. This may occur through either constitutive activation of the receptor or ligand-induced signaling via chemokines present during inflammatory conditions.

Streblow *et al.* demonstrated the role of US28 in cardiovascular diseases [48]. Using vascular SMC, they showed that the deletion of US28 from the HCMV genome impaired the cellular migration induced after viral infection. In addition, US28-transfected vascular SMC exhibited chemotaxis towards CCL2 and CCL5, providing a molecular link between HCMV infection and the development of cardiovascular diseases. Upon CCL5 binding, US28 activates $G\alpha_{12}$ proteins and its downstream effector RhoA kinase [49]. Furthermore, US28-mediated cell migration in HCMV-infected cells was sensitive to protein tyrosine kinase inhibitors [48]. Upon CCL5 and CX3CL1 stimulation, US28 interacts and activates Src kinase, which phosphorylates the focal adhesion kinase (FAK) and activates Grb2/Sos [50, 66]. Besides highlighting cell-specific phenotypes, the chemotactic responses elicited by CCL5 and CX3CL1 chemokines in SMC and macrophages, respectively, also provide several functions for US28 during the development of HCMV-related cardiovascular diseases [50]. US28 expression may on one hand induce macrophage infiltration into atherosclerotic plaques, while on the other hand, favor the recruitment of SMC to the inflamed lesion. As such, US28 expression may play an important role during the pathogenesis of cardiovascular diseases in HCMV-infected patients.

In addition, another function of US28 resides in its ability to facilitate HIV-1 entry. Pleskoff *et al.* demonstrated that US28 expression in $CD4^+$ cell lines is sufficient to

allow HIV-1 entry [67]. Since US28 presents homology to chemokine receptors, which have previously been to shown to act as co-receptors for HIV, US28 may possess common motifs that allow recognition by HIV and play a role in the interplay between HCMV and HIV-1 virus. The US28 inverse agonist VUF2274 is able to reduce US28-mediated HIV-1 entry [33], indicating US28 as a potential drug target in treatment of HIV infection.

9.3.1.2 The Constitutively Active Orphan Chemokine Receptor UL33

UL33 RNA transcripts are expressed in the early phase of HCMV infection [45] and its protein product has been found in viral particles [68, 69]. UL33 presents highest homology to the chemokine receptor CCR10 [70] but to date no ligands have been identified for this vGPCR. We have investigated the signaling abilities of this receptor and shown that UL33 can activate multiple G proteins in a ligand-independent manner. In COS-7 cells, expression of UL33 constitutively led to the production of inositol phosphates partially via $G\alpha_{q/11}$ and $G\alpha_{i/o}$. The modulation of CREB-driven gene transcription was mediated by the $G\alpha_{i/o}$, $G\alpha_s$ and $G\beta\gamma$ subunits [71], as well as the protein kinase p38 [57]. In addition, in a viral context UL33 was partially responsible for the HCMV-induced CREB activation in infected U373 cells [71].

While the roles of UL33 during viral life cycle or virus-induced diseases still remain undefined, some light has been shed on the importance of its rodent orthologs such as the mouse M33 and rat R33 receptors. Both rodent receptors belong to the UL33 gene family [57] and similarly to UL33, both M33 and R33 were shown to be dispensable for viral replication *in vitro* [72, 73]. However, these receptors are important for viral replication in the salivary glands of rodent CMV-infected animals [72, 74]. Also, M33 and R33 exhibit ligand-independent signaling towards NF-$\varkappa$B and CREB via $G\alpha_{q/11}$ and $G\alpha_{i/o}$ proteins [57, 75]. In contrast, the rodent orthologs present slightly different signaling abilities compared to their human counterpart UL33 [57, 71]. This is particularly true regarding the development of virus-associated diseases. We have discussed above the involvement of US28 in virus-induced cardiovascular diseases. In a similar manner, both R33 and M33 are important for the migration of respectively rat and mouse CMV-infected smooth muscle cells *in vitro* [73, 76]. In a physiological *in vivo* model, RCMV infection of immunocompromised rats led to a high mortality of heart-transplanted animals compared to non-infected recipients. Infection of animals with the R33 deletion mutant of RCMV resulted in a lower mortality compared to animals infected with WT RCMV, which was due to a milder development of transplant vascular sclerosis and subsequent slower graft rejection [76]. As such, the rodent UL33 genes present similar signaling properties to human UL33, but their functions and signaling abilities may compensate for the lack of US28 orthologs in these nonhuman viruses [57].

9.3.1.3 The Chemokine Receptor-Related US27 and UL78 Orphan Receptors

The US27 and UL78 HCMV-encoded GPCRs are the least characterized. US27 is considered a chemokine receptor presenting 23% homology to CXCR3 [70] but so far neither chemokine ligands [45] nor putative functions in viral life cycle or HCMV-

related pathologies have been identified. In virally infected cells, US27 is expressed during the late phase of infection and its protein product is heavily glycosylated [28, 45, 77]. Furthermore, several reports indicate that US27 protein is present in HCMV virions [69, 77, 78].

UL78 presents very low homology to chemokine receptors with only 13% to CXCR1 [70], and some other reports indicate that it may not be related to this family of GPCRs [40]. Michel and colleagues have investigated the importance of UL78 in HCMV viral life cycle and characterized it as an early gene that is also transcribed in the late phase of infection [79]. Deletion of the UL78 gene from the HCMV genome resulted in an unaltered ability of the virus to replicate both in human foreskin fibroblasts as well as in a renal artery organ culture system [79]. In contrast, the mouse M78 and rat R78 genes, which are also expressed during the early phase of infection, showed to be crucial for viral replication *in vitro* [80, 81] and the production of infectious RCMV particles in the spleen *in vivo* [82]. Interestingly, deletion of the R78 gene from the RCMV genome resulted in an increased survival of RMCV-infected immunocompromised rats, highlighting an important role for R78 in the pathogenesis of RCMV *in vivo* [80].

9.3.2
Roseoloviruses-Encoded Chemokine Receptors: The U12 and U51 Genes

U12 and U51 genes were first identified in the U1102 strain of the HHV-6A subfamily after DNA sequencing of the viral genome [26] and were later functionally characterized as chemokine receptors [83, 84]. Both chemokine receptors have different transcription kinetics in HHV-6-infected cells. U12 is expressed late during the viral replication phase [83], while U51 appears early after viral infection [85]. Homologues of the U12 and U51 genes have been found in HHV-7 [86, 87], but the U12 and U51 genes from HHV-6 and HHV-7 present different chemokine binding profiles. HHV-6A U51 has been shown to bind CCL2, CCL5, CCL7, CCL11, CCL13, CCL19, CCL22, CX3CL1 and XCL1 [84, 88], while HHV-7 U51 binds CCL17, CCL19, CCL21 and CCL22 but not CCL1, CCL2, CCL5 or CXCL8 [87]. Similarly, U12 from HHV-6B and HHV-7 bind CCL2, CCL3, CCL4, CCL5 [83] and CCL17, CCL19, CCL21, CCL22 [87] respectively. Furthermore, HHV-6A U51 can constitutively modulate signaling pathways [89]. While the constitutive activation of $G\alpha_q$ proteins by U51 in transfected COS-7 cells resulted in the production of inositol phosphate and the inhibition of cAMP responsive element (CRE)-mediated signaling, CCL2, CCL5 and CCL11 specifically modulated these signaling pathways in different manners. The three chemokines could reverse the constitutive CREB inhibition, acting as inverse agonist. However, CCL5 could also further increase U51-mediated PLC activation. The different chemokines were hypothesized to bind to different active states of U51, further modulating the downstream signaling of the receptor and acting as diverse pharmacological modulators [89]. Besides binding endogenous human chemokines and affect cellular chemotactic properties, HHV-6A U51 has been shown to down-regulate CCL5 and FOG-2 mRNA expressions [84, 88]. The transcriptional repression of these immunomodulatory genes may represent a way for HHV-6 to subvert the

immune system and avoid recognition by immune cells [88]. The function of U51 during HHV-6A viral life cycle has also been studied *in vitro*. With the use of siRNA against U51, it was shown that U51 is important for viral replication and induction of cytopathic effects after HHV-6A infection [90]. Furthermore, U51 can enhance cell–cell fusion but apparently not the virus–cell interaction [90]. So far, the signaling pathways activated by HHV-7 U51 and U12 from both HHV-6 and -7 have not been delineated and further studies are required to understand how these receptors may affect cellular functions and viral life cycles of their respective viruses.

9.3.3
EBV-Encoded Constitutively Active Orphan vGPCR BILF1

BILF1 is expressed as an early or immediate-early viral gene after EBV infection. BILF1 expression is restricted to the lytic phase of infection and is not associated to any of the latency programs [14, 91, 92]. This vGPCR is hypothesized to belong the class of chemokine receptors, presenting a low homology to CXCR4 [70], but so far no chemokines have been found to bind to this receptor. As such, BILF1 is still considered an orphan receptor. However, like other HHV-encoded chemokine receptors, BILF1 constitutively activates several signaling pathways. For instance, BILF1 expression has been shown to modulate CRE- and NF-$\varkappa$B-mediated gene activation in COS-7 and B cells via $G\alpha_{i/o}$ proteins [91, 93]. Interestingly, we have recently shown that BILF1 can heterodimerize with a wide variety of chemokine receptors, which could potentially affect their normal physiological functions and affect cellular migration capacities [94].

To this date, the functional consequence of BILF1 expression tends towards evasion from immune surveillance. Expression of BILF1 *in vitro* leads to the inhibition of phosphorylated RNA-dependent protein kinase (PKR) phosphorylation [91]. PKR plays an important function in cellular antiviral defense and the constitutive activity of BILF1 was postulated to help EBV to prevent the host immune response. Additionally, it was recently discovered that BILF1 can downmodulate the surface expression the MHC class I but not MHC class II proteins [95]. BILF1 was immunoprecipitated from transfected cells together with MHC class I molecules and these antigen presenting molecules were more rapidly internalized from the cell surface and subsequently degraded. In addition, overexpression of BILF1 in EBV-infected cells was able to reduce $CD8^+$ T cell activation [95]. As such, expression of BILF1 may provide a way for the virus to escape the immune system.

9.3.4
ORF74, the Tumorigenic Constitutively Active Chemokine Receptor Encoded by KSHV

The KSHV genome possesses a single chemokine receptor, ORF74, which was initially identified as a homolog of the IL-8 receptor [96, 97]. ORF74 binds a broad range of human CC and CXC chemokine ligands, namely CCL1, 5 and CXCL1, 2, 3, 4, 5, 6, 7, 8, 10, 12, as well as the KSHV-encoded viral chemokine vCXCL2 [98–103].

ORF74 expression is expressed during the early phase of lytic infection *in vitro* [104]. Its mouse ortholog has been shown to be dispensable for viral growth [105]. Interestingly, ORF74 can transactivate different promoters of KSHV viral genes, implying a potential regulatory function for ORF74 during viral infection [106]. In addition, ORF74 gene products have been detected in the main KSHV-related diseases, that is, KS, PEL and MCD [96, 97, 106, 107]. Initial studies on ORF74 rapidly demonstrated the oncogenic potential of this receptor [99, 108] and its signaling abilities were subsequently determined.

9.3.4.1 ORF74 Constitutively Activates Multiple Signaling Pathways in Different Cell Types

Initial characterization of ORF74 signaling properties demonstrated that this viral chemokine receptor signals in a constitutive manner via $G\alpha_{q/11}$ proteins. ORF74 expression leads to the constitutive formation of inositol phosphates [99, 109]. ORF74 also triggers constitutive signaling through $G\alpha_{i/o}$ and $G\beta\gamma$ subunits [109, 110] and through $G\alpha_{13}$ proteins [111]. In addition to classical trimeric G proteins, ORF74 constitutive activity can also be mediated by small G proteins such as Rac1 [112]. ORF74-induced G protein coupling appears to be cell type-dependent, highlighting the potential of ORF74 to adapt to different cellular context to perturb cellular homeostasis. The activation of a broad range of G proteins leads to the activation of various downstream kinases possessing different functions in ORF74-induced phenotypes. For instance, the constitutive stimulation of the protein kinase C (PKC) mediated by ORF74 [99] or the PMA-induced PKC stimulation results in an inhibition of $G\alpha_q$-mediated ORF74 constitutive signaling [99, 113]. Similarly, co-transfection of the GPCR-specific kinases GRK4, GRK5 or GRK6 inhibits ORF74 constitutive activity by desensitizing the receptor [113]. However, kinases traditionally associated with inflammation and proliferation were shown to be activated by ORF74 in a constitutive manner. This included members of the MAPK family such as p44/42 [109, 114, 115], p38 [108, 114, 115] and JNK [108, 114], but also Akt [109, 110, 115]. Other kinases involved in cell cycle regulation, for example, mTOR [116] and the p21-activated kinase 1 (PAK1) [112] are also important for ORF74-induced transformation. Different *in vitro* settings either inhibiting or overactivating these pathways highlighted the importance of these different kinases during ORF74-induced pathogenic conditions. Similarly, ORF74 can also constitutively activate several transcription factors that have been linked to inflammation and proliferation. Initially, a link between ORF74 and angiogenesis was established because of the presence of ORF74 in highly vascularized KS lesions. ORF74-expressing cells were shown to activate the hypoxia-inducible factor 1α (HIF1α) [114]. A wide variety of transfected cells also present a constitutive activation of different transcription factors, such as AP-1, NF-κB, CREB, NFAT and SRE [110, 111, 115, 117–119], which can further deregulate the expression of pro-angiogenic and inflammatory factors. A non-exhaustive list of molecules contributing to ORF74-derived pathologies includes the vascular endothelial growth factor VEGF and its receptor VEGFR2 [120], the inflammatory cytokines IL2, IL-4, IL-6 and GM-CSF [119, 121], the chemoattractant factors CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL8 [117, 121, 122] and adhesion molecules such as VCAM-1, ICAM-1 and E-selectin [121] (Table 9.3). It is important to

Table 9.3 Signaling abilities of viral chemokine receptors and determined functions in pathological settings.

Herpesvirus class	**β-HHV**	**β-HHV**		**γ-HHV**	
Viral chemokine receptor	**US28**	**UL33**	**U51**	**ORF74**	**BILF1**
Coupling G proteins	$\alpha_{q/11}$, $\alpha_{12/13}$, α_{16}, $\alpha_{i/o}$, $\beta\gamma$	$\alpha_{q/11}$, $\alpha_{i/o}$, $\beta\gamma$	$\alpha_{q/11}$	$\alpha_{q/11}$, α_{13}, $\alpha_{i/o}$, $\beta\gamma$	$\alpha_{i/o}$
Activated downstream kinases	MAPK p44/42, MAPK p38, FAK, RhoA	MAPK p38	?	MAPK p44/42, MAPK p38, MAPK JNK, PKB, PKC, JAK2, mTOR, PAK1	?
Activated transcription factors	NF-κB, CREB, NFAT, SRE	CREB	CREB	AP-1, NF-κB, CREB, NFAT, HIF1α, STAT3, SRE	NF-κB, CREB
Modulated target genes	?	?	CCL5, FOG2	Angiogenic factors (VEGF, VEGFR2, TNFα), inflammatory proteins (COX-2, IL-2, IL-4, IL-6, CXCL8, CCL5, GM-CSF), adhesion molecules (ICAM, VCAM), chemokines (CCL2, 3, 4, 7 and CXCL1, 9, 12)	PKR, MHC Class I
Potential diseases induced by the receptor	Atherosclerosis, oncogenesis	?	?	Oncogenesis	?

AP-1, activator protein 1; COX-2, cyclooxygenase-2; CREB, cAMP responsive element binding protein; FAK, focal adhesion kinase; FOG, zinc finger protein, multitype 2; GM-CSF, granulocyte-macrophage colony-stimulating-factor; HIF1a, hypoxia inducible factor 1a; ICAM, intercellular adhesion molecule; IL, interleukin; JKN, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; PKR, RNA-dependent protein kinase; SRE, serum responsive element; STAT3, signal transducer and activator of transcription 3; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

note that the secreted factors, along with the different modulated signaling pathways are highly cell type-dependent and highlight the ability of ORF74 to adapt to the cellular context to exert its pathogenic effects [117, 119, 121, 122].

The chemokines binding to ORF74 have all been pharmacologically characterized using G protein-coupling assays such as cAMP accumulation assays and IPx formation. Studies revealed that CXCL10, CXCL12 and vCXCL2 act as full inverse agonists; CXCL6 is a partial inverse agonist; CXCL4, 5, 7 and 8 act as neutral antagonists; CXCL2 is a partial agonist; and CXCL1 and CXCL3 are full agonists [98, 100–103]. The two most interesting and best characterized ligands for ORF74 are CXCL1 and CXCL10. Their pharmacological behaviors have been investigated beyond the scope of G protein coupling. The respective agonist and inverse agonist activities of CXCL1 and CXCL10 were highlighted during physiological relevant mechanisms such as DNA synthesis, activation of kinases (Akt and MAPK p44/42) and the NF-κB transcription factor, as well as in the release of CXCL8 and VEGF [101, 109, 111, 115, 117]. As such, these two chemokines may play prominent roles during the development of ORF74-related pathological conditions.

9.3.4.2 ORF74 Expression Mimics KSHV-Induced Transformation

Early studies indicated that the cells transformed with the KSHV-encoded vGPCR ORF74 show an increase in cell proliferation [99]. Furthermore, ORF74-expressing NIH-3T3 cells induced foci formation and injection of these cells in nude mice led to the formation of angiogenic lesions resembling those found in KS [108]. The strongest argument to date regarding the significance of ORF74 in inducing angiogenesis and inflammation in KS is derived from *in vivo* studies in mice. Mice expressing ORF74, either under the control of a segment of the CD2-promoter or by specifically targeting expression to endothelial cells using an *in vivo* retroviral-mediated infection system, develop angioproliferative lesions and tumors that strongly resemble those observed in KS patients [123, 124].

As discussed above, ORF74 is constitutively active but its activity can be enhanced by angiogenic chemokines and inhibited by angiostatic chemokines. To delineate the role of the ligand-dependent and -independent ORF74-mediated angioproliferation, transgenic mice were generated carrying mutant ORF74 [125]. Transgenic mice with a mutant ORF74 capable of ligand-binding and activation, but deficient in constitutive signaling, did not develop any angiogenic lesions by 90 days of age, despite cell-surface expression of this mutant receptor. To determine if chemokine binding was required for pathogenesis, a N-terminal deletion mutant ORF74 that was unable to bind chemokines was generated [98, 126]. Transgenic mice carrying this N-terminal deletion mutant of ORF74 did not develop any KS-like tumors, despite the fact that the mutant receptor still displayed unaltered high constitutive signaling activity. Finally, transgenic expression of a mutant ORF74, which preserved high constitutive activity but did not respond to the agonist chemokines CXCL1 and its mouse ortholog CXCL2, resulted in reduced penetrance and severity of the angioproliferative disease. These results indicate that modulation of the high constitutive activity of ORF74 by endogenous angiogenic chemokines is an important factor in the development of a KS-like angioproliferative disease in mice, since the constitutive activity of the receptor

alone is not sufficient. The modulation of ORF74 activity within tissues is probably mediated by chemokines expressed within the KS lesions, but the mechanisms of chemokine production and the kinetics of this interaction are presently unknown.

Both in human KS and in KS-like lesions found in ORF74-transgenic mouse models, expression of ORF74 is observed only in a subpopulation of spindle cells [123, 124, 127]. Therefore, it was suggested that the ORF74-mediated angioproliferation is due to paracrine mechanisms. As described above, ORF74-mediated signaling involves activation of for example, VEGF, AKT-mTOR, and NF-κB, leading to the induction of proangiogenic and proinflammatory factors [121]. Co-injection of ORF74-expressing endothelial cells with nontransformed cells greatly increased the tumorigenic potential of ORF74 compared to injection of ORF74-expressing cells alone. Although nontransformed endothelial cells do not proliferate by themselves, the presence of ORF74 on surrounding cells, through presumably a paracrine mechanism, leads to an increased tumorigenic potential of these cells [123]. Furthermore, ORF74-mediated neoplasia was shown to be dependent on activation of NF-κB and Akt. In human KS, both these pathways are over-activated, indicating their importance in the pathogenesis of human KS [128, 129].

There is also strong evidence that ORF74, besides activating a paracrine mechanism, plays a direct role angioproliferation and tumorigenesis. This was shown by using transgenic mice that express both ORF74 and β-galactosidase (LacZ) [130, 131]. Cells expressing ORF74 and LacZ within proliferative lesions were phenotyped, their distribution was mapped in early lesions and tumors and tests for their relevance to angioproliferation and tumorigenesis were evaluated. The ORF74/LacZ$^+$ cells were found to express markers of endothelial progenitor cells; they proliferated upon transgene activation (DOX treatment) and transferred disease to immunodeficient RAG1$^{-/-}$ mice. Furthermore, ORF74/LacZ$^+$ cells surrounded tumors but were scarce within them, mimicking what is observed in human KS. DNA profiling of tumor-derived laser-microdissected LacZ$^-$ cells suggested that they originated from the same population as ORF74/LacZ$^+$ cells. These findings suggested that ORF74 may induce tumorigenesis by a hit-and-run mechanism like other oncogenes. Together these results indicate that ORF74 induces an early autocrine component characterized by angioproliferation and inflammation followed by a second phase of tumorigenesis [130, 131].

Intervention with ORF74 expression or signaling during the early stages of the diseases (angioproliferative phase) has been effective [123, 129, 131, 132], indicating that ORF74 or ORF74-mediated signaling might be an attractive drug target in the treatment of KS. It remains unclear whether intervention during later stages of the disease, when few cells express ORF74, will be effective.

9.4 Concluding Remarks

The different herpesvirus-derived chemokine receptors have been hypothesized to be involved in a plethora of biological processes (Figure 9.1). One of their main

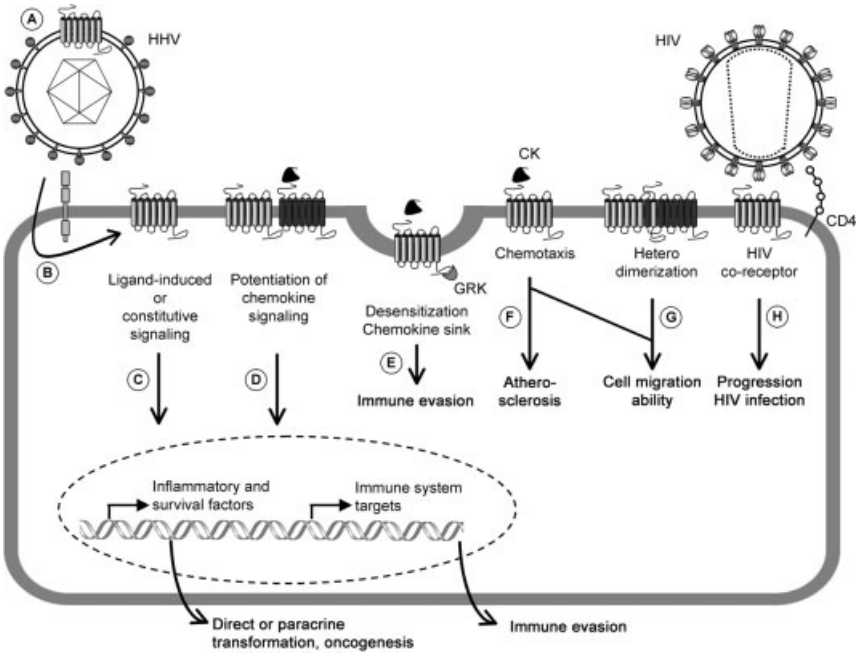

Figure 9.1 Schematic representation of the implication of viral chemokine receptors in various biological events and pathologies. Step A: Viral chemokine receptors are encoded in the viral genome but some of them are present on the virion and may participitate in cell adhesion during infection (US28, UL33, US27). Step B: Upon binding to its cellular coreceptor, the virus enters the cell by endocytosis and viral genes (e.g., viral chemokine receptors) are expressed on the surface of the infected cell using the host cellular machinery. Step C: Viral chemokine receptors signal in a ligand-independent or ligand-dependent manner. Constitutive activation of signaling pathways leads to the transcriptional modulation of several target genes, including inflammatory and survival factors, as well as targets of the immune system. Modulation of target genes have implications in transformation and oncogenesis (US28) or in the evasion of the immune system (BILF1, U51). Step D: Viral chemokine receptors also enhance ligand-stimulated signaling of coexpressed endogenous chemokine receptors. This leads to increased activation of transcription factors, for example, NF-κB (US28). Step E: Endogenous human chemokines are scavenged and can be constitutively internalized by viral chemokine receptors in order to reduce inflammatory signals and escape immune surveillance. Step F: Chemokine stimulation induces chemotaxis of cells expressing viral GPCRs, altering the normal migration ability of the targeted cell. In a pathophysiological setting, viral chemokine receptors may in turn play a role in the dissemination of the virus (all deorphanized viral chemokine receptors), or in the development of virus-induced cardiovascular conditions, for example, atherosclerosis (US28, M33, R33). Step G: Viral chemokine receptors were shown to heterodimerize with endogenous chemokine receptors, potentially modifying the natural migration abilities of the cell (BILF1). Step H: Besides using endogenous chemokine receptors, HIV-1 also utilizes US28 as cellular co-receptor in $CD4^+$ cells. This may in turn facilitate HIV infection.

hallmarks is to signal in a ligand-independent, that is, constitutive manner. As such, they can promote or repress the transcription of various cellular targets. For instance, the HHV-8-encoded ORF74 receptor upregulates various inflammatory and survival factors to promote viral oncogenesis. The constitutive activation of signaling pathways by BILF1 (from HHV-4) or U51 (from HHV-6) may help the virus to evade from the host immune system rather than to promote viral pathologies.

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Part Three
Targeting Chemokine Receptors

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

10
CCR5 Antagonists in HIV

David C. Pryde and Christopher G. Barber

10.1
Introduction

10.1.1
HIV and AIDS

Acquired immunodeficiency syndrome (AIDS) entered the public consciousness in the 1980s [1] with the identification of human immunodeficiency virus 1 (HIV-1) as the causative pathogen [2, 3]. HIV-1 infection and AIDS has since grown into epidemic proportions affecting millions of individuals across the globe. In 2007, some 33 million individuals were estimated to be infected, including some 2.7 million new infections in that year. Resulting deaths from AIDS in 2007 were estimated to be some 2.0 million [4].

Since the discovery of the HIV-1 pathogen, there has been much progress in understanding the virus, how it interacts with the immune system, its pathology and how to treat it. Highly active antiretroviral therapy (HAART) regimens introduced in the late 1990s have significantly reduced morbidity and mortality resulting from HIV-1 infection in the developed world, and there are currently 25 drugs spanning six classes approved for the treatment of HIV-1 infection. Current HAART regimens typically comprise three or more drugs selected until very recently from the protease inhibitor, nucleoside/nucleotide reverse transcriptase inhibitor, non-nucleoside reverse transcriptase inhibitor and fusion inhibitor classes. While effective, drug-related toxicity [5] and drug resistance [6] are severe shortcomings of current therapy. In one large study of HIV-positive adults who received treatment yet were viraemic with >500 HIV RNA copies/ml, within three years it was estimated that approximately 76% had resistance to one or more HIV drugs [7]. In addition, HIV resistance to one or more classes of drugs is increasingly transmitted to treatment-naïve patients, dramatically limiting their treatment options from the outset [8, 9]. This brings into sharp focus a continuing need for anti-HIV-1 agents which act through novel mechanisms.

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

10.1.2
Viral Entry as a Therapeutic Target

While the majority of the initial HIV-1 antivirals targeted essential viral proteins inside infected cells, the approval of enfuvirtide in 2003 [10] which targets the viral envelope glycoprotein and prevents the entry of virus into host cells, demonstrated viral entry to be a viable therapeutic target. During viral replication, the viral envelope glycoprotein, Env, is expressed as a 160-kDa precursor protein, which is processed intracellularly into surface and transmembrane subunits, termed gp120 and gp41, respectively (for a review, see [11]).

The first step in HIV-1 entry (see Figure 10.1) is the specific binding of gp120 to CD4 [12], the primary receptor used by HIV-1 on host cells such as T-lymphocytes and macrophages. This interaction provokes massive conformational change in gp120 to enable its further engagement of a co-receptor, most commonly the chemokine receptors CCR5 and/or CXCR4. This triggers conformational changes in gp41, which drives the remaining steps in viral entry and ultimately, fusion with the host cell. Generally, the vast majority of viral strains that are transmitted and establish new infections are R5-tropic and use CCR5 as the co-receptor for HIV-1 transmission, whereas the emergence of X4-tropic viral strains that use CXCR4 as a co-receptor has been associated with rapid CD4 T-lymphocyte decline and more advanced disease progression [13–15].

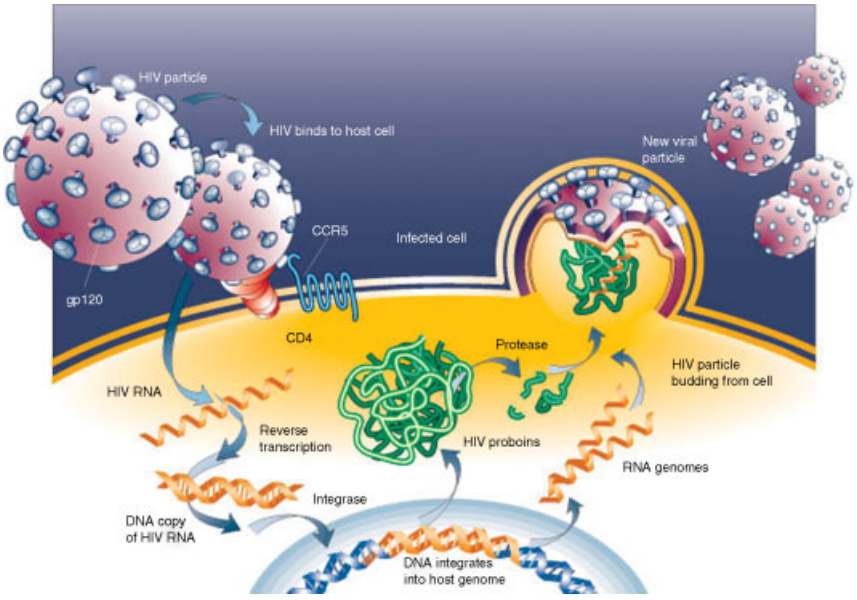

Figure 10.1 HIV lifecycle and points of intervention. Reprinted with permission from Macmillan Publishers Ltd [12].

10.1.3
CCR5 as a Therapeutic Target

Approximately 1% of the Caucasian population, lower in other populations, is homozygous for a 32-base-pair deletion in the gene encoding the CCR5 receptor which results in a complete absence of cell surface CCR5 expression. These individuals are highly resistant to HIV-1 infection. Heterozygotes for the CCR5-Δ32 allele have relatively low densities of cell surface CCR5 receptors and show delayed disease progression [16–19]. Significantly, there is no overt phenotype associated with CCR5-Δ32 homozygosity, and both homo- and heterozygotes are in good health, suggesting chronic functional blockade of the CCR5 receptor is a safe target for drug intervention.

CCR5 is a 352-amino-acid 7-transmembrane G-coupled receptor. It consists of three extracellular loops (ECL1-3) towards the N-terminus which engage chemokine ligands and three intracellular loops towards the C-terminus which mediate signal transduction. CCR5 is expressed on the cell surface of a wide range of cell types, and through engagement of its chemokine ligands, is believed to mediate the activation and trafficking of leukocytes to sites of inflammation. Activation of CCR5 also results in a number of typical GPCR-mediated cellular responses which include inhibition of cAMP production, and the release of Ca^{2+} [20]. The natural ligands of CCR5 are the macrophage inflammatory proteins CCL3 (MIP-1α) and CCL4 (MIP-1β), and the protein CCL5 [RANTES (regulated on activation, normal T cell expressed and secreted)], CCL8 [MCP-2 (monocyte chemoattractant protein 1)], CCL14 [(HCC-1) (hemofiltrate CC chemokine 1)], and CCL3L1 (the MIP-1α minor isoform variant LD78β) [21, 22]. CCL4 is selective for CCR5 and individuals with elevated levels of this chemokine have a reduced risk of HIV-1 infection, lending further weight to the potential of CCR5 antagonism as a viable mechanism [23]. Additionally, some 30% of all prescription drugs exert their effects through selectively targeting a cell-surface-accessible GPCR, and the CCR5 receptor would therefore be considered a “druggable” target [24].

10.1.4
Enabling Drug Discovery Programs Targeting CCR5

Armed with the above weight of evidence that antagonism of the CCR5 receptor could be an effective, safe and achievable therapeutic approach for the treatment of HIV-1, many major pharmaceutical companies have pursued CCR5 antagonist small molecule programs throughout the past decade. While aminergic GPCRs recognize small basic molecules, peptidic GPCRs such as CCR5 recognize short polypeptides, which are highly challenging to mimic with rationally designed small molecules [25]. As such, the vast majority of small molecule lead matter has emerged from random high-throughput screening of corporate compound collections. While not reviewed here, a number of alternative biological approaches based on analogs of endogenous peptide ligands or antibodies to the receptor have also been pursued (for an advanced example, see [26]). The majority of small molecule drug discovery programs were

initially based upon ligand displacement assays, using labeled chemokines [20] to identify leads. To study the interaction of the viral envelope protein with CCR5, a number of investigators described binding assays between labeled gp120 [27–29] and recombinantly expressed receptor, while more recently the binding of gp120/CD4 complexes to CCR5 has been measured [30, 31] using a gp120-targeted monoclonal antibody which is amenable to screening small chemical libraries. Technologies with higher throughput potential and greater correlation with antiviral activity in native systems have since been developed to interrogate fusion events occurring between transiently transfected cell lines [32, 33]. An assay able to detect both CCR5- and CXCR4-dependent fusion using inducible Env constructs has recently been reported [34, 35]. The above surrogate assays have now been complemented by the availability of a fully replicative high-throughput virus replication assay [36] which has made antiviral screening of large compound collections feasible.

Through this body of work, knowledge of the structure, pharmacology and virology of CCR5 has increased markedly, as has the advancement of drug discovery enabling technologies to find effective blockers of the CCR5 receptor, particularly those with accompanying potent antiviral activity. This has culminated in the first approval from within the class, for the small molecule maraviroc, in 2007 (see Figure 10.2, compound **10**) 11 years after the initial genetic validation evidence of CCR5 as a viable therapeutic target was published (for an excellent recent review of the area, see [37]).

10.2
CCR5 Antagonist Programs

The leading programs directed towards the discovery of oral small molecule CCR5 antagonists are described below. Notable common features of many of the programs are the absence of potent antiviral activity in the early, lead phase of the program, and the degree of overlap between the CCR5 pharmacophore and that of the hERG ion channel, activity against which has been linked to QTc interval prolongation and severe cardiac arrhythmias, and therefore became a key selectivity requirement for many of the programs.

10.2.1
Merck

In an extensive series of papers spanning several years, researchers at Merck have described the evolution of several distinct series of CCR5 antagonists [38]. The initial Merck lead emerged from a high-throughput screen of the corporate compound collection using a CCL4 binding assay [39]. **1** was originally synthesized as part of a neurokinin-directed medicinal chemistry program.

This initial lead showed potent binding affinity to CCR5 (IC_{50} 35 nM), but only weak, micromolar activity in a PBMC viral replication assay, with a close analog also showing very low oral bioavailability in rat [40, 41]. Development of SAR through

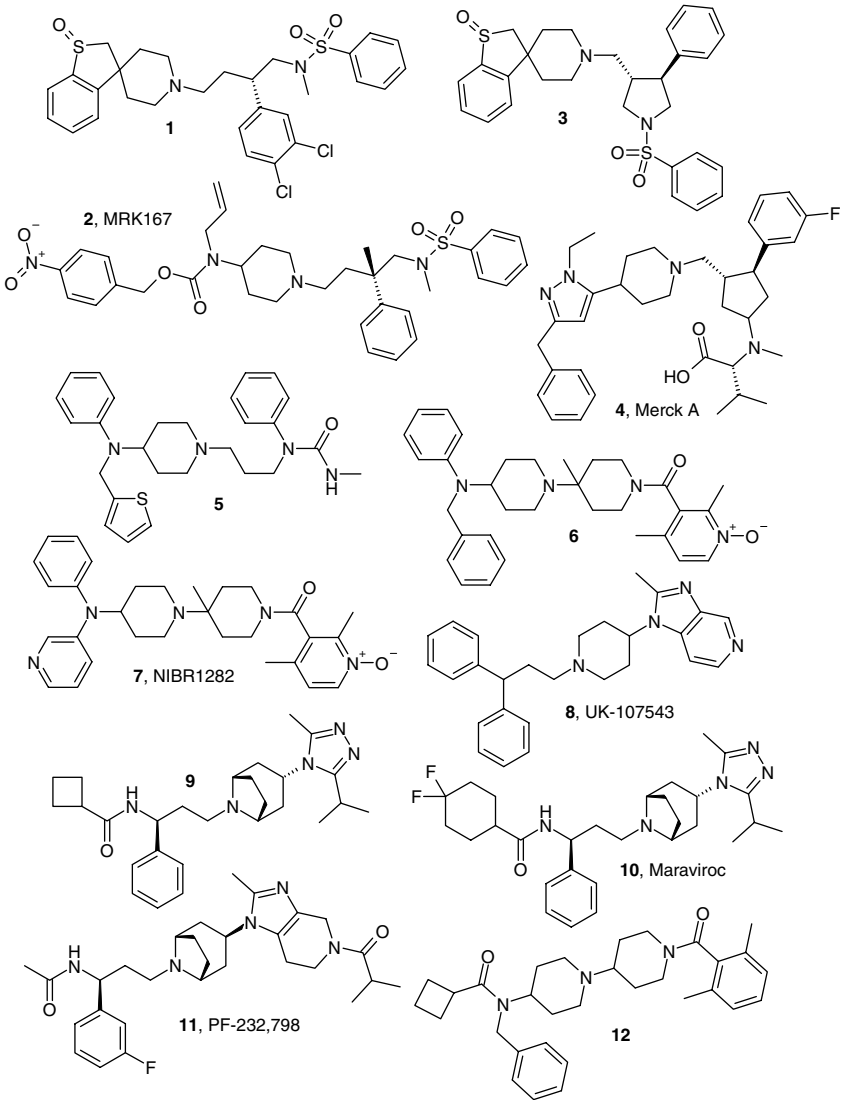

Figure 10.2 CCR5 antagonists as described by Merck, Novartis and Pfizer Inc.

a series of carbamate piperidine derivatives [42] and substituted versions thereof [43, 44] led ultimately to MRK-167 **2** (IC_{50} 0.1 nM). This showed encouraging antiviral activity (EC_{95} 13 nM) and modest oral bioavailability (F 29%) in rat. Interestingly, MRK-167 was shown to protect macaques from infection by an R5 strain of simian HIV-1 following topical vaginal administration [45] and has since been licensed to the International Partnership for Microbicides where it is being investigated as a topical HIV-1 prophylactic agent. MRK-167 also demonstrated

efficacy in a cynomolgus monkey model of organ transplantation when dosed intravenously [46]. Despite the promise of this work in delivering a potentially useful drug product, the Merck program was still configured around the discovery of an optimized orally active compound.

A parallel medicinal chemistry effort was ongoing at this time around the initial lead to constrain the sulfonamide motif into a pyrrolidine ring [47–49], driven largely by synthetic accessibility. Activity was found to be enantiospecific, with the eutomer represented by the 3-(*S*),4-(*S*)-*trans* enantiomer **3** (IC_{50} 26 nM). In an attempt to improve the oral pharmacokinetics of the series, a combinatorial approach was taken [50] to the evaluation of a range of piperidine substituents. Interestingly, the presence of acidic functionality, such as a carboxylate group or a tetrazole ring was found to enhance antiviral activity [51], while also providing a means to ameliorate offtarget activity against the L-type calcium channel. This work with acidic substituents precipitated a range of investigations by the Merck group on zwitterionic derivatives of the pyrrolidine series [52–54].

Through extensive SAR evaluation [55–57] a cyclopentane scaffold was chosen for maximum access to diversity, upon which to incorporate the accumulated SAR knowledge. Further optimization of PBMC antiviral activity and rat pharmacokinetics prompted a move into an amino acid basic zwitterion series, from which the prototype Merck development candidate, Merck A **4**, emerged. This agent possessed potent PBMC-based antiviral activity ($EC_{95} < 8$ nM), had no significant offtarget effects, and displayed a good cross-species pharmacokinetic profile in rat, dog and rhesus monkey ($F > 40\%$ in all three species) [38].

This concluded an impressive and comprehensive SAR survey around the initial HTS hit through to a development candidate. Subsequent publications from the Merck group have indicated the compound to be hepatotoxic in preclinical rat studies at elevated doses, implying a mechanism of mitochondrial inhibition and explaining the discontinuation of Merck A [58].

10.2.2 Novartis

A research team at the Novartis Institute for BioMedical Research has disclosed a series of highly potent and selective *bis*-piperidine amides with a favorable pharmacokinetic profile in both rat and cynomolgus monkey and which are crossreactive with cynomolgus monkey CCR5 [59]. The Novartis team's interest in CCR5 was based on literature observations of the role which CCR5 plays in human kidney allograft rejection through the prolonged graft survival experienced by CCR5Δ32 homozygotes. While not apparently targeted specifically as an HIV therapeutic approach, the Novartis series is included here for completeness and due to the structural similarity of the compounds with the Schering and Pfizer compounds highlighted below.

A high-throughput binding screen of the Novartis compound file identified a potent hit, **5**, which the authors hypothesized had a close structural similarity to the Schering CCR5 antagonist, SCH-C. Crossover compounds were prepared and

evaluated in both binding and CCR5 functional assays. A feature of the Novartis work was the routine testing of compound binding to both human and monkey CCR5, in which the majority of compounds showed balanced cross-species activity and similar levels of binding and functional activity. These studies allowed the identification of **6** which showed an acceptable *in vivo* pharmacokinetic profile in both rat and monkey. Interestingly, removal of the central rigidifying quaternary methyl group provided compounds which, in some cases, were significantly weaker against the monkey receptor than the human. A detailed structural analysis revealed the quaternary methyl group of **6** to occupy an equatorial position, which drives an axial linkage between the two piperidine rings, a structural feature which was proposed to be critical for high binding affinity. In a follow-up publication, **6** was revealed as carrying a cardiac safety liability, with a measured hERG IC_{50} of 1200 nM [60]. Through a systematic process of polarity distribution, the closely related analog NIBR-1282 **7** was found to have a much improved hERG window ($IC_{50} > 30\,000$ nM) and promising pharmacokinetic profile, particularly in dog and monkey ($T_{0.5}$ approx. 8 h in both species) with a similar pharmacologic profile to **6** (human binding IC_{50} 5 nM, monkey IC_{50} 8 nM, calcium mobilization IC_{50} 64 nM). NIBR-1282 has demonstrated *in vivo* efficacy in a monkey model of kidney transplantation, but only in combination with cyclosporine A. No antiviral data is available for NIBR-1282.

10.2.3
Pfizer

Maraviroc was granted regulatory approval in the United States and Europe in 2007 as the first prescribed CCR5 antagonist for the treatment of CCR5-tropic confirmed HIV-1 infection in treatment-experienced patients. Maraviroc is sold under the tradename Selzentry (Celsentri in Europe and Canada) [61].

The Pfizer CCR5 antagonist program [62] began with a high-throughput screen of the corporate screening file based on inhibition of the binding of radiolabeled CCL4 to human CCR5 stably expressed on HEK-293 cells. Several hits emerged from the screen, most prominently UK-107543 **8** [63]. **8** was found to be a potent (IC_{50} 40 nM) inhibitor of the polymorphic CYP2D6 enzyme, putatively via coordination of the pyridine N atom to the haeme group. Two strategies were adopted to circumvent CYP2D6 inhibition. First, simple replacement of the pyridine N atom with carbon retained potent CCR5 receptor binding but with much reduced CYP inhibition and secondly, steric crowding of the piperidine N atom by incorporation into a tropane ring gave further improvements in CCR5 antagonist activity and effectively ablated CYP inhibition [64]. Further replacement of one phenyl group from the benzhydryl moiety with an amide function drove a significant reduction in lead lipophilicity and, in combination with the tropane ring design, instilled potent antiviral activity within the series. It was proposed that reducing the conformational flexibility of the propylamino linking group through *syn*-pentane interactions contributed to the enhanced performance of the tropane group. SAR studies showed activity in the series to reside within the *S*-phenyl configuration, while both the *endo*- and the *exo*-benzimidazole diastereoisomers showed largely equivalent binding potency

and antiviral efficacy. The Pfizer program then focused on overcoming high and unacceptable affinity for the hERG potassium channel and deployed a number of strategies directed towards all locations of the lead structure [65]. The most successful of these sought to introduce polarity in peripheral aromatic regions and optimize substitution patterns for CCR5 binding affinity. This led to the triazole **9**, a series within which antiviral activity of the *exo* and *endo* tropanes began to very much favor the *exo* diastereoisomer. Evolution of the cyclobutyl group, powered by parallel chemistry techniques then provided the difluorocyclohexyl group in maraviroc **10**, which retained excellent antiviral potency (cross-clade primary isolate $IC_{90} = 1$ nM) with low activity against the hERG channel (IC_{50} and $K_i > 10\,\mu M$). Modeling studies suggest this group was not tolerated within the hERG channel by virtue of the steric bulk of the cyclohexyl ring, coupled with the dipole created by the difluoro moiety.

Maraviroc has relatively poor membrane permeability and transcellular flux is enhanced in the presence of P-glycoprotein inhibitors. It is 20–30% absorbed in rat, but well absorbed in the dog and shows nonlinear pharmacokinetics in oral dose escalation studies in human. This profile is indicative of P-glycoprotein transport, with nonproportional pharmacokinetics resulting from the saturation of the efflux transport process at higher doses [66]. The pharmacokinetic profile in man is closest to the situation in the rat, with modest oral bioavailability of approximately 30% and a terminal half-life of 15–18 h following multiple dosing [67]. Notably, drug levels in cervicovaginal fluid exceed those in plasma, raising the prospect of maraviroc being a useful prophylactic agent [68]. Studies on *ex vivo* clinical samples, coupled with modeling studies highlight that CCR5 receptor occupancy is in excess of 99% at steady state at the therapeutic dose, and remained high for several days after cessation of drug treatment. The compound is a substrate for CYP3A4, and its dosing requires adjustment in the presence of inhibitors or inducers of this enzyme. Maraviroc is exquisitely selective for the CCR5 receptor and has broad spectrum antiviral activity against a range of primary viral isolates of various clades and geographic origin (geometric mean EC_{90} 1 nM of unbound compound) and is active against a wide panel of clinically-derived pseudoviruses, including those originating from viruses resistant to existing drug classes [69].

Maraviroc was well tolerated in three clinical Phase 1 studies at doses up to 1200 mg, with postural hypotension being the dose-limiting event [67]. In Phase IIa studies, treatment näive patients who received drug monotherapy at doses ranging from 25 mg *qd* up to 300 mg *bid* over 10 days experienced viral load reductions of up to 1.6 $\log_{10}$ copies/ml [70], providing positive proof of concept for the approach. The Phase 2b/3 development program for maraviroc centered around two long-term randomized, placebo-controled double-blind trials [Maraviroc plus optimized therapy in viremic antiretroviral treatment experienced patients (MOTIVATE 1 and 2)] [71, 72] in treatment-experienced patients with persistent viremia. Patients with R5-tropic virus receiving a 300-mg *qd* or *bid* dose of maraviroc within an optimized background regimen achieved an approximately 1 $\log_{10}$ drop in viral load compared with background therapy [73] and significantly increased frequency of achieving decreases in HIV-1 RNA levels to <50 copies/ml at the 24-week timepoint, which persisted through 48 weeks. The compound continued to be well tolerated, with no

compound related effects on immune function or hepatic transaminase levels. An enhanced $CD4^+$ cell count was observed with maraviroc, in accordance with compound-mediated viral load reduction. A safety study to investigate the effect in patients harboring CXCR4-utilizing HIV-1 showed no effect on viral load, but a small yet statistically significant increase in CD4 count, the origins and implications of which are under investigation [74]. A Phase 3 study [multicentre randomized double-blind comparative trial of a novel CCR5 antagonist maraviroc versus efavirenz both in combination with combivir (MERIT)] [75] has been carried out to compare maraviroc dosed 300 mg *bid* to a 600-mg *qd* dose of efavirenz both in combination with zidovudine/lamivudine in treatment-naive patients. Detailed virology and safety analysis at the week 48 timepoint showed similar response rates in both groups. Maraviroc appeared to be better tolerated and non-inferiority of the maraviroc group was shown for the primary end point of <400 copies/ml. However, the proportion of subjects who responded with a viral load <50 copies/ml was smaller (by approximately 4%) in the maraviroc 300-mg *bid* treatment group compared to the efavirenz 600-mg *qd* treatment group, which failed the non-inferiority criterion of the lower 97.5% confidence interval boundary being above −10% relative to efavirenz although this was subsequently found to be below the −10% value following a retrospective study that used the Trofile assay to enable identification of CXCR4-utilizing HIV-1 in patients with higher sensitivity [76]. Virologic analyses have identified two phenotypic pathways associated with virologic failure in the Phase 3 studies of maraviroc. Either CXCR4-using virus was detected on maraviroc treatment or virus was selected that was able to infect cells using maraviroc-bound CCR5 [77]. Mutation to gain entry via CXCR4 has not been observed in any clinical or preclinical setting, and resistance to maraviroc and CCR5 inhibitors in general appears a relatively high hurdle to HIV-1.

Pfizer has recently disclosed clinical data on a second CCR5 receptor antagonist from their tropane series, PF-232798 **11**, which is currently in Phase 2 trials. This compound arose from an alternative means of circumventing the CYP2D6 and hERG activity associated with the original high-throughput screening hit UK-107543 by simple reduction of the pyridine ring to give an imidazopiperidine. Incorporation of the SAR learning from the maraviroc medicinal chemistry program and optimization of the amide and imidazopiperidine substituents, in particular maintaining small amide substitution, allowed the identification of PF-232798. It is notable that greatest activity in this series resides within the *endo* tropane diastereoisomer, distinct from the maraviroc series. This compound has an equivalent pharmacological and virological profile to maraviroc, but has the potential to deliver a once daily dosing regimen by virtue of superior absorption and pharmacokinetic properties. Importantly, PF-232798 has shown activity against laboratory-generated maraviroc-resistant virus, suggesting potential for multiple-line therapy using different CCR5 antagonists [78]. The Pfizer group have proposed that the imidazopiperidine moiety is capable of making additional contacts within the ECL2 hinge region of the receptor, sufficient to escape resistance.

The Pfizer group has also disclosed the structure of a range of distinct CCR5 antagonists **12** related to the Schering compounds depicted below. A combination of

modeling and deliberate targeting of structural diversity within a range of templates identified both pyrrolidine-piperidine and *bis*-piperidine compounds, which are notable for their structural similarity to the Novartis series, but suffered significant CYP-mediated metabolism *in vitro* [79].

No further pharmacokinetic or virologic information has been disclosed on these compounds.

10.2.4
Incyte

Incyte have reported promising clinical data for INCB9471 (**13**), derived from a piperazine-piperidine structural class, related to the Schering CCR5 antagonist series described above (Figure 10.3) [80, 81]. INCB9471 is a potent, selective and reversible CCR5 antagonist with an *in vitro* potency of IC_{90} 8–9 nM, and an extremely long pharmacokinetic half-life in man of some 60 h, which has been shown to be extended significantly through coadministration with ritonavir. The compound had shown comparable *in vitro* antiviral activity to maraviroc, although saturation binding assays suggested the compound bound to a different region of the CCR5 receptor [82]. Very well tolerated in a 10-day Phase 1 trial, INCB9471 was progressed through a 14-day placebo-controled Phase 2a trial in which treatment naïve patients received a 200 mg *qd* dose [83]. The compound delivered potent and prolonged viral suppression, which persisted after cessation of drug treatment, with a viral load suppression of 2.1 $\log_{10}$ being achieved at day 20. Despite this encouraging profile, in March 2008, Incyte announced they would focus their resources on other programs and were seeking to outlicense INCB 9471, and that they would therefore not initiate two planned six-month Phase 2b trials in treatment-experienced patients [84].

10.2.5
Schering Plough

Schering Plough have identified a number of clinical candidates from a piperidine-based template that originated from a muscarinic M2 receptor antagonist **14** that showed modest antagonism of CCR5 (K_i 1 μM) [85]. A subsequent program to improve CCR5 potency, selectivity over the M2 receptor and oral bioavailability led initially to the introduction of an oxime moiety that enhanced both potency and oral bioavailability. The *Z*-isomer was preferred and was shown to be stable to acidic hydrolysis and to isomerization. *Ortho*-substituted aryl amides proved essential for potency and resulted in the identification of a 2,6-disubstitution pattern as optimal. Metabolic stability was assured with a pyridyl *N*-oxide amide leading to the identification of SCH-C **16** [86]. SCH-C showed good potency at the CCR5 receptor (*K*i 2 nM) and a strong oral pharmacokinetic profile in the rat (*F* 63%, $T_{0.5}$ 5 h). In PBMCs infected with M-tropic HIV-1 it showed strong activity with a mean IC_{50} of 2 nM against a range of viral isolates. However, the steric congestion around the 2,6-disubstituted amide moiety leads to the formation of very slowly interconverting atropisomers, which complicates the spectroscopic analysis, and

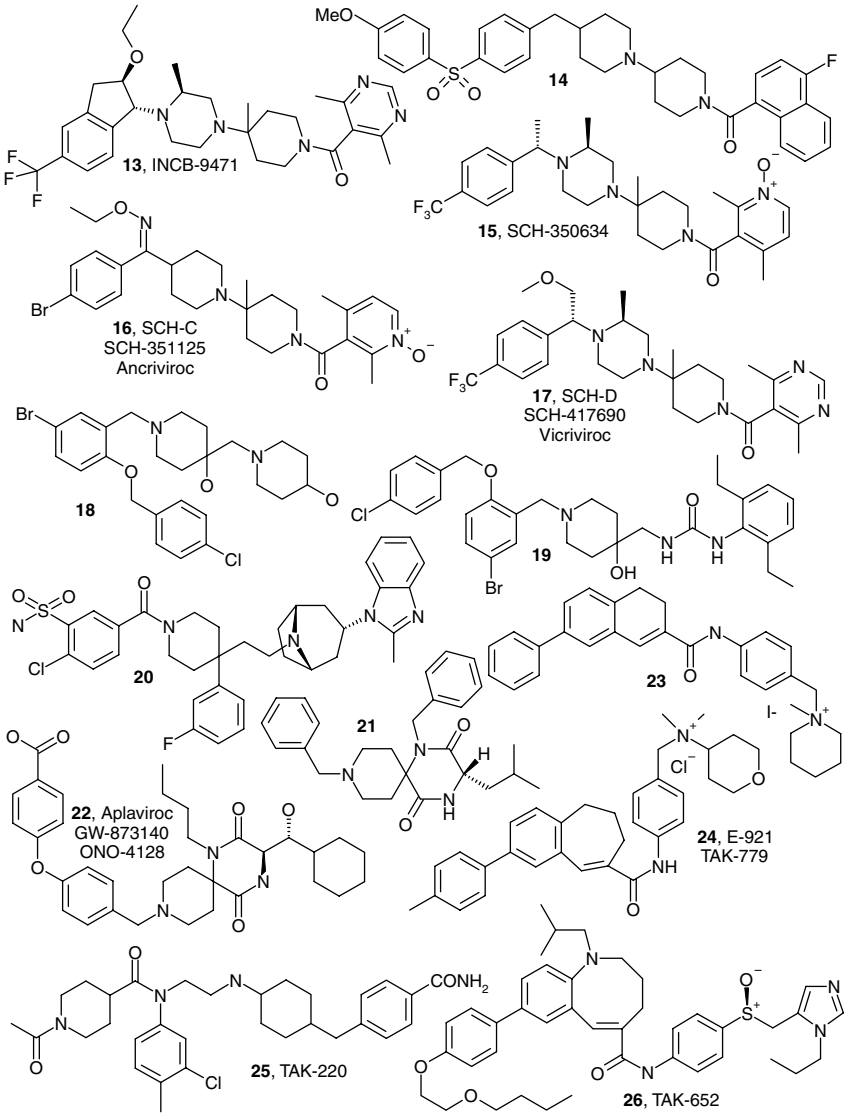

Figure 10.3 CCR5 antagonists as described by Incyte, Schering-Plough, Berlex, GlaxoSmithKline/Ono and Takeda.

indeed pharmacology and pharmacokinetic analysis of this structural type. At equilibrium, a mixture of four isomers of SCH-C exist at room temperature as a result of restricted rotation about both the N−CO and the CO−aryl bonds. Some elegant work was undertaken to separate these isomers, demonstrate that they have similar activities, and measure their interconversion rates [87]. Palani *et. al.* demonstrated that all isomers exist in approximately equal proportions in the solvents

examined. Equilibration rates in saline were $T_{0.5}$ values of 5.6 h (N−CO bond) and 23 h (CO−aryl bond).

Phase 1 trials in 2003 showed a >0.5 log drop in viral load following doses of 50–200 mg/day over 10 days [88], although some significant prolongation of the QTc interval was observed [89]. No further development has been reported.

The identification of a new subseries was exemplified by SCH-350634, **15**. The additional structural complexity of this series was essential for potency, and again the 2,6-dimethyl pyridyl *N*-oxide moiety gave metabolic stability resulting in an excellent pharmacokinetic profile in rat, dog and monkey (e.g., *F* 65%, $T_{0.5}$ 6 h in dog) [90]. Antiviral activity was demonstrated against a range of isolates showing IC_{50} values in the sub-20 nM range. A more potent derivative was identified in SCH-D **17** which incorporated a symmetrical pyrimidyl aryl amide substituent which reduces the synthetic complexity of rotamer formation within the unsymmetrical amides described above [91]. Across a range of clinical isolates SCH-D was 2–40 times more potent than SCH-C and showed excellent oral bioavailability (F 89% in monkey) and a $T_{0.5}$ of 3–4 h [92]. It showed a sixfold drop in activity against the hERG ion channel compared to SCH-C, and in a two-week clinical trial showed 1.1- and 1.6-log drops in viral load after two weeks monotherapy dosing at 10 and 50 mg twice-daily [85–94]. Human pharmacokinetics are impressive with rapid absorption, a long $T_{0.5}$ (28–33 h) and linear pharmacokinetics across the 10–50 mg *bid* dosing regimes [94]. SCH-D is currently in Phase 3 trials.

10.2.6
Berlex

Berlex have developed a series of hydroxypiperidines starting from an IC_{50} 1 μM high-throughput screening hit resulting in the identification of two potent compounds (**18**, **19**) that demonstrated good levels of activity (CCL3 IC_{50} 49 nM and 11 nM respectively) [95]. Unfortunately, a lack of oral bioavailability and rapid clearance following intravenous administration in the rat resulted in termination of work within this series [96].

10.2.7
GlaxoSmithKline/Ono

GlaxoSmithKline have been active in developing CCR5 antagonists. A representative from a recent patent is shown in structure **20** [97]. No activity data have been published for this compound series although a publication describing synthesis of potential precursors on a kilogram scale is suggestive of some perceived potential [98].

Spiropiperidines have the ability to orient substituents in mimicry of a peptidic β-turn which could explain their activity against a range of peptidic GPCR targets. Ono Pharmaceuticals developed a solid phase combinatorial library based upon the Ugi reaction to generate potential lead matter for a range of chemokine receptors. From their initial 576-compound collection, several compounds showed low

micromolar levels of activity in a human CCL3/CCR5 binding assay, for example the IC_{50} 3.2 μM hit **21** which led them to systematically optimize each substituent with further libraries [99]. Interestingly, information gained through the Ono lead optimization program was enriched through identifying biologically active metabolites [100] of some early leads. This work led eventually to the identification of ONO-4128 **22**, which was subsequently licensed to GlaxoSmithKline and named GSK873140 (aplaviroc) [101]. This spirocyclic carboxylic acid showed early promise with high antiviral potency (IC_{50} 0.1–0.6 nM across a range of clinical isolates) and the potential for a PK/PD split as a result of slow offset from the CCR5 receptor [102, 103]. However, aplaviroc was withdrawn in 2006 during Phase 2b/3 trials as a result of hepatotoxicity [104]. Elevated levels of liver enzymes aspartate aminotransferase (AST) alanine aminotransferase (ALT) and total bilirubin levels were observed in some individuals after four weeks of dosing, initially promoting suggestions of mechanism-based toxicity but these seem less likely now given the success of maraviroc and the significant difference in their respective chemical structures [105, 106].

10.2.8
Takeda

Takeda started their CCR5 antagonist program when an internal high-throughput screen identified a series of positively charged sub-1 μM hits from a binding assay. SAR development based upon a benzocyclohexene lead **23**, led ultimately to the identification of TAK-779 **24**, the first reported small molecule CCR5 antagonist. TAK-779 showed exquisite potency against CCR5 (IC_{50} 1 nM) and high selectivity over other chemokine receptors with the exception of CCR2b which it inhibits with an IC_{50} of 27 nM which may be indicative of the origin of the series. Its good *in vitro* potency against clinical isolates (IC_{90} 7–27 nM) aided its progression into clinical trials [107, 108]. Since the physical properties of the quaternary salt precluded oral administration it was developed as a subcuteous agent, but trials were terminated in 2001 as a result of irritation at the injection site [109]. More recently it was shown that TAK-779 also acts as an antagonist at CXCR3 receptors and that it has potential use in a number of other applications, including allergen-induced asthma, atherogenesis and intestinal ischemia/reperfusion injury [110–112].

Takeda continued to develop new CCR5 antagonists targeting an oral profile, by removing the permanent charge engendered by a quaternary salt. Simply removing a methyl group to give the equivalent tertiary amine of TAK-779 resulted in a 700-fold drop in potency to give an IC_{50} of 0.95 μM. However, in contrast to the SAR for the quaternary derivatives, the tertiary amines were sensitive to changes in the benzocycloheptene [113]. Analogs from the tertiary amine series showed good activity in a cell fusion assay (IC_{50} 1 nM) and oral bioavailability in the rat [114]. Interestingly Takeda have reported some discrepancies in the translation between CCR5 binding and cell fusion determinations between enantiomers within this series of up to two orders of magnitude. The reasons for this are not clear but changes in translation

between binding and antiviral activity have been seen by others (for example in Pfizer's tropane series) [115].

Further optimization led to the identification of TAK-652 **26** which had good inhibitory efficacy against a range of clinical isolates (IC_{90} 0.1–0.4 nM). Oral pharmacokinetics showed a bioavailability of 10% in the rat, 89% in the dog and 16% in the monkey together with a moderate $T_{0.5}$ across these species (rat 1.3 h, dog 2.7 h, monkey 2.0 h) [116]. While there is some off-target activity (CCR2b IC_{50} 6 nM), this compound was progressed into clinical studies and is currently in Phase 1. Early clinical trial data has shown TAK-652 to be well tolerated, has good bioavailability and low plasma clearance giving a $T_{0.5}$ of 12 h following an oral dose of 25 mg which gave a plasma concentration at 24 h of some 9 nM [117]. In 2007, Tobira Therapeutics negotiated the worldwide rights to develop TAK-652 for the treatment of HIV infection.

A weak hit from a high-throughput screen identified a new starting point for Takeda. This was subsequently optimized to give TAK-220 **25**, which showed excellent *in vitro* inhibition against six R5 clinical isolates (mean IC_{90} 13 nM) and no activity against a range of other chemokine receptors including CCR2b up to 10 μM concentration [118]. The pharmacokinetic profile in monkey was also encouraging with a modest oral bioavailability of 29% and a $T_{0.5}$ of 6 h leading to its selection as a clinical candidate [119, 120]. TAK-220 is also currently in Phase 1 clinical trials with Tobira Therapeutics.

10.2.9
AstraZeneca

AstraZeneca's search for CCR5 antagonists started with micromolar hits from a binding assay, and through a versatile 4-aminopiperidine template based on structure **27**, several hundred analogs were prepared which identified amide and urea derivatives **28** with good activities. Representative examples of both subseries have shown good selectivity over chemokine receptors but weak micromolar activity against other peptidic GPCRs such as M1 and 5HT1A as well as the hERG ion channel (Figure 10.4) [121].

While optimizing metabolic stability [122], SAR studies yielded **29** that demonstrated potency and metabolic stability could be achieved in the same compound. Interestingly the 3,5-difluorophenyl motif gave enhanced stability *in vivo* ($T_{0.5}$ 2.6 h, *F* 38% in the rat) compared to the corresponding monofluoro analog. An encouraging pharmacokinetic profile was also seen in the dog ($T_{0.5}$ 3.9 h, *F* 86%) [123].

AstraZeneca currently have one agent, AZD-5672 (structure not disclosed) in Phase 2 clinical trials for rheumatoid arthritis, kidney function and atherosclerosis.

10.2.10
Recent Disclosures

Several series of CCR5 antagonists have been disclosed in the journal and patent literature, for which there is little detail available.

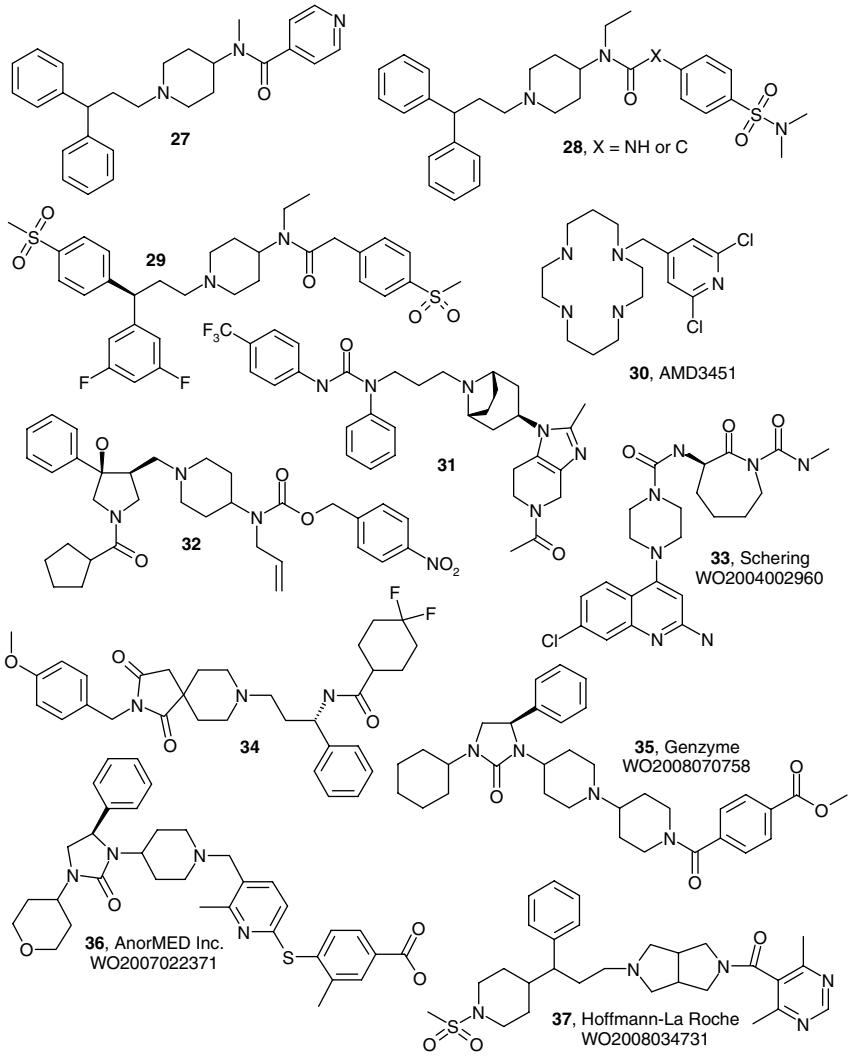

Figure 10.4 CCR5 antagonists as described by AstraZeneca, AnorMed, Kemia, Shanghai Institute, Schering-Plough, Genzyme and Hoffman La Roche.

AnorMED have developed a series of cyclams that inhibit X4 HIV strains through antagonism of the CXCR4 receptor, the other most common co-receptor used by HIV-1 besides CCR5. One of the most advanced agents was AMD3100 which was then discontinued as a potential HIV therapy as a result of cardiac effects [124]. More recently, AnorMed have extended this class to include activity against the CCR5 receptor with dual CCR5/CXCR4 antagonists such as AMD-3451 **30**, which has demonstrated low micromolar activity against both X4- and R5-tropic clinical isolates [125].

Kémia have disclosed a series of *bis*-aniline urea-based derivatives of the Pfizer tropane series including **31** which showed good antiviral activity HIV-1 (EC_{90} 6 nM) and encouraging pharmacokinetics in the rat (F 52%, $T_{0.5}$ 7.2 h) [126].

Starting from the Merck pyrrolidine series, a number of hydroxypyrrolidines have been developed by the Shanghai Institute of Bioorganic and Natural Products Chemistry. **32** shows strong binding to the CCR5 receptor (IC_{50} 3 nM), which translated into efficacy against seven clinical isolates of R5-tropic HIV-1 with a mean EC_{50} of 7 nM. Oral bioavailability was seen in rat (F 41%) and dog (F 22%) with modest pharmacokinetic half-lives in both species [127].

Examples of other series recently disclosed in the patent literature are shown below in structures **33–37** [125–128].

10.2.11
Overview of Published CCR5 Antagonists

Most companies started their internal programs with chemotypes identified from file screening using a binding assay. Progression of these leads into potential development candidates was achieved through the optimization of both antiviral potency and pharmacokinetics. Figure 10.5 shows the relationship between the leads and the most advanced agents from each company.

It is interesting to note that in almost all cases, lead potency was increased by orders of magnitude while lead LogD was correspondingly reduced. The majority of the advanced, optimized compounds occupy a small region of lipophilicity space. In turn, dose size is governed by potency and rates of systemic clearance with the latter

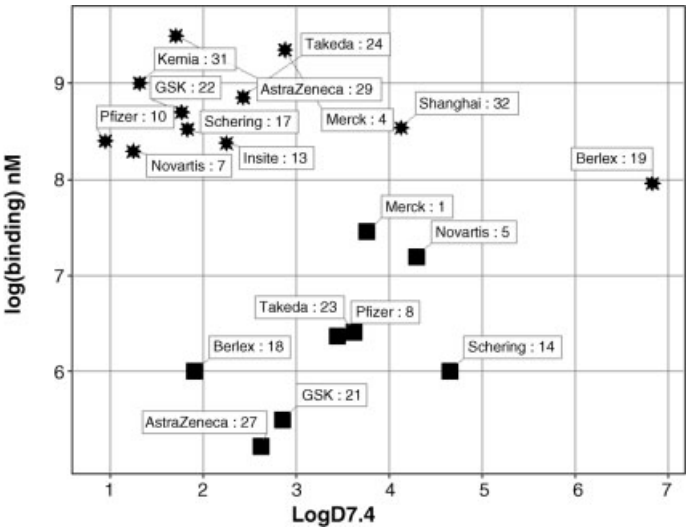

Figure 10.5 Calculated LogD versus published CCR5 binding data (squares = leads; stars = most advanced compound disclosed). For compound structures, see Figures 10.2–10.4

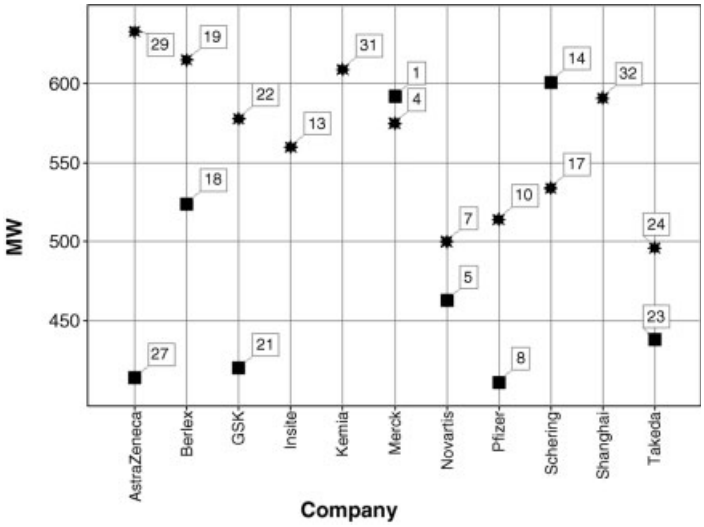

Figure 10.6 Molecular weight of leads and candidates across companies (squares = leads, stars = most advanced compound disclosed). For compound structures, see Figures 10.2–10.4.

showing a strong correlation with lipophilicity [129]. In this analysis the vast majority of development candidates fall within a small region of property space. A notable exception is the leading compound from Berlex, but equally notable, this compound was dropped because of a poor pharamacokinetic profile. While for most programs, optimization led to a reduction in lipophilicity, this has also resulted in a general increase in molecular weight as shown in Figure 10.6, to a region outside that typically found for compounds exhibiting good oral bioavailability [130].

This is most likely to be a consequence of the peptidic GPCR gene family which CCR5 belongs to, which is generally challenging for small molecule intervention [131], but clearly targeting this "expanded" window of target chemical space does not preclude the identification of development candidates, or in the case of maraviroc, a launched drug. This is particularly true when a cap is maintained on target lipophilicity, as evidenced in Figure 10.5.

10.3
Molecular Interactions and Binding Modes of CCR5 Receptor Antagonists

Effective means of probing and visualizing molecular interactions of ligands with membrane-bound receptors is an emerging science based on developing technologies such as surface plasmon resonance [132] which are beginning to add an extra level of sophistication to our understanding of ligand recognition by GPCRs such as the CCR5 receptor. The majority of studies thus far aimed at elucidating ligand-CCR5 interactions have been based on site-directed mutagenesis [133–137]. These studies have indicated the binding site for all small-molecule antagonists to be

a lipophilic pocket buried within the transmembrane region of the receptor, while interaction of gp120 with the receptor occurs on the extracellular surface at the N-terminus of the receptor [25, 138–140]. Initial attempts to model the small molecule antagonist binding region were based on the crystal structure of the transmembrane domain of bovine rhodopsin upon which the primary sequence of human CCR5 was aligned and provided some structural insights which were consistent with the apparent slow-offset of these molecules from the receptor [141–143]. While a single glutamic acid residue, E283, in the transmembrane region of the receptor appears to be essential for the binding of small molecule antagonists, all of which thus far have featured a basic group towards the center of their structure, mutagenesis at other sites surrounding the putative binding pocket produce different results [133, 137, 144]. This suggests that different antagonist chemotypes share broadly similar, but only partially overlapping binding regions, producing distinct conformations of the receptor and in some cases, different ligand binding inhibition profiles [136]. This is most dramatically seen with TAK-220 and aplaviroc, which potently inhibit the binding of CCL5 and CCL4, but not CCL3 and CCL3 and CCL4, but not CCL5, respectively although pan-chemokine signaling inhibition is observed [143]. More significantly from a therapeutic perspective, the different conformations each antagonist stabilizes indicates potential for non-overlapping resistance profiles for strains that eventually overcome a given antagonist through mutation. Such mutation may not enable recognition of receptor occupied by other antagonists, as observed in numerous studies that is, class resistance is not inevitable. The slow dissociation of antagonists from a buried pocket within the receptor is widely believed to contribute to antiviral activity *in vitro* and in the clinic, in which an inhibitor-receptor complex needs to persist throughout the timeframe within which gp120 binds to CCR5, and thereby prevent the early stages of membrane fusion. It is notable that many of the leads from the CCR5 antagonist programs described above did not show accompanying antiviral activity until they were developed into more optimized structures which were capable of contacting the receptor more efficiently, and that the translation between binding and antiviral activity within any given chemical series can be variable. Given that chemokines occupy both the extracellular regions and the transmembrane pocket, whereas the gp120 protein only contacts the extracellular region, it is also conceivable that specific antagonists could inhibit chemokine signaling, yet stabilize a conformation that is recognizable by gp120.

A fully mapped binding pocket model of the antagonist transmembrane region, built using five different clinically precedented chemotypes, has been recently described and shown in Figure 10.7 [144].

Slow receptor dissociation brings the potential for extended pharmacodynamics beyond that suggested by pharmacokinetic predictions. In monotherapy trials of CCR5 antagonists the rebound in viral load after treatment discontinuation does not appear to be immediate, but is delayed by 1–2 days for vicriviroc [145] and aplaviroc [146] and up to 5 days for maraviroc [147]. It seems likely that a combination of pharmacokinetic half-life and slow receptor offrate is required for optimal efficacy [148].

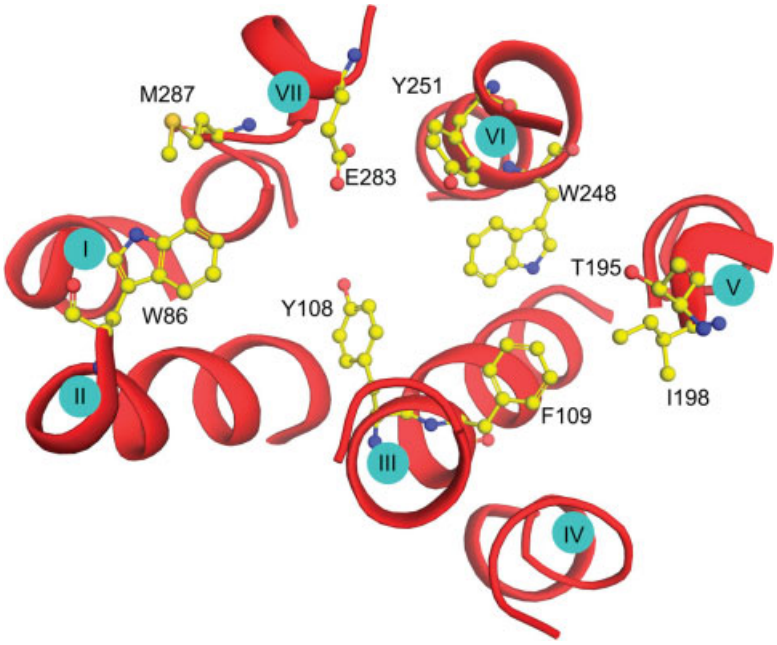

Figure 10.7 Residues on the CCR5 receptor which define the small molecule binding region [144].

10.4
Resistance to CCR5 Receptor Antagonists

As described above, the emergence of resistance to antiretrovirals is one of the primary challenges facing the current range of drug therapies. In theory, targeting an immutable host protein such as CCR5 should present a significantly higher hurdle for virus to acquire resistance. This can happen in one of two ways; either the virus can continue to gain entry and replicate via inhibitor bound CCR5 receptors, or the virus can switch to using CXCR4 as its preferred co-receptor. There has been only one report of an R5-tropic virus switching tropism *in vitro* in the presence of a CCR5 antagonist based on data generated with close analogs of the agonist, CCL5 [149]. This implies a difference between agonists (which internalize the receptor) and antagonists (which block the receptor and are held at the cell surface), and the requirement for CCR5 to be blocked rather than internalized to prevent co-receptor switching. Some caution should be exercised in making this conclusion however, as co-receptor switching is observed with specific isolates during passage without CCR5 antagonist pressure, where the strains used require single point mutation rather than the more usual multiple mutation in envelope. Conversely, there have been several reports of the *in vitro* selection of R5-tropic viruses which are resistant to a CCR5 antagonist, but retain R5 tropism [150–153]. In all cases, resistance appears to emerge slowly [150], and requires multiple mutations in the variable regions of the viral envelope proteins.

The emergence of X4-tropic virus during treatment with a CCR5 antagonist has been described for a minority of treated patients undergoing short term monotherapy. In the Phase 2a trials of maraviroc, X4-tropic virus was detected in two of 63 patients with R5-tropic virus at baseline, following ten days of monotherapy [154]. Phylogenetic analysis indicated that the X4-using virus most likely emerged from a pre-existing X4-using reservoir which was undetectable at baseline/patient screening rather than by a tropism shift of an R5-tropic virus. Encouragingly, maraviroc-resistant strains retain sensitivity to other CCR5 antagonists [155] indicating that not only do they still rely on CCR5 for entry, but their ability to gain access to antagonist-associated receptor is limited to the compound used to select for them in the first instance [137, 155–159]. Similar data have been presented for vicriviroc [160] and aplaviroc [161]. It thus appears based on evidence to date that the likelihood of cross resistance between different chemical classes of CCR5 antagonists is low.

10.5 Outlook

The publication of genetic evidence a decade ago pointing towards CCR5 antagonism as a promising therapeutic approach to the treatment of HIV-1 infection has spawned an enormous amount of fundamental and applied research against this target. This chapter summarizes the output of these endeavors, from a small-molecule drug discovery perspective, and how this collective work has resulted in advanced clinical development candidates and one launched drug. This work has been of great importance in addressing the toxicity and resistance liabilities of current HAART regimens through targeting a host cell surface receptor, and current clinical data indicates maraviroc to be both very well tolerated and to present a high barrier to resistance, with no loss of efficacy observed against pre-existing resistant virus in treatment-experienced patients. Further, it appears based on data available thus far that susceptibility to cross-resistance among different CCR5 chemotypes is low. Only time will inform possible emergence of cross-resistant viruses among the CCR5 antagonist class, or indeed if the antagonist class itself will expand to other distinct binding regions of the CCR5 receptor.

Maraviroc is currently approved for use in treatment-experienced patients, and is the subject of ongoing trials in treatment-naive individuals, which would expand the eligible patient population for this agent towards a patient cohort which in general displays a higher incidence of R5-tropic virus. Maraviroc is also being investigated for use as a topically applied prophylactic agent which would extend its usage further. It should be noted that CCR5 antagonism is being investigated for other nonviral indications, including transplant rejection (see [59] and references therein) and rheumatoid arthritis [162].

One limitation to the use of CCR5 antagonists in HIV therapy is the need to carry out expensive viral tropism testing due to the ineffectiveness of these agents against X4-tropic viruses. This has already been highlighted in the clinical studies with maraviroc in which a small percentage of patients underwent a tropism shift in their

virus between screening and receiving the first dose of drug. Recently implemented advances in diagnostics offer a more sensitive tropism screen, and a more cost-effective test of viral tropism is anticipated. An alternative strategy to tackle tropism shift is to develop agents that combine inhibition of both CCR5 and CXCR4 by either one drug or through a combination. This has been the approach taken by AnorMED although it is too early to know whether this approach will be effective and safe.

With the withdrawal of aplaviroc in 2005 following observations of severe hepatotoxicity, concerns had been raised of potential class effects of CCR5 antagonism, which have thus far been unfounded. Nonetheless, until extensive long-term exposure to CCR5 antagonists has been achieved, it is likely that careful and regular monitoring of liver enzyme levels will continue for all members of the class.

The analysis of CCR5 antagonist programs presented in this chapter indicate a quite defined CCR5 "target property space" which the most advanced agents fall within. This property space is not conducive with high compound penetration of the central nervous system (which favors much smaller compounds), which can harbor viral reservoirs and it remains to be seen the link between CNS penetration and antagonist efficacy under chronic exposure. However, the prolonged occupancy of CCR5 by antagonists and the phenomenon of immune trafficking into the brain may enable antiviral activity in the CNS.

It is also notable that the majority of advanced agents from the class fall outside of the *rule of five* property space targeted by many modern drug discovery programs, primarily on molecular weight grounds, and offers additional examples of a more appropriate target property space for medicinal chemistry programs targeting the peptidic GPCR gene family.

The work reviewed above bears testament to the modern drug discovery process in which early epigenetic data was translated into a launched small molecule drug in approximately one decade. The fundamental research, enabling screening technology, drug design and synthesis and clinical development components of this process make CCR5 antagonism an outstanding case study of the drug discovery engine in action. It is anticipated that the next decade will be characterized by refinements and additions to current levels of knowledge of the target, additional drug launches and the embedding of CCR5 antagonists within the arsenal of HIV therapies.

Acknowledgment

The authors would like to thank Pat Dorr for critical reading of this manuscript and for very useful discussions relating to its content.

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11
CXCR4 as a Therapeutic Target

Ken Y.C. Chow and Françoise Bachelerie

11.1
Biology and Physiological Role of CXCR4

11.1.1
General Introduction

The paradigmatic function of chemokines is to promote the chemotaxis of leukocytes. Chemokines are able to form gradient that induces polarization of leukocytes through the reorganization of actin cytoskeleton and finally their oriented migration. Importantly, chemokines can also regulate the differentiation, proliferation and survival of various types of cells. Chemokines accomplish these numerous functions through the binding of chemokine receptors. Like other chemokine receptors, the CXC chemokine receptor 4 [CXCR4; previously known as CD184/fusin/LESTR (leukocyte-derived seven transmembrane domain receptor)] belongs to the large class A family of seven transmembrane (7TM) G protein-coupled receptors (GPCR). CXCR4 binds to its ligand CXCL12/SDF-1 (Stromal cell-derived factor-1) and mediates infection of T-tropic human immunodeficiency virus (HIV) strains by acting as a co-receptor for cell entry [1–3]. By using degenerated primers that target the rabbit interleukin (IL)-8 receptor, the human *CXCR4* cDNA was cloned from a human spleen-derived cDNA library and was mapped to chromosome 2 [4]. The *CXCR4* gene contains two exons separated by an intronic sequence and a promoter region that contain a TATA box and a transcription start site located 2.6 kb upstream of the *CXCR4* ORF [5–7]. Although the CXCR4 receptor displays a wide expression profile in different types of mammalian tissues (e.g., endothelial cells, brain, lung), yet its expression is tightly regulated. In cells of haematopoietic origin such as leukocytes, expression of CXCR4 is dependent on the stage of differentiation, developmental state and their originated tissue, whereas preferential expression of the receptor has also been described on the surface of naïve T-lymphocytes [8]. Expression of CXCR4 is also reported to be strongly upregulated

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

in many types of tumor cells [9]. Various cellular factors have been shown to bind different regions of the CXCR4 promoter in order to regulate expression of the receptor transcript.

11.1.2 Regulation of CXCR4 Expression

11.1.2.1 Transcriptional Regulation

Initial characterization of the *CXCR4* promoter sequence identified two transcriptional factors that have opposing action on *CXCR4* transcription in various types of leukocytes – the nuclear respiratory factor-1 (NRF-1) that positively modulates the promoter activity [4–6], and the ying yang 1 (YY1) transcription factor that possesses a negative effect [10, 11]. It has also been reported that calcium can negatively modify the cAMP (cyclic adenosine monophosphate)-mediated upregulation of *CXCR4* mRNA expression in T-lymphocytes [12, 13]. Other cellular factors such as interleukin (IL)-2 [5], IL-4, -7, -15 [14], IL-10 and TGF (transforming growth factor)-β1 [15] have also been shown to upregulate CXCR4 transcript in different types of human leukocytes including monocytes. In contrast, inflammatory cytokines such as tumor necrosis factor (TNF)-α [16–18], interferon-γ (IFN- γ) [17, 19, 20] and growth factor independence-1 (Gfi-1) [21] are capable to negatively regulate the expression of CXCR4, although TNF-α can have opposite function in other cell types [22]. Recently, it has been identified that the notch ligand delta-like 4 (DLL4), an important vascularization regulatory factor, can also downregulate CXCR4 expression in endothelial cells by modulating the promoter activity of the receptor [23]. Under pathological condition, it has been demonstrated that the trans-activator protein (Tax) of Human T-lymphotropic virus type I (HTLV-1) can trans-activate the *CXCR4* promoter by associating with NRF-1 [24]. In contrast, human herpesvirus 6 (HHV-6) downregulates expression of *CXCR4* mRNA by interrupting the interaction between the proto-oncogene c-Myc with the CXCR4 promoter suppressor YY1 [11, 25]. In cancer cells, several cellular factors can positively modulate CXCR4 expression that in turn affect the progression of tumor formation, including vascular endothelial growth factor (VEGF) [26, 27], nuclear-factor kappa-light-chain-enhancer of activated B cells (NFκB) [28, 29], PAX (paired box) 3-FKHR (forkhead in human rhabdomyosarcoma) [30, 31] and RET/PTC (rearranged in transformation/papillary thyroid carcinomas) [32, 33]. Recently, inhibition of CXCR4 expression in Kaposi's sarcoma (KS)-associated herpesvirus (KSHV)-infected cells has been shown to be associated with microRNA miR-146a activity [34], probably through the binding of miR-146a to the 3′-untranslated region of the *CXCR4* mRNA [35]. Under ischemia, the hypoxia-inducible factors-1 (HIF-1) transcription factor is stabilized in a post-transcriptional manner and in turn upregulates expression of *CXCR4* transcripts. Cumulative evidence also suggests that HIF can upregulate CXCR4 transcripts in cancer cells [36–39]. It is believed that these processes are mediated by the down-regulation of von Hippel–Lindau tumor suppressor (pVHL), which degrades HIF under normoxic conditions [37, 38, 41]. Of note, CXCR4 expression in tumor cells can also be modulated at a post-transcriptional level (see Section 11.1.2.2)

11.1.2.2 **Post-Transcriptional Regulation**

Like other GPCRs, CXCR4 undergoes post-translational modifications upon stimulation by its ligand, which involve modifications of residues of the cytoplasmic domains of the receptor: namely the phosphorylation of serine (Ser) and threonine (Thr) residues and the ubiquitination of lysine (Lys) residues. These changes lead to receptor internalization and the interrelated processes of homologous desensitization, that is, the specific uncoupling of agonist-bound GPCR from its cognate G proteins (see Section 11.1.4.3). At this stage, receptors are either recycled back to the cell surface or are sorted to lysosome for degradation [41, 42]. Studies have demonstrated that the CXCR4 receptor is poorly recycled [43]. Evidence also indicate that the lysosomal sorting of CXCR4 involves ubiquitination of the C-terminal domain (C-ter) of the receptor (Ser 330, 324, 325) by the E3 ubiquitin ligase AIP4 (atrophin 1-interacting protein 4) [44], the activity of which depends on interaction between AIP4 and β-arrestin-2 [45]. In a more recent analysis, AIP4 was shown to directly interact with the C-ter region with phosphorylated Ser residues [46]. Such role of C-ter ubiquitination of CXCR4 is further highlighted by a recent study showing that the Ubiquitin carboxyl-terminal hydrolase 14 (USP14) can interact with that region of receptor, which leads to the de-ubiquitination of CXCR4 and consequently inhibition of receptor degradation [47]. Furthermore, it has been shown that the activation of the HER2/neu (human epidermal growth factor receptor 2; also known as ErbB-2 (erythroblastic leukemia viral oncogene homolog 2)-mediated phosphatidylinositol 3-kinase (PI3K)-dependent (HER2/PI3K) pathway can also inhibit CXCR4 receptor degradation [48]. Such process is proposed to depend upon PI3K-mediated activation of the cytokine-independent survival kinase (CISK), which directly interacts with AIP4 and inhibits its function [49]. With the fact that activation of the CXCL12/CXCR4 axis could transactivate the HER2/PI3K pathway [50], these results thus suggest for the existence of a positive feedback loop involving the activation of HER2/PI3K and CXCR4. As the PI3K pathway is robustly activated in most types of cancers [51, 52], such findings may therefore also account for the strong upregulation of the receptor in cancer cells.

Other post-translational modifications of CXCR4 that affect the receptor activity include glycosylation and tyrosine sulfation. For example, glycosylation in the N-terminal (N-ter) part of the receptor – the asparagine (Asn) residue at position 11 (Asn 11/g1) [53] – alters the binding affinity for CXCL12 and the downstream signaling properties [54, 55], while binding of the selective CXCR4 antagonist AMD3100 remain unaffected [56]. Glycosylation of the receptor is reported to enhance binding of the gp120 envelope of HIV isolates using CXCR4 as a co-receptor (see Section 11.2.1) and consequently viral infection [54, 57]. However, the observation that viruses using CCR5 as a co-receptor for cell entry are similarly affected remains unexplained [53, 54]. In addition to glycosylation, tyrosine (Tyr) residues Tyr7, Try12 and Tyr21 in the CXCR4 N-ter domain are subjected to sulfation [58, 59]. The process involves the addition of a sulfate group to Tyr residue when it passes through the Golgi apparatus. Apparently, sulfation of CXCR4 does not modify its activation [60, 61] nor its co-receptor function [59, 61], although it affects CXCL12-binding properties [58–61].

11.1.3 Physiological Functions of the CXCR4 Receptor

11.1.3.1 Role in Leukocytes Trafficking and Development

The tight control of expression of CXCL12 and CXCR4 and its signaling capacities contribute to the pleiotrophic but yet specific function of the chemokine/receptor couple. Originally, CXCL12 was found to be secreted by bone marrow stromal cells in addition to endothelial cells, as implicated by its original acronym – stromal cell-derived factor 1. CXCL12 serves as chemoattractant for immature and mature myeloid (monocytes, macrophages, neutrophils, basophils, eosinophils, megakaryocytes/platelets, dendritic cells) and lymphoid haematopoietic cells (T lymphocytes, B lymphocytes, natural killer cells). Such function determines the trafficking and retention of leukocytes between the blood stream and secondary lymphoid tissues during both maturation of progenitor cells and homeostatic condition. This process, which is referred as "homing," involves the transient and reversible interaction between leukocytes and vascular endothelium (adhesion), as well as the transendothelial migration of leukocytes into tissues where chemokine gradients are established [63]. The CXCL12/CXCR4 couple mediates these functions by triggering arrays of signaling cascade that lead to the adhesion, cytoskeletal rearrangements, polarization and thus the directional migration (chemotaxis) of leukocytes [64–66]. During the development of T lymphocytes, lymphoid progenitors arose from the bone marrow (BM) enter the circulation and migrate towards the thymus [67–70]. It has been shown that the CXCL12/CXCR4 interaction is non-redundant for the proper cortical localization and developmental arrest of the lymphoid progenitors in post-natal thymus [71]. More recently, a positive role for CXCR4-mediated signaling in the proliferation, survival and differentiation of immature thymocytes (T cell precursor) by modulating the activity of the pre-T cell antigen receptor has also been proposed [70]. Consequently, as the cells mature, they progressively loss their responsiveness to CXCL12 [72, 73], probably due the downregulation of CXCR4 [74]. During B lymphocytes development in BM, B lymphopoiesis takes place following three steps of maturation – pro-B cells, pre-B cells and immature B cells and the final maturation step that occur in the periphery. Migration, survival and differentiation of these cells are tightly regulated by the CXCL12/CXCR4 signaling axis. Early studies have shown that CXCL12, which has been originally identified as a cellular factor in supporting B cell development [75, 76], induces the proliferation [77–79] and chemotaxis [75, 80–85] of conventional B lymphocytes and their precursors. Furthermore, studies of CXCL12 expression in human embryos and mice, as well as *in vitro* adhesion assays, suggest that interaction between the chemokine and CXCR4 are critical for the residence of B lymphocytes lineage and its precursors in the BM [86–91]. It has also been suggested that CXCR4 expression in endothelial cells mediate translocation of circulatory CXCL12 into the BM, which in turn enhances the homing of progenitor cells [92]. Together, these results echo previous findings in which *Cxcl12*$^{-/-}$ or *Cxcr4*$^{-/-}$ knockout mice display a reduced number of B cell progenitors in the BM and deficits in B-lymphopoiesis [76, 93], and support the critical role of the CXCL12/CXCR4 axis in B-lymphopoiesis in both pre-natal life and adult stage for host defense.

Under pathological condition, the number of neutrophils present in the peripheral circulation, which is controlled by its release from the BM and its clearance in the circulation via the spleen, liver and BM [94, 95], is an indicator of disease progression. In attempt to control mobilization of neutrophils under stress, it has been well documented that cellular factors including G-CSF and GM-CSF could induce the release of these cells from the BM to the periphery [96–99]. Various chemokines such as CXCL8 (IL-8) and CXCL2 [macrophage inflammatory protein 2 (MIP-2)/KC] have also been implicated in neutrophil release in response to inflammation [99–101]. Subsequent studies revealed a dynamic relationship between CXCR4 and CXCR2 that controls both the release and clearance of neutrophils in the BM [102, 103]. Such crosstalk between CXCR2 and CXCR4 via heterologous desensitisation is proposed to mediate the release of neutrophils from the BM stimulated by CXCR2 chemokines, while endogenous CXCL12 in the BM acts as a retention factor. From this, the blockade of the CXCR4-dependent pathway (i.e., by neutralizing antibodies and AMD3100) thus results in the mobilization of neutrophils. A more recent investigation using neutrophils with a myeloid-restricted deletion of *CXCR4* further confirmed the role of CXCR4 in regulating the neutrophil release from the BM [104]. As the neutrophil ages, expression of CXCR4 is upregulated and thus confers these senescent cells with the capacity to migrate towards CXCL12 gradients back to the BM, before the cells became apoptotic [102–104]. Such CXCR4-dependent homing of senescent neutrophils to the BM also represent a novel site for clearance of these cells [95].

11.1.3.2 **Role in Neovascularization and Angiogenesis (Pre- and Post-Natal)**

The CXCL12/CXCR4 couple also plays important role in other homeostasis process during adult life, like angiogenesis, which involves the formation of blood vessels from the existing ones. In contrast to that of the pre-natal life, which involves vasculogenesis/neovascularization (*de novo* formation of blood vessels), it involves circulating endothelial progenitor cells (EPCs) that egress form the BM. These cells migrate to and proliferate in target tissues and organs [105, 106], after the degradation of the extracellular matrix and the adjacent basement membrane as well as the formation of endothelial cords that penetrate the surrounding tissue [107, 108]. Such processes are fundamental to tissue repair upon injury. In this regard, a number of angiogenesis mediators have been identified, including members of the fibroblast growth factor 1 (FGF-1) family, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), TNF-α, angiopoietins, ephrins and certain members of the CXC chemokine family [109, 110]. Among them, VEGF is the best characterized angiogenic factor, which displays mitogenic activity on endothelial cells by stimulating their elongation and branching, as shown in numerous *in vivo* and *in vitro* models [26, 111–113]. The role of CXCL12/CXCR4 signaling axis in angiogenesis has received intensive attention [114] after the original study of Salcedo and colleagues showing that stimulation of endothelial cells with VEGF upregulates both CXCR4 transcripts and protein levels in endothelial cells [115]. In supporting the functional upregulation of CXCR4, endothelial cells stimulated by VEGF migrate toward CXCL12 gradients, and *in vivo* administration of CXCL12 can induce

formation of local small blood vessels [115]. Subsequent studies also indicate that the CXCL12/CXCR4 axis principally mediates the migration of EPCs [86, 116, 117], EPC progenitors [118] and differentiated endothelial cells [119], probably through the activation of extracellular signal-regulated kinase (ERK)1/2 and PI3K signaling pathways [120]. Under ischemic condition, CXCL12/CXCR4 axis-mediated EPCs recruitment is thought to play an important role during angiogenesis, by enhancing the adhesion, migration and homing of EPCs to the affected ischemic tissue. Under such stress conditions whereby cellular oxygen supply is greatly reduced (i.e., ischemia/hypoxia), HIF-1 is stabilized and in turn upregulates the expression of CXCL12 [86]. Inhibition of this process affects the capacity of EPCs to migrate toward CXCL12 and results in an impairment of wound healing [121]. In an experimental model of epithelial wound healing following burns, a remarkable increase of CXCL12 levels were observed in human burn blister fluids, hair follicles and in blood vessel endothelium surrounding the injured area [122]. Detailed characterization of CXCL12 productive cells has revealed an increased number of fibroblasts expressing CXCL12 in the recovering dermis. Interestingly, interfering *in vitro* with CXCL12/CXCR4 interactions blocks the mitogenic effect of fibroblast-expressed CXCL12 excerted on adjacent keratinocytes [123], as well as the wound healing of intestinal epithelial cells monolayers [124]. Together, these results emphasize the essential role that the CXCL12/CXCR4 axis plays in skin homeostasis and wound healing through the control of endothelial cells recruitment and the proliferation of keratinocytes by paracrine mechanisms.

Apart from EPCs, CXCL12 also acts on other important players of the angiogenesis process, namely the BM-derived circulating cells (RBCCs)/hemangiocytes [125, 126]. These group of cells are recruited to angiogenic sites due to the VEGF expression and serve a function in angiogenesis that is distinct from that displayed by EPCs. Such retention of RBCCs in the adjacent area of sprouting vessels is mediated by CXCL12, which enhances *in situ* the proliferation of recruited EPCs.

11.1.3.3 Role in Embryonic Development: Phenotypes of CXCL12 and CXCR4-Knockout Mice

Development of tissues and organs during embryogenesis relies on the appropriate and directed migration of stem cells from the area in which they are produced towards the developing organ where they proliferate and differentiate. Mechanisms governing the directed migratory processes of stem cells and progenitor cells in postnatal life as described above are thought to be similar to those during embryonic development. The essential activity of the CXL12/CXCR4 couple in the development has been illustrated upon invalidation of either genes in *Cxcl12*$^{-/-}$ and *Cxcr4*$^{-/-}$ mice. Similar phenotypes were displayed by both types of mice, in which the vast majority of embryo died *in utero* between 15 and 18 days of gestation. Owing to these observations, CXCL12 and CXCR4 were long thought to form a monogamous pair. Defaults in B-lymphopoiesis and BM myelopoiesis as stated above, in addition to impaired cardiovascular development [76, 93, 127, 128] and neurogenesis [129–131], have been observed in both types of mice. It is generally believed that such observations rely on the role of CXCR4-mediated signaling in the regulation of the

migration of different type of stem/progenitor cells [132–134]. Consistent with this, studies have depicted that the expression profile of CXCL12 and CXCR4 during the development of embryo is tightly controlled [135–137], suggesting that the CXCL12/CXCR4 couple plays distinct roles during different developmental stages of different tissues. In developing embryos, the initial hematopoietic activity known as primitive hematopoiesis appears in the blood island of the yolk sac, whereby the adult-type definitive hematopoiesis take place in a structure called aorta–gonad–mesonephros (AGM), an embryonic site that produce the definitive haematopoietic stem cells (HSCs) that forms erythroid cells and endothelial cells [138]. As the hematopoiesis progresses, HSC leave the AGM and migrate to the fetal lever, an early organ of hematopoiesis. At the late stage of the gestation process, HSCs leave the fetal liver and migrate to BM. In either *Cxcl12*$^{-/-}$ or *Cxcr4*$^{-/-}$ mice, migration of HSC from liver to BM is greatly impaired, while early migration from AGM to fetal liver remains unaffected [93, 139, 140]. Although the mechanisms controlling such a differential role of the CXCL12/CXCR4 axis remain to be determined, the dependency of migration of HSCs towards CXCL12 gradient has been well documented [141], and is consistent with the constitutive expression of CXCL12 in BM. The CXCL12/CXCR4 axis can also regulate haematopoiesis by modulating HSCs proliferation and survival [142]. As being identified originally as a pre-B cells growth stimulation factor, it is thus not surprising that CXCL12 could also provide survival signal to HSCs. Indeed, defects in haematopoiesis in *Cxcl12*$^{-/-}$ or *Cxcr4*$^{-/-}$ transgenic mice involve a reduced number of B cell progenitors and myeloid progenitors in BM [76].

During cardiovascular development, a cardiac neural crest cell subpopulation migrates and aggregates at the endocardial cushions prior to septation, which consequently give rise to the development of the ascending aorta and the pulmonary trunk [143]. In both *Cxcl12*$^{-/-}$ or *Cxcr4*$^{-/-}$ deficient mice, the cardiac septation process is greatly impaired [76, 93]. As CXCL12 expression has been observed in the developing heart tissue [136] and that of CXCR4 is found in migrating cells of the neural crest [144], the impaired migration of these cells mediated by the CXCL12/CXCR4 axis in *Cxcl12*$^{-/-}$ or *Cxcr4*$^{-/-}$ deficient mice is thus likely to account for the above phenotype. Furthermore, it should also be noted that *Cxcl12* or *Cxcr4* knockout mice manifest a deficiency in angiogenesis [128]. In keeping with this, it has been shown that CXCR4 is expressed by hemangioblasts, the earliest common precursor of hematopoietic and endothelial stem cells found in yolk sac blood islands [145], and also by endothelial cells derived from embryonic stem cells *in vitro* [146]. These cells expressing CXCR4 migrate towards CXCL12 gradients. As it is well documented that CXCL12 could enhance angiogenesis *in vitro*, it is believed that the CXCL12/CXCR4 axis in endothelial progenitors is critical for the neovascularization process during embryogenesis. Such important properties are indeed recapitulated during the adult life under physiological and pathological conditions as described above.

11.1.3.4 **Role in Embryonic Development: Insights from the Zebra Fish Model**

Apart from the knockout mice models described above, other animal systems may also be considered in view of the CXCR4 and CXCL12 orthologs in other

organisms [147, 148], the presence of which highlights the primordial and evolutionary conserved role of the CXCL12/CXCR4 couple. In zebrafish (*Danio rerio*), CXCR4 homolog is expressed by two genes, *cxcr4a* and c*xcr4b*, which show different tissue distribution [147] and functions. The optical clarity, characteristic of extrauterine development and the available tools for genetic modification, make the zebrafish an attractive model to dissect the role of the CXCL12/CXCR4 axis in different developmental processes in an unprecedented resolution [149, 150]. The zebrafish *cxcr4a* gene has been implicated in the formation of muscle [151, 152], while *cxcr4b* is involved in the movement of primordial germ cells (PGC) [135, 153, 154], sensory cells [155–157] and myosin contraction [158]. Interestingly, apart from CXCL12/CXCR4 interaction, a third player in the CXCL12 axis, that is, the RDC1/CXCR7 receptor [159, 160], is found to be expressed in somatic cells. The receptor sequesters the chemokine and shapes the CXCL12 gradient, thus providing guidance cues for CXCL12/CXCR4-dependent migration of PGC [161]. In the development of the lateral line, the restricted expression of CXCR7 in rear cells of the primordium is also consistent with a role of the receptor in CXCL12 clearance [162], while others proposed that active signaling through CXCR7 is needed for cells migration [163]. In human, CXCR7 has been shown to be a second receptor for CXCL12 [159, 160], while mice lacking CXCR7 die perinatally [164]. Collectively, the above results challenge the monogamous relationship between CXCL12 and CXCR4. However, how CXCR7 contributes the biological functions of CXCL12 remains elusive, as some studies suggest a signaling activity of the receptor, while others propose that the receptor modulates CXCR4-dependent signaling. (see Section 11.1.4.5).

11.1.4 Signal Transduction via the CXCR4 Receptor

11.1.4.1 Interaction with the Cognate Ligand – CXCL12

Chemokines mediates signaling by engagement on their cognate receptors that belong to the class A of the superfamily of GPCR. The structural basis of class A GPCR-mediated signal transduction is mostly extrapolated from crystal structures of the rhodopsin [165] and more recently of the human β adrenergic receptor (βAR) [166, 167]. Indeed, although these two paradigmatic examples display low sequence homology with chemokine receptors, they share striking common features: a core seven transmembrane domains (7TM) connected by three extracellular loops (ECL), three intracellular loops (ICL), and with a N-ter extracellular domain and a C-ter intracellular domain at each end of the 7TM. The two cysteine (Cys) residues (one in ECL1, one in ECL2) are conserved in most of the GPCRs including chemokine receptors, and are thought to be important for the stabilization of the conformation of 7TMs. For the structure–activity relationship of CXCL12/CXCR4 interactions, a two-step binding model was proposed to take place at two independent binding sites at both the chemokine and receptor levels [168]. In this model, the docking site involves the RFFESH motif of the CXCL12 N-ter (amino acid 12–17) and the N-ter of CXCR4, whereas signal initiation relies on the interaction between the

first eight residues of the N-ter of CXCL12 to the ECL2 of CXCR4. This model was supported by data obtained with CXCR4 chimeras and mutants [61, 62, 169], by nuclear magnetic resonance (NMR) study on CXCR4 fragments associated with dimeric CXCL12 [170], and more recently, by using a modified highly sensitive NMR method [171]. In this last report, a loss of NMR signal from region of CXCL12 that binds to the second ECL of CXCR4 was observed in the presence of AMD3100, a CXCR4 antagonist that binds to the transmembrane region of the receptor [172], whereas the docking of CXCL12 to the N-ter of CXCR4 was maintained. These findings provide the first structural evidence for the existence of two independent interactions domains between CXCL12 and CXCR4.

11.1.4.2 **Activation of Heterotrimeric G Proteins**

Signals activated downstream this CXCL12/CXCR4 axis are the subject of an extensive interest [173]. Like for other GPCRs, CXCR4 is coupled to and activates heterotrimeric G proteins ($\alpha\beta\gamma$ subunits). The β- and γ-subunits (Gβ and Gγ, respectively) are assembled as dimers that act as a functional unit, while the α-subunits (Gα), which binds to guanine nucleotides, are activated when the exchange of GDP into GTP takes place [174]. The Gα proteins are classified into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$. Although CXCR4 can signal through different Gα families that lead to distinct signaling pathways and biological effects [173], most of CXCR4 functions involve the $G\alpha_i$ family of proteins as they are sensitive to PTX [175, 176]. Upon binding of CXCL12, activation of the $G\alpha_i$ subunit leads to a lowered level of cellular cAMP through inhibition of adenylyl cyclase and activates the Src family of tyrosine kinases [177, 178]. Meanwhile, the Gβγ subunit is released from the heterotrimeric $\alpha\beta\gamma$ complex and activate various effectors including PI3K and phospholipase C (PLC)-β pathways [179]. The PI3K pathway further activates PAK (p21-activated kinase) and Akt, which are important for cell polarization and actin rearrangement respectively [176, 180, 181]. PLC activation activates various components critical for cell adhesion, including calcium release, PKC activation, as well as focal adhesion kinase (FAK) and ERK, although some of them may also be activated independently of PLC activation upon CXCL12 stimulation [182–185]

11.1.4.3 **Regulation of CXCR4 G Protein-Dependent Signaling**

To avoid a prolonged and potentially deleterious signaling, stimulation of CXCR4 by CXCL12 initiates adaptive responses that arrest G protein activation, a process that is referred to as desensitisation. Such process is typically associated with internalization of the activated receptor [41, 186–188], which involves phosphorylation of Ser and Thr (threonine) residues of the receptor C-ter [186, 187, 189] through the action of protein kinase C and/or specialized GPCR kinases (GRK). Such modifications of the intracellular domain of the CXCR4 receptor in turn promote the recruitment of the adapter proteins β-arrestins (βarr) [186, 187, 189, 190]. β-Arrestins negatively regulate G protein dependent signaling by promoting the desensitization, recycling and degradation of the receptor [191, 192]. Upon ligand stimulation, CXCR4 is rapidly phosphorylated and internalized [42, 186, 187] in a C-ter-dependent

fashion [186, 187, 189]. Although GRK that is responsible for the phosphorylation of CXCR4 has not yet been identified, more recent results that suggest that GRK3 and GRK6 as candidates that regulate differentially the functions in CXCR4-mediated signaling [190, 193]. Recruitment of βarr to CXCR4 desensitizes the receptor and subsequently target it to clathrin-coated pits for internalization and lysosomal degradation [173].

G protein-dependent signaling can also be regulated by proteins that accelerate the intrinsic GTPase activity of Gα subunits, namely the regulator of protein signaling proteins (RGS) [194]. These proteins can inactivate members of the $G\alpha_i$ family, $G\alpha_q$, and $G\alpha_{12/13}$ [195] and thus can affect the biological activities of chemokines. For example, it has been shown that RGS1, 3, 13, and 16 can downregulate CXCL12-mediated chemotaxis [196, 197], whereas the expression of RGS13 has also been implicated in the inhibition of G protein activation downstream CXCR4 [198]. RGS16 has also been identified as another negative regulator of CXCL12/CXCR4 signaling, which plays a critical role in the maturation process of megakaryocytes [199] and regulation of CXCL12 levels [200].

11.1.4.4 Activation and Regulation of β-Arrestin-Dependent Signaling Pathways

Apart from mediating the regulation of G protein activation, recent evidances have indicated that the scaffolding βarr can also dynamically assemble a wide range of signaling complexes in response to ligand stimulation [191, 201, 202]. These signaling pathways include the mitogen-activated protein kinase (MAPK)/ERK, Jun N-terminal kinase (JNK), p38, PI3K and the Ras homolog gene family, member A (RhoA) [191, 203]. For CXCR4, downstream pathways that can also be activated without the involvement of G protein coupling include ERK1/2 and the Janus kinases (JAKs)/signal transducers and activators of transcription (STATs) (JAK/STAT) pathways [204, 205], which positively modulate CXCL12-induced chemotaxis [189, 206–209]. These results are in accordance with the observation that leukocytes from βarr2-knockout animal display an impairment of the CXCL12-induced chemotaxis, in which the activation of G protein downstream of CXCR4 is preserved [210]. Interestingly, it has been shown that C-ter deleted forms of CXCR4, which show impaired desensitization, could still associate with βarr and transduce βarr-dependent signaling [189, 211, 212], as suggested by the fact that βarr can associate with a peptide encompassing the ICL2 apart from the receptor C-tail [189]. Furthermore, mutation of CXCR4 ICL2 disrupts βarr-dependent ERK1/2 activation, and leads to a receptor that is constitutively desensitized and internalized, certainly as a result of the interaction of the βarr with the receptor C-ter [211]. These results accord with a model whereby βarr-mediated signaling and -receptor desensitisation involve βarr-association with distinct region of the receptor [213].

11.1.4.5 Other Factors Affecting CXCR4 Signaling: Role of Receptor Oligomerisation

Analysis of ligand binding and signal transduction properties of the receptor was made based on the assumption that GPCRs exist and function as monomers. A number of class C GPCRs have been shown to form dimers at the plasma membrane,

and accumulating evidence supports the hypothesis that class A GPCRs also form dimers or higher-order oligomers. It is believed that such oligomerisation processes could impact different aspects of GPCR biology including receptor folding, ligand affinity, signaling capacity, selectivity of G protein activation and receptor internalization [214]. For CXCR4, it has been shown that the receptor display the propensity to form constitutive (i.e., ligand-independent) homodimers [204, 215, 216] as well as heterodimers with another chemokine receptor CCR2 [217–221] and other GPCRs such as the δ-opoid receptor [222, 223]. Resonance energy transfer (RET) technique that consists of the measurement of energy transfer between a donor and an acceptor when the two molecules are in close proximity (<100 Å) [224], have been instrumental to show that CXCR4 can form constitutive homodimers [218, 225] and oligomers with CXCR7 [164, 226, 227]. Interestingly, although CXCR7 *per se* cannot mediate $G\alpha_i$-dependent signaling [228], the receptor was found to constitutively interact with $G\alpha_i$ proteins and to induce conformational changes within the pre-assembled CXCR4/$G\alpha_i$ protein complexes that consequently impair CXCL12/CXCR4-mediated responses ($G\alpha_i$ protein activation, calcium influx and chemotatic response of primary T cells) [226]. Emerging evidences indicate that engagement of CXCR7 with CXCL12 can induce the recruitment of βarr to the receptor [229, 230] and consequently the activation of MAP kinases [231]. However, these data further emphasize that CXCR7 does not behave as a classical chemokine receptor and that it also act as a scavenger for CXCL12 in human cells [232].

11.2
Patho-Physiological Role of CXCR4: Potential as a Therapeutic Target

11.2.1
Role in HIV Infection

A large number of reviews highlight the co-receptor role of CXCR4 and CCR5 for HIV type 1 (HIV-1) infection [233–238]. HIV isolates are classified based on the use of CXCR4 (X4) or CCR5 (R5) as the co-receptor to infect target cells [239]. Although other chemokine receptors including CXCR7 may also function as co-receptors in experimental cell systems [234, 240, 241], CXCR4 and CCR5 are the only co-receptors that mediate HIV-1 infection *in vivo* [236, 242]. In line with this, it was demonstrated that the ligand of CCR5 (CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) and CXCR4 (CXCL12) could effectively block the entry of R5 and X4 strains respectively [243, 244]. Moreover, polymorphisms of CCR5 [245] or CXCL12 [246–248] that affect expression of the receptor at the cell membrane [245] or stability of the chemokine [249, 250], respectively, are associated to resistance or higher susceptibility to HIV infection. Transmission and early progression of HIV infection mostly involve R5 viruses [240, 251]. Although the presence of X4 viruses is detectable at virtually all stages of infection, their early propagation is rare [252]. At later stage, X4 viruses emerge and coexist with the R5 types, and such emergence is often accompanied by a rapid loss of CD4 + T lymphocytes and progression of the disease [253, 254].

11.2.2 Role in Inflammatory Diseases

Chemokines and receptors are regulator of leukocytes trafficking, the perturbation of which can lead to the development of inflammatory diseases. In this regard, inflammatory diseases that involve the CXCL12/CXCR4 pair include allergic airway disease (e.g., asthma), arteriosclerosis and rheumatoid arthritis. For example, in different mouse models of allergic eosinophilic airway inflammation, the involvement of the CXCL12/CXCR4 axis has been demonstrated by either the usage of competitive antagonists [255, 256] or antibodies against CXCR4, as well as neutralizing antibodies against CXCL12 [257]. These treatments significantly lower leukocytes infiltrate in the lung, which is accompanied by a significant decrease in airway hyper-responsiveness. In arteriosclerosis, which refers to an abnormal hardening of arteries due to extensive accumulation of inflammatory infiltrates (monocytes, neutrophils, platelet cells, endothelial progenitor cells), both CXCL12 and CXCR4 are highly expressed in arteriosclerotic plaques compared to normal blood vessels [258, 259]. In a transplant arteriosclerosis model, expression of *CXCL12* mRNA increases as the disease progresses [260]. Such upregulation may enhance recruitment of neutrophils to the atherosclerotic plaques, as suggested by the important role of these leukocytes in a mice model of diet-induced atherosclerosis reconstituted with *Cxcr4*-knockout BM [261]. In keeping with this, individuals carrying *Cxcl12* gene polymorphism that are highly susceptible to HIV infection have a lower incidence of arteriosclerosis, probably due to a lower plasma concentration of CXCL12 in the blood [262]. More recently, it has been proposed that the cytokine macrophage migration inhibitory factor (MIF), which is suggested to play a critical role in inflammatory diseases and arteriogenesis, could compete with cognate ligands for binding to CXCR4 and CXCR2, raising the possibility of a chemokine-like function of MIF in the disease [263]. For rheumatoid arthritis (RA), which is a chronic persistent inflammatory disease affecting limb joints, accumulation of T lymphocytes and monocytes at defined areas in synovial tissue that involves the CXCL12/CXCR4-axis are observed. $CD4^+$ T lymphocytes within the rheumatoid synovium express constitutively CXCR4 in a TGF-β and/or CD40-dependent manner [264, 265]. These findings suggest that the upregulated CXCR4 expression in $CD4^+$ T cells following their entry into inflamed tissue might play an important role in disease progression. Further, it has been observed that CXCL12 is abundantly expressed by the stromal and lining fibroblasts in the RA synovial microenvironment [265, 266] and may be associated with the expression of angiogenic marker such as αvβ3 integrin [266]. In mouse, ectopic expression of CXCL12 induces pathological lymphoid tissue development with prominent dendritic and plasma cell accumulation, features that are commonly observed in rheumatoid synovium [267]. Collectively, the above results pinpoint a multifaceted function of the CXCL12/CXCR4 signaling axis in the increased migration, infiltration and the differentiation of the recruited cells that ultimately leads to the development of airway allergies and arteriosclerotic lesions. For CXCR7, expression of the receptor in adult osteocytes and chondrocytes in joints leads to changes in gene expression that are associated with chondrocyte

hypertrophy, angiogenesis and increased matrix degradation [268]. These results suggest that the receptor may play a role in homeostasis and pathology of connective tissues including development of osteoarthritis.

11.2.3 Role in Cancer Development

Studies on chemokine/chemokine receptor interaction have received much attention in the domain of tumorigenesis (see Chapter 8) since the early report demonstrating the implication of the CXCL12/CXCR4 axis in breast cancer. Apart CXCR4, CXCR2 and CCR7 receptors were found to be over-expressed in tumor cells, with CXCR4 and CCR7 being the most abundantly expressed in malignant breast cells, disseminated cancer cells in auxiliary lymph nodes, pulmonary and hepatic metastases, as well as in melanoma cell lines when compared to normal mammary tissue and primary melanocytes [269, 270]. Functionally, the enhanced expression of CXCR4 is proposed to mediate dissemination of tumor cells to specific secondary sites, which in turn leads to the promotion of organ-specific metastasis. In support of this possibility, *in vitro* migration and invasion assays, which measure the metastastic potential of cells, have shown that some breast cancer cell lines are indeed responsive to CXCL12 [269]. *In vivo*, the extent of metastasis in severe combined immunodeficient (SCID) mice xenografted with human breast cancer cells could be greatly reduced upon injection of anti-CXCR4 antibodies [269]. Later studies using small interfering RNA (siRNA) [271–273], small peptides [274] and microRNA [275] against the CXCR4 receptor confirmed these observations. Moreover, it has also been shown that over-expression of this receptor in B16 melanoma cells could increase the metastatic potential of the cells to the lung [276].

Apart from the receptor, CXCL12 has also been reported to be abnormally expressed in various types of cancer cells, including breast, ovary, glioma and prostate cancer [277, 278]. These studies gave the early suggestive evidence on the association of the CXCL12/CXCR4 axis in the primary growth of cancer in a paracrine and/or autocrine manner. Indeed, a mitogenic role of the CXCL12/CXCR4 signaling pathways in tumor cells has been demonstrated first by Sehgal and coworkers, by showing that the proliferation of glioma cells *in vitro* can be inhibited by the application CXCR4 siRNA [279]. Such proliferative effect of the signaling axis was correlated with the activation of ERK1/2 and Akt pathways [280, 281], which can be extended to other tumor cell types [282, 283] and was found to be inhibited upon CXCR4 blockade (e.g., AMD3100, 12G5 monoclonal antibody) [284, 285]. Abnormal expression and secretion of CXCL12 by fibroblast-associated carcinoma is critical for the promotion of tumor growth and the angiogenesis through the recruitment of endothelial progenitor cells (EPCs) into carcinomas [286]. A similar paracrine role of the chemokine has also been observed in the transformation of prostate epithelial cells [287]. More recently, the CXCL12 signaling axis was implicated in the survival of disseminated breast cancer cells in bone [288]. Of note, recent evidences suggest that CXCR7 can also mediate such effect of the chemokine [289, 290], and thus implying that the receptor should also be considered when studying of role of CXCL12 in tumor development.

11.2.4
The WHIM Syndrome (WS)

The syndrome of neutropenia, hypogammaglobulinemia, myelokathexis (WHIM) is a rare primary immunodeficiency associated to a marked leucopenia, as well as recurrent, severe bacterial infection of the aero-digestive tract and lung. The infection is frequently observed in WS patients and can be life-threatening in both early and adulthood life [291–294]. Majority of the patients also suffer from human papillomavirus (HPV) infection (W for warts), while there is no increase in general susceptibility to other viruses [295–297]. Apart from the neutropenia and myelokathexis, the immuno-hematological symptoms of WS are heterogeneous [298], since hypogammaglobulinemia is absent in some cases [299]. The clinical picture of the syndrome is often completed with a profound lymphopenia. In this regard, although T cell responses to vaccine antigens are preserved in WS patients [293, 300, 301], the lymphopenia severely affects both B and T cell subpopulations in most affected individuals, in particular the $CD4^+$ T cell subset [293]. Neutropenia is associated with myelokathexis, which is referred as an abnormal retention of senescence neutrophils in the BM [302]. Whether these observations are due to the defect in neutrophil egression to the periphery or by increased homing of senescent neutrophils to the BM is undetermined. To date, there is no specific treatment for WS, although intravenous immunoglobulin is often the first therapy that is prescribed together with prophylactic antibiotic therapy. Administration of GM-CSF or G-CSF may be proposed, as it transiently normalizes BM cytology and the peripheral neutrophil counts, although it fails to improve HPV-related disease [297]. The discovery that this syndrome is linked in most cases to autosomal-dominant heterozygous gain of function mutations of *CXCR4* [301] has opened promising lead regarding the role of the CXCL12/CXCR4 axis in the pathogenesis and as a potential therapeutic target. Indeed, WS is the first reported inherited immuno-hematological disease due to dysfunction of the CXCL12/CXCR4 axis (i.e., impaired desensitization and enhanced G protein-dependent signaling and chemotaxis upon CXCL12 stimulation), whether it is associated with a mutated or a wildtype *CXCR4* gene [193, 293, 301, 303, 304]. All *CXCR4* mutations identified so far lead to the loss of the last 10–19 amino acid residues of CXCR4 C-tail [212, 297, 305]. In the genetic forms associated with a wildtype *CXCR4* gene, other chemokines receptors, including CXCR7, were shown to function normally, suggesting anomalies selective of the CXCL12/CXCR4 signaling axis [193].

11.2.5
Potential of CXCR4 as Therapeutic Target

11.2.5.1 Application in HIV-Infection

The pathological role of CXCR4 in many diseases has boosted the search of therapeutic molecules targeting the receptor. Notably, the discovery of CXCR4 as a co-receptor for HIV has initiated this research area over the past two decades and lead to identification of various molecules that alter CXCR4 functions. Such large

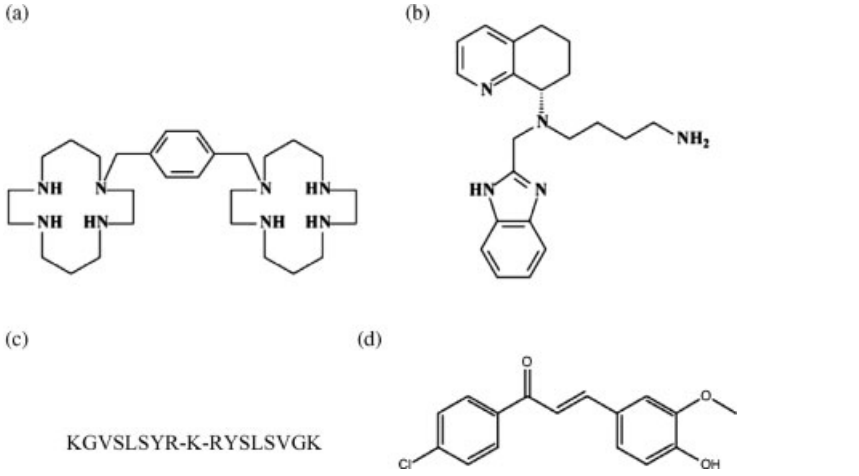

Figure 11.1 Chemical structure/peptide sequence of selected inhibitors against CXCR4 and CXCL12 as described in the text. Chemical structure of: (a) the bicyclam CXCR4 inhibitor AMD3100 {1, 1′-[1,4-phenylenebis (methylene)]-bis 1,4,8,11-azatetradecane} and (b) another CXCR4 antagonist – AMD11070/AMD070. (c) Sequence of CTCE-9908, a peptide inhibitor against CXCR4 that is derived from a modified dimer of the eight-amino-acid N-terminal sequence of CXCL12. (d) Chemical structure of the CXCL12 neutraligand: chalcone 4, one of the intermediate products during flavonoid synthesis.

body of work has fertilized the search for therapeutic strategies for cancers as well as treatment for autologous transplantation based on allogeneic stem cell mobilization by the bicyclam AMD3100 (also known as JM3100, plerixafor or Mozobil; Figure 11.1a). Among the compound such as peptides, antibodies, and small interfering RNA that disrupt CXCR4 functioning in various model systems [306, 307], AMD3100 is the first and the best characterized small molecule that antagonize CXCR4 functions [308, 309]. The inhibitor consists of two cyclam rings connected by an aromatic linker that interacts with the transmembrane region of CXCR4 [172, 310] and is suggested to behave as an agonist for CXCR7 but at very high concentration [229]. Indeed, functional studies have clearly demonstrated the specificity of the AMD3100 for CXCR4 [160, 229, 311]. Although this compound has the ability to block infection of X4 and R5/X4-HIV strains [308, 312–314] and thus the viral load [315, 316], chronic administration of the drug is associated with some adverse effects, including cardiac toxicity [315] and leukocytosis [316–318]. Moreover, the lack of oral bioavailability was also an obstacle for these trials. Some of these shortcomings have also been observed with other recently developed CXCR4-targeting noncyclam compounds, such as AMD070 (also known as AMD11070; Figure 11.1b) [319, 320]. Nevertheless, the identification of CXCR4 inhibitor as HIV entry inhibitor remains important in the repertoire of anti-HIV strategies owing to the emergence/re-emergence of X4-tropic HIV subtypes observed upon the treatment with inhibitors against CCR5. Collectively, although CXCR4 inhibitors can effectively hamper HIV

infection *in vivo*, these observations call for a biologically safe and orally available anti-HIV entry inhibitor, as well as the importance of monitoring of HIV receptor usage in patients under disease management.

11.2.5.2 Application in the Development of Cancer and Stem Cell Mobilization

As mentioned above, proof of principle application of antibody- and small molecule-based approaches against CXCR4 in various *in vivo* tumor models of solid and hematological malignancies have been reported. Among these agents, antibodies suffer from some limitations such as the crossing of biological barriers, selectivity, *in vivo* stability and the accessibility of the neutralizing epitope [321–324]. Nevertheless, several compounds have successfully progressed from these preclinical studies to clinical trials. For example, CTCE-9908 (Figure 11.1c), a cytostatic drug that is a derivative of the N-ter of CXCL12 developed by the Chemokine Therapeutics Corp., has been reported to undergo clinical trial in attempt to eliminate various types of cancers including ovarian, breast and osteogenic sarcoma [306, 325, 326]. *In vivo*, application of the compound can also be used in conjunction with treatments such as chemotherapy, radiation and anti-VEGF therapy to reduce tumor size [306]. In 2008, the CTCE-9908 Phase Ib/II clinical trial in late-stage cancer patients with no/mild dose-limiting toxicities has been reported [326].

For treating blood-related cancers, early study has shown that AMD3100 can inhibit not only the transmigration but also the proliferation of acute myeloid leukemia (AML) cell [327, 328]. In this type of cancer that involves an uncontrolled proliferation of myeloid progenitors within the BM, treatment with CXCR4 inhibitors like AMD3100, TC140012 and AMD3465 can sensitize the AML cells to cytotoxic and antiproliferative effects of cytotoxic agents such as vincristine and dexamethasone, through the increased release of AML cells from the BM [329–331]. Pretreatment of primary human AML cells with neutralizing CXCR4 antibodies can also block their homing into the BM [332, 333]. Base on these findings, pilot clinical trials with AMD3100 in AML patients are ongoing [334]. For other types of leukemia, like chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL), a clinical trial is in process which employs AMD3100 in conjunction with Rituximab (commercially known as Rituxan/MabThera; a monoclonal antibody against the cell surface CD20 protein of B cells to treat lymphoma, leukemia and some autoimmune disorders [335]; Phases I and II; clinicaltrials.gov identifier: NCT00694590). Similar trials also include those against nonHodgkin's lymphoma (NHL; Phase II; clinicaltrials.gov identifiers: NCT00444912, NCT00665314) and a Phase II study in multiple myeloma [336]. Interestingly, a recent finding has shown that the combined administration of AMD3100 and G-CSF could specifically induce the egression of HSCs population from the BM [337]. With the clinical importance of HSCs in the treatment of various types of leukemia, and a relatively lower dosage requirement for AMD3100 to mobilize enough HSCs for early allogeneic transplantation [338], the above results may eliminate the concern about the toxicity of this compound as observed in clinical trials against HIV infection [313]. Indeed, such usage and AMD3100 in combination with G-CSF for stem cell mobilization for treating NHL and multiple myeloma has recently been approved by the United States Food and

Drug Administration [339, 340], which represents a groundbreaking advancement in the development of therapies that target the CXCR4 receptor. For CXCR7, the successful application of antagonists and RNA interference against the receptor in reducing tumor development and metastasis in animal models [160, 341] also underscore CXCR7 as another therapeutic target against malignancies induced by CXCL12.

The above reagents neutralize the biological activity of CXCR4 by interacting to the receptor. New pharmacological approaches by targeting the CXCL12 (i.e., CXCL12 neutraligand) were recently proposed [342] and represent promising alternative therapeutic approaches to the traditional competitive antagonists that bind to the receptor. One such characterized compound, the flavonoid precursor chalcone 4 (Figure 11.1d), binds to the CXCL12 chemokine and so disrupts its interaction with receptors and the downstream signaling events. Of note, chalcone 4 does not alter the basal/resting levels of receptor-associated responses [342].

11.3 Concluding Remarks

CXCR4 has received intensive attention in the research domain of chemokine and chemokine receptors owing to its multifunctional nature. Reported efficacies in numerous preclinical studies underscore the possibility to control CXCR4-related diseases by directly targeting the interaction between the receptor and its ligand. Nevertheless, the critical and multifaceted physiological role of CXCR4, as well as the poorly defined genetic basis of most CXCR4-associated diseases, including cancer and inflammatory diseases, impose hurdles in evaluating potential deleterious effects of inhibitors against the receptor and the development of relevant animal models. In this regard, the WS-associated CXCR4 dysfunctions that are mostly attributed to mutation of the *CXCR4* gene and the conserved CXCL12/CXCR4 axis between human and mice opens the possibility of engineering transgenic mice to study a disease attributed to CXCR4 dysfunction *in vivo*. Furthermore, thanks to the recent advance of biophysical techniques such as RET and knowledge from other GPCRs, studies on the molecular pharmacology and the downstream effectors of CXCR4 have greatly improved our knowledge on the unprecedented complexity of CXCR4-mediated signaling in various disease settings and cell types. The identification of CXCR7 as a second receptor for CXCL12 that displays differentially a higher affinity for the ligand [159, 160] and the capacity to regulate the functioning of CXCR4 through receptor oligomerization and CXCL12 sequestering call for a re-examination of previous works in which a mutually exclusive interaction between CXCL12 and CXCR4 is involved and the necessary caution in analyzing the role of the CXCL12/CXCR4 axis. In this regard, the future direction will focus on a detailed understanding of the structural and pharmacological properties of both CXCR4 and CXCR7 and the downstream signaling in different disease settings, especially when both receptors coexist. Such information is fundamental for developing biased strategies/inhibitors that target/activate the specific downstream effectors

of CXCR4 for disease treatment without affecting the normal physiological role of the receptor.

Acknowledgments

We are grateful to all previous and current members of the Viral Pathology Unit of the Paris Pasteur Institute, for their ultimate technical support, insightful discussions and contribution in research on CXCR4, CXCL12 and CXCR7, especially Drs B. Lagane, K. Balabanian, A. Levoye and the team of Prof. F. Arenzana and Dr. A Amara. We deeply apologize to other coworkers and researchers in the domain of CXCL12 and its receptors that we could not cover all the excellent work in this field due to space limitation.

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12
Low Molecular Weight CXCR2 Antagonists as Promising Therapeutics

Katsuhiro Mihara and Jac Wijkmans

12.1
Introduction

The chemokine receptor CXCR2 recognizes endogenous chemokines that possess a N-terminal Glu-Leu-Arg (ELR)$^+$ amino acid sequence immediately adjacent to their CXC motif. CXCR2 and the closely related receptor CXCR1 are expressed on the cellular surface of leukocytes, notably neutrophils, endothelial cells and a range of other cell types throughout the human body. It has been firmly established now that CXCR2 plays a critical role in the development of numerous inflammatory disorders, wound healing and tumor progression [1–5]. As classically defined for chemokine receptors, CXCR2 mediates cell migration but has also been recognized as a key angiogenic factor [6, 7]. These multifunctional roles have drawn increased attention to CXCR2 as a potentially successful drug target for therapeutic intervention in a range of diseases. The first half of this chapter summarizes the biological role of CXCR2 and its involvement, particularly in inflammatory disorders, in order to illuminate the potential clinical impact and relevance of CXCR2 blocking strategies. A review of all currently known low molecular weight (LMW) CXCR2 antagonists is addressed in the second half of this chapter.

12.2
CXCR2 Ligands and Signal Transduction

CXCR2, which shows 78% overall amino acid identity with CXCR1, was first cloned in 1991 out of human promyelocytic leukemia HL60 cells [8]. Subsequent research demonstrated that CXCR2 is the cognate receptor for at least seven structurally related (ELR)$^+$ chemokines, which are growth-related protein (Gro)-α, -β and -γ (CXCL1–CXCL3), epithelium-derived neutrophil attractant-78 (ENA-78; CXCL5), granulocyte chemotactic protein-2 (GCP-2; CXCL6), neutrophil-activating peptide-2 (NAP-2; CXCL7) and interleukin-8 (IL-8; CXCL8) [9, 10]. In contrast,

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

only two chemokines (CXCL6/8) are known to activate CXCR1. In addition to these CXCR2 ligands, proteolytic cleavage products of chemokines such as CXCL8 (7–77), CXCL8(8–77) and CXCL8(9–77) and several other N-terminally truncated ligands have also been identified to possess potent chemoattractant activity through CXCR2 [11–13].

CXCR2 activation induces receptor coupling with the G_i family of guanine nucleotide-binding proteins [14, 15]. Upon ligand binding, CXCR2 is activated to stimulate release of intracellular inositol phosphates, increase in intracellular calcium [16] and to induce a variety of subsequent cellular responses (see Section 12.3). Once activated, CXCR2 is phosphorylated and is rapidly internalized through β-arrestin/dynamin-dependent mechanisms, which subsequently results in receptor desensitization and removal of the ligand and ultimately degradation or re-expression of the receptor on the cell membrane [17–19].

Recently, it was reported that CXCR2-dependent chemotactic activity is impaired in mice lacking transducer and activator of transcription 3 (STAT3) [20], a protein that has been linked to cellular adhesion and migration functions [21–23]. The impaired chemotaxis of STAT3-deficient neutrophils was associated with an enhanced actin polymerization response to mouse CXCL2/3 (MIP-2), a CXCR2 acting chemokine, indicating that STAT3 operates in pathways that mediate CXCR2 signaling to the cytoskeleton.

12.3 Biological Functions

12.3.1 Leukocytes

CXCR2 is expressed on a broad range of leukocytes, including neutrophils, monocytes/macrophages, eosinophils, mast cells, basophils and lymphocytes [24–28]. The physiological function of CXCR2, however, has been most extensively studied in neutrophils. Using genetic and pharmacological tools, a critical role of CXCR2 in mediating neutrophil recruitment has been demonstrated across different species including human [29], nonhuman primates [30], rodents [31–35] and rabbits [36, 37]. Furthermore, mice lacking CXCR2 showed altered leukocyte rolling capabilities and impaired neutrophil recruitment into the inflamed peritoneal cavity, lung and skin [38–43]. These findings suggest that CXCR2 plays a central role in neutrophil trafficking under a variety of inflammatory conditions.

Interestingly, CXCR2 chemokines were shown to inhibit spontaneous human neutrophil apoptosis [44]. This response was abrogated by a selective CXCR2 antagonist, suggesting a role of CXCR2 in neutrophil survival. Additionally, recent results suggest a negative crosstalk between CXCR2 and CXCR4 to induce neutrophil mobilization from the bone marrow [45]. When the mouse homolog of CXCL1, KC, is administered, the release of neutrophils from the mouse bone

marrow was observed. This response was enhanced by the administration of CXCR4 antagonists. These results indicate that maximal mobilization of neutrophils, induced by CXCR2-acting chemokines, is controlled by a CXCR4-dependent pathway that promotes the retention of neutrophils in the bone marrow. Furthermore, activation of CXCR2 was shown to induce various responses in neutrophils including phagocytosis, degranulation and respiratory burst [46–48]. Taken together, these findings underline the pleiotropic and complex functions of CXCR2 in neutrophils.

Although less understood, roles of CXCR2 in cell recruitment have also been suggested in other immune cells. The GRO family chemokines were shown to induce monocyte arrest on endothelium under flow conditions, but to have a limited role in mediating monocyte chemotaxis, suggesting a potential role of CXCR2 in mediating macrophage accumulation to the inflamed vasculature [49–51]. The recruitment of eosinophils into the airways was impaired in CXCR2-deficient mice sensitized with *Aspergillus fumigatus* [52]. Further, in an OVA sensitization and aerosol challenge model, a significant reduction in the number of pulmonary mast cells was demonstrated in the CXCR2-deficient mice [53]. While physiological relevance remains unclear, the expression of CXCR2 has been reported on subpopulation of circulating human CD8 T lymphocytes and natural killer cells but not on CD4 T cells or B lymphocytes [28]. In order to fully delineate the involvement of CXCR2 in the biological functions of the aforementioned leukocytes in both normal and disease conditions, additional studies are certainly required.

12.3.2
Nonhematopoietic Cells and Cells of the Central Nervous System

It has become evident that CXCR2 is expressed on many other, nonhematopoietic cells, including endothelial cells [54], airway epithelial cells [55] and cells of the central nervous system (CNS) [56]. *In vivo* mouse studies have demonstrated a role for endothelial CXCR2 in angiogenesis and promoting tumor growth [57, 58]. Moreover, involvement of CXCR2 expressed on endothelial cells in leukocyte recruitment has been suggested in several mouse models of inflammation [41, 53]. Further, the expression of CXCR2 on bronchial epithelial cells has been demonstrated *in vitro* [59] as well as in pulmonary disease patients [55]. However, the biological functions and pathophysiological importance of CXCR2 on the bronchial epithelial cells remain to be further studied.

Recent studies further indicate functions of CXCR2 in different types of cells in the CNS and its involvement in diseases. For example, CXCR2 has been shown to play an important role in the development and maintenance of the oligodendrocyte lineage, myelination and white matter in the vertebrate CNS [60]. In addition, it was shown that CXCR2 activation might directly contribute to motor neuron degeneration [61]. Since the expression of CXCR2 has also been detected in neurons, astrocytes and microglia [62–64], more work is required to understand the role of CXCR2 in the CNS both in normal and disease conditions.

12.4
CXCR2 in Inflammatory Disorders

12.4.1
Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality throughout the world. It is projected to be the third leading cause of death worldwide by 2020 due to an increase in smoking rates and demographic changes around the world [65]. The chronic airflow limitation characteristic of COPD is caused by a mixture of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), in which neutrophilic inflammation plays a key role. Recent studies revealed that both levels of CXC chemokines and expression of CXCR2 are increased in bronchial biopsies and sputum of COPD patients [55, 66]. Furthermore, increased expression of CXCR2 mRNA present in bronchial biopsy specimens was shown to correlate with the presence of tissue neutrophils [55]. In several animal models of COPD induced by smoke inhalation or by pulmonary challenge with virus, an important role of CXCR2 in mucus hypersecretion and goblet cell hyperplasia has been demonstrated following CXCR2 blockade [30, 35, 67, 68]. These preclinical and clinical observations strongly support that targeting CXCR2 is a novel and potential therapeutic approach to COPD. Indeed, this strategy is currently being pursued by several pharmaceutical companies with small molecule antagonists [69].

12.4.2
Asthma

Asthma is an inflammatory disorder of the airways associated with airflow obstruction and bronchial hyperresponsiveness that varies in severity across the spectrum of the disease. Whereas mild to moderate asthma patients are usually treated with a combination of inhaled corticosteroids and beta-adrenergic bronchodilators, severe asthmatic patients respond poorly to these therapies. Histologically, neutrophilic lung inflammation accompanied by extensive airway remodeling is an important feature of severe asthma. In fact, in severe asthmatic patients, a positive correlation between increased expression of the ELR^+ CXC chemokines and the presence of neutrophils in lung tissue and BAL fluid was established [70, 71]. Recently, it was demonstrated that CXCR2 is involved in airway goblet cell hyperplasia [30, 67, 68] that leads to hypersecretion of mucus and which is highly associated with asthma [72]. Moreover, a potential angiogenic role of CXCR2 in hypervascularity, which is known to be an important element of airway remodeling in bronchial asthma, has been suggested [73]. Finally, CXCR2 expression is also found on airway smooth muscle cells and is suggested to be involved in the smooth muscle cell contraction, though physiological relevance in asthma remains to be further investigated [74]. Collectively, these results implicate a potential role for CXCR2 in the lung inflammation, lung histopathology and abnormal physiology seen in asthma.

12.4.3
Acute Lung Injury and Acute Respiratory Distress Syndrome

Acute lung injury (ALI) and its more severe form called acute respiratory distress syndrome (ARDS) are characterized by an excessive inflammatory response of the lung usually caused by sepsis, pneumonia, trauma and/or aspiration. The accumulation of activated neutrophils in the lungs has been recognized as an early step in the pulmonary inflammatory process that leads to ALI and ARDS [75]. In preclinical animal models of ARDS [76, 77], an important involvement of CXCR2 has been defined. In these models, pathological features representing lung injury such as airway microvascular leakage, pulmonary neutrophilia and lung edema were reduced in animals treated with neutralizing antibodies to CXCR2 but also in CXCR2-deficient mice. Aspiration of gastric content is a common clinical event associated with the development of ARDS. In a mouse model of acid aspiration, features of lung injury such as edema formation, increased lung vascular permeability and recruitment of neutrophils into the bronchoalveolar lavage were clearly reduced when animals were pretreated with an antiCXCL8 antibody [78]. This clearly indicates a critical role of CXCR2 and/or CXCR1 in the pathology of ALI and ARDS.

12.4.4
Atherosclerosis

Substantial evidence is available now to conclude that atherosclerosis could be regarded as an inflammatory disease of the artery wall. Arrest and transmigration of monocytes as well as continuous immigration and infiltration of activated macrophages into and within atherosclerotic lesions have been recognized as prominent features in both human and experimental atherosclerotic disease. Recent studies have revealed that CXCR2 and CXCR2 chemokines are involved as crucial mediators of atherosclerosis. For instance, in a mouse model of atherosclerosis, it was demonstrated that deficiencies of either CXCL1 or macrophage CXCR2 expression were associated with a loss of intimal macrophages and attenuated disease progression throughout time within established fatty streak lesions [79]. In addition, the repopulation of atherosclerosis-prone LDLR knockout mice with bone marrow deficient in CXCR2 resulted in a substantial reduction of atherosclerotic lesions [80]. Thus, indicating that CXCR2 on hematopoietic cells and its ligands play a critical role in promoting atherosclerosis. Moreover, oxidized LDL, a critical factor in atherogenesis, was shown to induce CXCR2 expression on human monocytes and enhances chemotaxis and adhesion to endothelial cells [81]. Altogether, these data strongly support a pivotal role of CXCR2 in atherosclerosis.

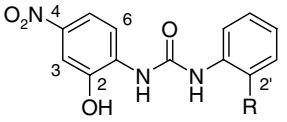
12.4.5
Other Inflammatory Disorders

Based on the variety of roles that CXCR2 plays in neutrophil functions under inflammatory conditions, it has been proposed that this receptor is associated with

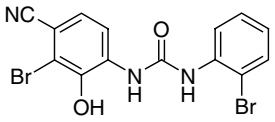
many other inflammatory disorders. For example, an active involvement of CXCR2 has been suggested in different forms of pulmonary fibrosis including idiopathic pulmonary fibrosis and cystic fibrosis and other inflammatory disorders in the lung [69]. In addition, recent research has acknowledged CXCR2 as a promising target in reperfusion injury [82–84]. As a matter of fact, reperfusion injury was one of the first indications for the evaluation of CXCR2 inhibitors in clinical trials (see Section 12.5.9). Furthermore, in a mouse model of cecal ligation and puncture (CLP)-induced sepsis, functional blockade or lack of CXCR2 resulted in a substantial decrease in liver injury and mortality, suggesting therapeutic relevance of targeting CXCR2 in the treatment of sepsis [85]. Finally, several studies in preclinical animal models of rheumatoid arthritis showed that CXCR2 antagonists or CXCR2 antibody significantly reduced arthritis development [86, 87]. Taken together, these observations indicate that antiCXCR2 strategy might be applicable for a broad range of inflammatory disorders.

12.5
Low Molecular Weight CXCR2 Antagonists

As discussed in the previous Section, controlling CXCR2-mediated signaling provides a promising avenue for therapeutic intervention in a range of acute and chronic diseases. To this end, peptide-derived antagonists, which were created by modification of the N-terminal part of CXCL1, CXCL4 and CXCL8 [48, 88], and humanized CXCR2 monoclonal antibodies [89] have been investigated. However, of special interest are LMW CXCR2 antagonists, since these compounds hold the promise for developing therapeutic treatments via oral or topical administration. This section discusses all currently known classes of LMW CXCR2 antagonists. Although most of these compounds (and others) have been disclosed in numerous patent applications [90–93], this review focuses on reports from the public domain.



1 (SK&F 83589): R = H
2 (SB-225002): R = Br



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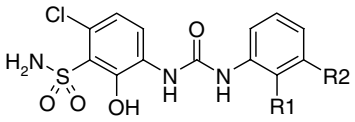


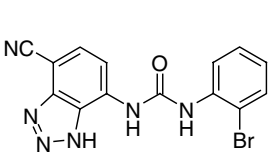
4: R1 = Br, R2 = H
5 (SB-332235): R1 = R2 = Cl

12.5.1
2-Hydroxyphenyl Ureas

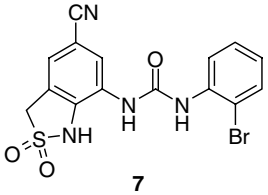
In the mid1990s, a HTS campaign at SmithKline Beecham (now GlaxoSmithKline) produced SK&F 83589 (**1**), the first LMW CXCR2 antagonist ever described [94]. Compound **1** was shown to compete with [^{125}I]-CXCL8 in a binding assay using CHO-cell membranes expressing CXCR2 with IC_{50} of 0.5 μM. Subsequent optimization

focusing primarily on *in vitro* potency led to the identification of SB-225002 (**2**), which showed much improved binding affinity ([^{125}I]-CXCL8 IC_{50} 22 nM). Moreover, **2** displayed excellent antagonistic activity in a Ca^{2+}-mobilization (Ca^{2+} IC_{50} 30 nM) and CXCL8-mediated neutrophil chemotaxis (IC_{50} 20 nM) assay. In addition, SB-225002 was shown to ameliorate the effects of acute experimental colitis in mice [95].

One of the crucial structural elements of the series in terms of potency was identified to be the phenolic hydroxyl group which could categorically not be removed or blocked by, for instance, methylation [96]. Measuring binding of **1** and close analogs as a function of pH indicated that these ureas bind most strongly in their anionic form [97]. As a result, electron-withdrawing substituents in the 3- and 4-positions were preferred. Furthermore, SAR evaluation of the right-hand side (RHS) phenyl ring indicated that substitution of the 2′-position increased affinity. Thus, modification of **2** by replacement of the 4-nitro group with a cyano functionality and introduction of a bromine at the 3-position furnished **3** with an [^{125}I]-CXCL8 IC_{50} of 6 nM and equally potent antagonistic activities [98]. Testing **3** in a topical rabbit model of delayed type hypersensitivity showed significant inhibition (33% inhibition at a dose of 0.5 mg). Unfortunately, this compound was rapidly cleared upon systemic administration due to glucuronidation. In order to improve the poor pharmacokinetic (PK) properties, varying substitution at the 3-position was explored [99]. Consequently, introduction of a blocking sulfonamide group, as in compound **4**, produced small potency improvements. However, more importantly, sulfonamide **4** demonstrated substantially enhanced PK properties as gauged by the low clearance rate (*Cl* 16 ml min^{-1} kg^{-1}) and excellent oral bioavailability (*F* 86%) in rat. *In vivo* evaluation of the related derivative **5** (SB-332235) in acute and chronic models of rheumatoid arthritis in rabbit furnished significant anti-inflammatory effects upon oral dosing at 25 mg kg^{-1}, twice daily [37].



6 (SB-265610)



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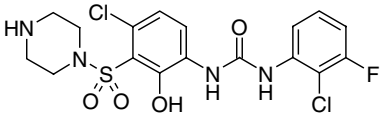
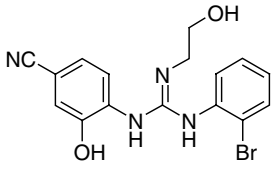


8 (SB-656933)

Equally good *in vivo* characteristics were obtained by bioisosteric replacement of the phenol moiety with acidic heterocycles. For example, benzotriazole **6** (SB-265610), which retained good *in vitro* potencies ([^{125}I]-CXCL8 IC_{50} 39 nM, Ca^{2+} IC_{50} 5.2 nM, CXCL8-mediated neutrophil chemotaxis IC_{50} 4.5 nM), showed high oral bioavailability (71% in rat, 25% in rabbit) and moderate clearance [100, 101]. In addition, **6** demonstrated oral activity in a mouse atherosclerosis model as well as *in vivo* efficacy in rat and rabbit lung inflammation models [102, 103]. A second phenol bioisostere, cyclic sulfonamide **7**, reportedly displayed *in vitro* potencies comparable to **5** [104]. Moreover, **7** was shown to be orally active in rabbit models of rheumatoid arthritis and a mouse COPD model. Currently, GSK is developing sulfonamide **8** (SB-656933) for the potential treatment of COPD and cystic fibrosis [105].



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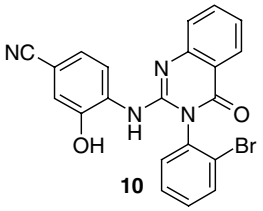
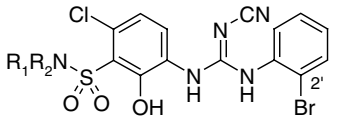
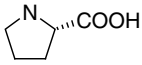


10

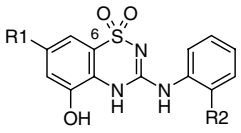


11: R1R2N = Me_2N

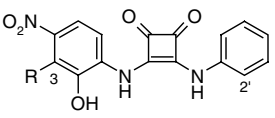
12: R1R2N = 

12.5.2
Urea Isosteres

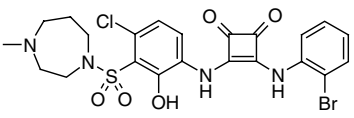
In 2000, Li reported GSKs efforts to replace the 5-hydroxyphenyl ureas with appropriately decorated diarylguanidines, as represented by structures **9** and **10** [90]. As a logical extension of this work, a series of significantly less basic cyanoguanidines were prepared [106]. The SAR in this series largely followed that of the urea series. However, in contrast to the urea derivatives, cyanoguanidines were much less selective versus CXCR1. For instance, dimethyl sulfonamide **11** gave only 10-fold selectivity of CXCR2 relative to CXCR1 ([^{125}I]-CXCL8 IC_{50} CXCR2 5 nM, CXCR1 55 nM) whereas urea **3** displayed >400-fold selectivity. Additional introduction of a carboxylic acid function yielded the completely nonselective receptor antagonist **12** (CXCR1/CXCR2 ratio 1.4). Ensuing SAR studies revealed that the 2′-bromine appeared to be optimal for CXCR1, however, larger substituents at this position resulted in a sharp drop in affinity for CXCR1 but not CXCR2. Thus, while the binding regions seem to be relatively similar in structure, CXCR1 is relatively restrictive whereas CXCR2 can accommodate a wider range of pharmacophores. Interestingly, cyclization of the guanidine core, as exemplified by compound **13**, gave again very selective CXCR2 antagonists (CXCR1/CXCR2 ratio >100, [^{125}I]-CXCL8 IC_{50} 65 nM, Ca^{2+} IC_{50} 0.4 μM) [107]. The left-hand side (LHS) and RHS SAR of this series closely mirrored that of the diarylurea series. However, it should be noted that the cyclic sulfonamide is now attached to the aryl moiety in what is referred to as the 6-position in the urea series. Previously, it was shown that this position is highly intolerant of substitution [98], however, this may be due to the 6-substituent perturbing the coplanarity of the aryl ring with the urea moiety. In **13**, the sulfonamide actually enforces this coplanarity. Preliminary rat PK studies with nitro analog **14** revealed a moderate clearance (*Cl* 23 ml min^{-1} kg^{-1}) but low oral bioavailability (*F* 5%).



13: R1 = Cl, R2 = Br
14: R1 = NO_2, R2 = Cl



15: R = H
16: R = $CONMe_2$

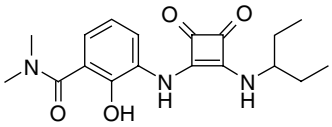


17

Researchers at Schering–Plough postulated that a squaramide would be a good mimic of the central urea moiety. Indeed, they found that compound **15**, which is the squaramide analog of **1**, potently inhibited [^{125}I]-CXCL8 binding to CXCR2 with an IC_{50} of 36 nM [108]. Subsequent optimization of the LHS afforded dimethylamide **16** ([^{125}I]-CXCL8 IC_{50} 2 nM). SAR studies again underlined the importance of the OH but also of both NH functions as critical structural determinants of potency. McCleland and coworkers, however, found that diarylsquaramides were less sensitive to the ionization state of the phenol than the corresponding ureas [109]. In addition, the SAR of the RHS phenyl ring was quite different. Whereas introduction of a 2′-bromo substituent in the urea series led to a substantial increase in receptor affinity (cf. **1** versus **2**), the effect in the squaramide series was negligible. However, like in the urea series, substitution of the 3-position with a sulfonamide, for example, entry **17** ([^{125}I]-CXCL8 IC_{50} 10 nM), improved the PK characteristics substantially. In this respect it is important to mention that the clearance of the squaramide series as a whole was lower than that of the urea series. The observed differences in SAR (and PK) are perhaps not surprising when taken into account that the dioxocyclobutene core of the squaramides pushes the anilino moieties approximately 1 Å further apart. Hence, it is reasonable to expect that the two series will experience slightly different binding environments. The fact that both series are able to attain high-binding affinity may be seen as evidence that the CXCR2 binding pocket is quite flexible.

Further research showed that replacement of the RHS aryl moiety of **16** with (cyclo) alkyls provided potent compounds such as **18** ([^{125}I]-CXCL8 IC_{50} 5 nM) [108]. Additional *in vitro* and *in vivo* evaluation of squaramide **18** confirmed it to be a potent CXCR2 antagonist (chemotaxis IC_{50} 190 nM) possessing modest oral bioavailability (AUC 6.4 μM h, dose 10 mg kg^{-1}) [110]. Further optimization focused on improving *in vitro* potency and oral bioavailability via modification of the RHS. Initial substitution of the 2-amino-2-ethylpropyl group with benzylamine led to a substantial reduction in CXCR2 binding affinity. However, α-branching of the benzylamine with an (*R*)-ethyl group, to give **19**, restored binding affinity ([^{125}I]-CXCL8 IC_{50} 6.8 nM). In addition, **19** showed 35-fold selectivity versus CXCR1 and improved oral exposure in rats (AUC 17.4 μM h). Notably, the corresponding (*S*)-enantiomer showed much reduced CXCR2 affinity and very poor rat PK. Subsequent bioisosteric replacement of the RHS phenyl ring with a 5-methyl substituted 2-furyl motif yielded **20** (SCH 527123) with excellent CXCR2 and CXCR1 potencies ([^{125}I]-CXCL8 IC_{50} CXCR2 2.6 nM, CXCR1 36 nM) and vastly improved rat plasma levels after oral administration (AUC 49 μM h). Alternatively, appropriate di-substitution of the RHS phenyl ring of **19** afforded potent compounds, for instance **21**, exhibiting equally good oral PK profiles as **20** [111]. Additional binding experiments using [^{3}H]SCH 527123 confirmed the high CXCR1 affinity of SCH 527123 (K_d 3.9 nM) but also showed that this compound is effectively a CXCR2-selective binder (K_d 0.049 nM) [112]. Consistent with this high affinity, SCH 527123 binds CXCR2 with impressive avidity, dissociating with a $t_{0.5}$ of ~22 h at room temperature. In contrast, binding at CXCR1 was also reversible but dissociation was relatively rapid, with $t_{0.5}$ ~5 min. SCH 527123 inhibited at 3 nM the potency and efficacy of CXCL1-induced chemotaxis of human polymorphonuclear leukocytes (PMN) significantly, with almost complete

inhibition of cell movement with 10 nM compound. *In vivo*, SCH 527123 displayed oral efficacy in a variety of animal models of pulmonary inflammation [30]. Currently, SCH 527123 is being studied in Phase II clinical trials for the potential oral treatment of COPD, asthma and psoriasis [105].



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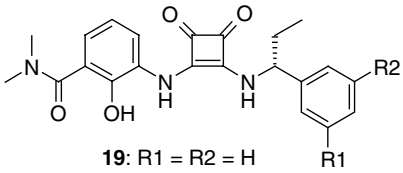
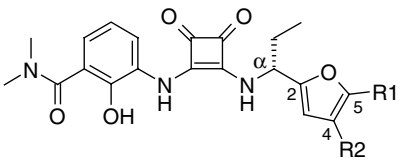


19: R1 = R2 = H

21: R1 = Me, R2 = F



20 (SCH 527123): R1 = Me, R2 = H

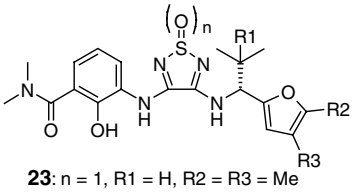
22: R1 = H, R2 = 

Further SAR studies of the squaramide series revealed that moving the furanyl substituent from the 5-position to the 4-position could improve CXCR1 inhibition while maintaining CXCR2 potency as exemplified by 4-isopropylfuranyl derivative **22** ([^{125}I]-CXCL8 CXCR2 K_i 1 nM, CXCR1 K_i 3 nM) [113]. **22** antagonized potently CXCL1- but also CXCL8-stimulated neutrophil migration, IC_{50} 0.5 and 37.0 nM, respectively. In addition, this dual inhibitor displayed excellent animal PK and equally good efficacy in rat and mouse neutrophilic inflammatory models. Very recently, Schering–Plough reported that replacement of the α-ethyl substituent with appropriate small fluoroalkyl groups provided also dual CXCR2-CXCR1 antagonists with similar potencies and good oral bioavailability [114].

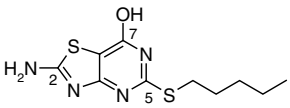
To further explore the role of the furan motif on chemokine receptor binding, functional activity as well as oral exposure in rats, Yu *et al.* found that the furyl series, and in particular 4-halo analogs, possessed superior *in vitro* and *in vivo* properties compared to a broad panel of other heterocyclic ring systems [115]. Interestingly, the SAR obtained in these studies illustrated once more the tolerant nature of CXCR2 versus the more sensitive CXCR1 with respect to structural but also electronic factors.

Another recent advancement has been the replacement of the squaramide by a thiadiazole-1-oxide ring, affording novel potent selective but also dual-acting CXCR2 antagonists like entry **23** ([^{125}I]-CXCL8 CXCR2 K_i 2.7 nM, CXCR1 K_i 50 nM) [116]. **23** showed good functional activity in a CXCL1- and CXCL8-mediated neutrophil MPO release assay, IC_{50} 54 and 13 nM, respectively, but also excellent blood levels in a rat PK screen after oral administration (AUC 119 μM h). In contrast, the closely related thiadiazole-1,1-dioxides, such as **24**, turned out to be functionally inactive despite showing potent CXCR2 binding. In contrast, the nonoxidized thiadiazole derivative

25 afforded nice binding affinities ([^{125}I]-CXCL8 CXCR2 K_i 14 nM, CXCR1 K_i 91 nM) but also functional activity comparable to **23** [117].



23: n = 1, R1 = H, R2 = R3 = Me
24: n = 2, R1 = Me, R2 = R3 = H
25: n = 0, R1 = R2 = Me, R3 = H



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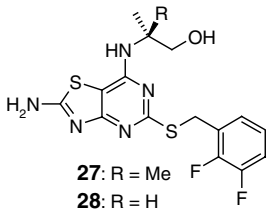
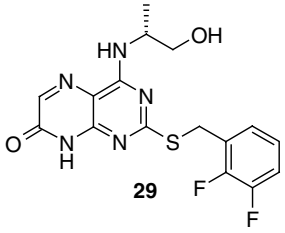


27: R = Me
28: R = H

12.5.3
Thiazolopyrimidines

Researchers at AstraZeneca identified thiazolopyrimidine **26** as one of the hits from a CXCR2 HTS campaign showing micromolar binding affinity ([^{125}I]-CXCL8 IC_{50} 10 μM) and comparable functional efficacy in a calcium mobilization assay (Ca^{2+} IC_{50} 2.0 μM) [118]. Hit optimization particularly focused on the 7-hydroxyl and lipophilic 5-thiopentyl moieties since both were seen as the metabolic hot-spots causing poor microsomal stability. Preliminary results indicated that the hydroxyethylamino and benzylthio groups were suitable substituents for the 7- and 5-positions, respectively. Minor but subtle modification of these groups led to the discovery of lead compound **27**, which showed high *in vitro* potencies ([^{125}I]-CXCL8 IC_{50} 14 nM, Ca^{2+} IC_{50} 40 nM) but only moderate oral bioavailability in rat (*F* 15%). Subsequent potency-driven optimization furnished chiral thiazolopyrimidine **28** with 10-fold improved CXCR2 affinity ([^{125}I]-CXCL8 IC_{50} 4 nM) [119]. Unfortunately, oral bioavailability in rat was again rather low (*F* 9%). A likely explanation for the observed bioavailabilities is poor absorption, which may be caused by the lipophilic nature of **28** and **27** and consequently a low aqueous solubility, 0.5 and 1.5 μg ml^{-1}, respectively. However, while less lipophilic compounds appear to be more attractive in this respect, it was found that compounds with lower lipophilicity showed a significant trend towards reduced antagonism in the cellular calcium mobilization assay [120].

In order to improve the oral biovailability of the series, a range of bicyclic analogs were prepared in which typically the annulated amino thiazole ring in **28** was replaced with alternative heterocycles [121]. This approach afforded several new scaffolds that indeed showed improved bioavailability while retaining high CXCR2 affinity (e.g., pteridinone **29**; [^{125}I]-CXCL8 IC_{50} 1 nM, F_{rat} 44%). Thiazolopyrimidine-based antagonists like **30** were found to possess the best combination of potency and PK properties. Compound **30**, which furnished the same binding affinity as **29**, exhibited low metabolic clearance of only 4 ml min^{-1} kg^{-1} and an impressive rat oral bioavailability of 81%. Surprisingly, selectivity profiling of **28** and **30** revealed that both compounds possess correspondingly high CCR2 activity, that is, CCR2 pA_2 6 nM and 16 nM, respectively.



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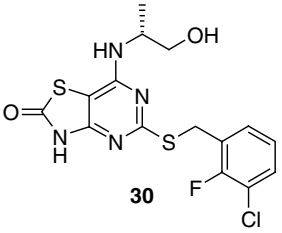
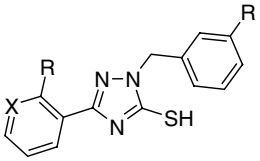


30



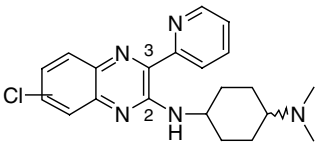
31: X = N, R = H
32: X = C, R = Cl

12.5.4
Triazolethiols

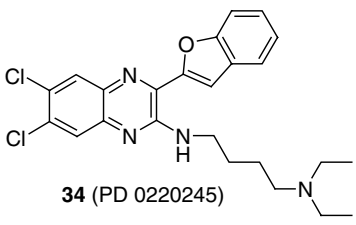
A second CXCR2 HTS hit identified by AstraZeneca was triazolethiol **31** ([^{125}I]-CXCL8 IC_{50} 4.6 μM, Ca^{2+} IC_{50} 2.4 μM) [122]. Optimization of the two phenyl rings afforded the chloro-substituted derivative **32** which displayed double-digit nanomolar *in vitro* potencies ([^{125}I]-CXCL8 IC_{50} 28 nM, Ca^{2+} IC_{50} 48 nM) but also good rat oral bioavailability (*F* 61%). Regarding the thiol group, it is tempting to compare this acidic hydrogen donor with the phenol moiety of diarylureas and the corresponding squaramides, however, attempted tuning of the thiol group by replacement with a hydroxyl or (substituted) amino group led to inactive compounds.

12.5.5
2-Amino-3-Heteroaryl Quinazolines

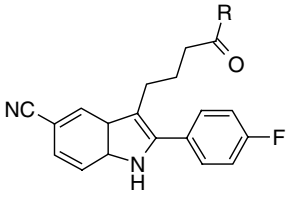
A collaborative research program between Pfizer and Millenium furnished quinazoline derivative **33** as a CXCR2 screening hit ([^{125}I]-CXCL8 IC_{50} 3.0 μM) [123]. This compound is different than all other described CXCR2 ligands in that they are basic and would be positively charged at physiological pH. Optimization of the amino and heterocyclic side-chains provided compound **34** as a fairly modest, dual-acting CXCR1 and CXCR2 antagonist. Activity of **34** in a CXCL8-mediated neutrophil chemotaxis assay nicely corresponded with its receptor affinity as assessed using a neutrophil binding assay (IC_{50} 0.17 and 0.11 μM, respectively). Further investigations revealed that **34** also binds to some other GPCRs including 5HT1a, NK2 and the chemokine receptor CX3CR1.



33 (PD 068238)



34 (PD 0220245)



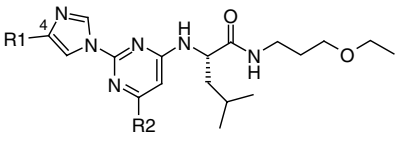
35: R = OH
36: R = $NHSO_2NMe_2$

12.5.6
Indolylbuteric Acid Derivatives

In search of orally bioavailable CXCR2 antagonist, researchers at Johnson & Johnson investigated carboxylic acid bioisosteres of **35**, a compound which reportedly is a submicromolar inhibitor of CXCR2 [124]. Most successful proved to be a series of acylsulfonamides as exemplified by compound **36**. This compound demonstrated high binding affinity ([^{125}I]-CXCL8 IC_{50} 40 nM) and potent inhibition of rabbit neutrophil chemotaxis (IC_{50} 0.7 μM). PK studies in rat showed very low *in vivo* clearance (*Cl* 3.8 ml min^{-1} kg^{-1}) and excellent oral bioavailability (F ~100%). In addition, **36** was active in a rat lung injury model after intraperitoneal dosing.

12.5.7
2-Imidazolyl Pyrimidines

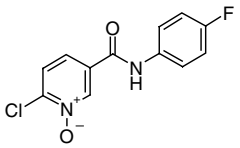
HTS library hit **37** was identified as a high affinity CXCR2 binder ([^{125}I]-CXCL8 K_i 60 nM) by Pharmacopeia and Organon (now Schering–Plough) [125]. Imidazolyl pyrimidine **37** suffered though from poor microsomal stability and consequently high *in vivo* clearance (*Cl* 29 mm min^{-1} kg^{-1}) and lack of oral bioavailability in rat. Substitution of the oxidation prone *n*-octyl thioether by, for example, a *n*-butyl group (**38**) improved *in vitro* stability significantly, however, this stability gain was offset by reduction in potency ([^{125}I]-CXCL8 K_i 1.7 μM). A marked improvement in potency was obtained by substitution of the 4-position of the imidazole ring. Consequently, trifluoromethoxyphenyl derivative **39** was identified showing high CXCR2 affinity ([^{125}I]-CXCL8 K_i 44 nM) combined with improved *in vitro* and *in vivo* stability and oral bioavailability (F_{rat} 22%). Interestingly, binding studies using the labeled urea derivative [^{3}H]SB-265610 (cf. Section 12.5.1) indicated that the imidazolyl pyrimidines and diaryl ureas as well as thiazolopyrimidines bind to distinct binding sites [126].



37: R1 = H, R2 = *S

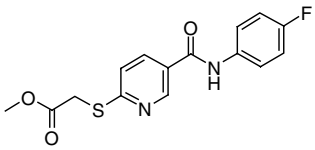
38: R1 = H, R2 = *

39: R1 = * OCF$_3$, R2 = Me



40: R = Cl

41: R = SO_2Me



42

12.5.8
Nicotinamide *N*-Oxides

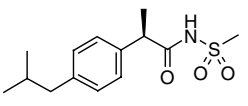
Researchers at Celltech identified a novel series of nicotinamide *N*-oxides as CXCR2 antagonist [127]. For instance, 6-chloro derivatives **40** was shown to inhibit CXCR2

binding ([^{125}I]-CXCL8 IC_{50} 1.0 μM) and was able to moderate CXCL1-driven neutrophil chemotaxis (IC_{50} 1.1 μM). Interestingly, SAR studies indicated that altering the substitution of the anilide moiety led to compounds that blocked neutrophil chemotaxis with similar efficacy as **40** but were inactive in the CXCL8 ligand binding assay. In contrast, replacement of the 6-chloro substituent with sulfonyl groups improved binding affinity while functional efficacy at best stayed the same, as illustrated by **41** ([^{125}I]-CXCL8 IC_{50} 0.13 μM, chemotaxis IC_{50} 2.0 μM). Further SAR studies showed that thioglycolate esters, like **42**, displayed improved inhibition of chemotaxis (**42**: IC_{50} 42 nM) but were, like **40**, unable to block [^{125}I]-CXCL8 binding [128].

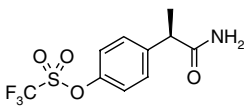
12.5.9
2-Arylpropionic Acid Derivatives

Researchers at Dompé discovered that known nonsteroidal anti-inflammatory drugs that belong to the class of 2-arylpropionic acids, like ibuprofen, potently blocked CXCL8-induced PMN chemotaxis in a COX-independent manner [129]. On the basis of this, a program was initiated to find novel and potent inhibitors of CXCL8-driven human PMN chemotaxis. As a result, acylsulfonamide reparixin **43** was identified as an exceptional inhibitor of human PMN migration induced by CXCL8 (IC_{50} 1 nM) [130]. CXCR2-mediated chemotaxis to CXCL1 was only inhibited at very high concentrations of **43** (IC_{50} 400 nM). Surprisingly, like the thioglycolate esters identified by Celltech (see above), **43** did not compete with the binding of [^{125}I]-CXCL8 despite displaying high functional activity. Additional biochemical and molecular modeling studies suggest that reparixin interacts with CXCR1 in a noncompetitive allosteric manner. Animal studies showed that **43** was able to protect organs against reperfusion injury and attenuate acute lung injury [131]. Currently, reparixin is under Phase II clinical investigation for the potential intravenous prevention of ischemia reperfusion injury in organ transplantation [105].

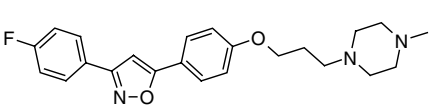
In a more recent publication, Dompé discussed their efforts to identify follow-up compounds that produce more favorable PK characteristics than reparixin [132]. One such compound is trifluoromethanesulfonate **44**, which inhibited both CXCL8- and CXCL1-induced PMN chemotaxis with equal potencies (IC_{50} 8.4 and 26 nM, respectively) [133] and in addition gave excellent rat PK properties (*Cl* 4.1 ml min^{-1} kg^{-1}, *F* 72%) [134]. Extensive *in vivo* profiling showed **44** to be orally efficacious in a range of inflammatory disease models such as the rat adjuvant-induced arthritis model.



43 (reparixin; DF-1681)



44 (DF-2162)



45

12.5.10
Diaryl Isoxazoles

Weidner-Wells and coworkers at Johnson & Johnson reported a series of diaryl isoxazoles [135]. Compounds were profiled in a neutrophil binding assay and a functional assay measuring the CXCL8-induced elastase release from neutrophils. One of the most potent compounds described is isoxazole **45** showing comparable single-digit micromolar activity in both assays. **45** was characterized in a rat adjuvant arthritis model where it was found to reduce swelling up to 30% when dosed orally at 10 mg kg^{-1} day^{-1}. On the basis of the reported data it is not known whether these compounds antagonize CXCR1, CXCR2 or both.

12.6
Challenges and Future Perspectives

The currently known LMW CXCR2 antagonists present a wide range of chemotypes displaying different structural elements. Apparently, these CXCR2 ligands are very distinct from the more typical chemokine receptor antagonist 2-D pharmacophore as postulated in Figure 12.1 [136]. In contrast, CXCR2 antagonists, like other chemokine receptor antagonists, have demonstrated differences in species crossreactivity [137]. These species differences, that are not uncommon for GPCRs, may likely be a result of the antagonists binding to allosteric sites, which indeed has been shown for a number of CXCR2 ligands [118, 124]. Allosteric binding sites need not have evolved to accommodate an endogenous ligand and are thus more likely to show sequence divergence between subtypes and between species. To add to this, species difference have also been observed in the expression of the chemokine receptors and their natural ligands. For instance, mice do not express CXCL8 while humans appear to have a more diverse array of CXCR2 ligands and, while the existence of a homolog of CXCR2 in mouse is well established, the existence of a CXCR1 homolog remains rather controversial. Indeed, Moepps and coworkers described a mouse CXCR1-like receptor, however, unlike mCXCR2, this receptor could not be activated by a large panel of known CXC chemokines of human or mouse origin [138]. Further complexity is introduced by a recent report claiming that CXCR1-CXCR2 heterodimers are likely to form in cells coexpressing these two chemokine receptors [139]. Provided that CXCR1-CXCR2 heterodimers have physiological and pathophysiolog-

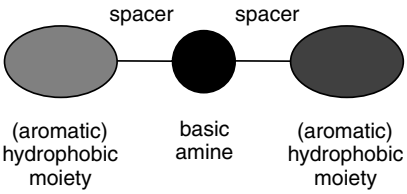


Figure 12.1 Prototypical chemokine antagonist 2-D pharmacophore.

ical consequences as reported for several examples of GPCR heterodimerization [140–142], studies in mice will, however, likely be of limited value because of the CXCR1 controversy. These species differences may hamper a straightforward translation of preclinical findings obtained in animal models into the human system. Therefore, additional approaches may be foreseen to ascertain preclinically defined therapeutic potentials or safety concerns with CXCR2-based therapeutic interventions before entering clinical studies. To partly circumvent this issue, a model of human CXCR2 knock-in mice has been developed [143] and used to better understand the role of human CXCR2 in the development of atherosclerosis [144]. Also, this animal model – given the crossreactivity of mouse ligands with human CXCR2 – would allow for testing novel pharmaceuticals designed to specifically antagonize human CXCR2.

A considerable number of small molecule CXCR2 ligands have now been identified that show nanomolar antagonistic potencies *in vitro* as well as convincing efficacy in a range of animal disease models. Three of these compounds are presently being evaluated in clinical trials for COPD (SCH 527123, SB-656933), cystic fibrosis (SB-656933) and reperfusion injury (reparixin) [105]. In the coming years, results of these trials will surely emerge and will, to a large extent, determine whether the therapeutic promise of CXCR2 blocking strategies with LMW antagonists can eventually be realized.

Acknowledgments

We would like to thank our SPRI colleagues Wim Dokter and Daniel Lundell for their critical review of this chapter.

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13
Therapeutic Targeting of the CXCR3 Receptor

Maikel Wijtmans, Iwan J. P. de Esch, and Rob Leurs

13.1
The CXCR3 Receptor

A search for T lymphocyte-specific chemokine receptors led to the discovery of the CXCR3 receptor in 1996. A novel cDNA was isolated from a human $CD4^+$ T cell library and the encoded GPCR proved to have affinity for chemokines [1]. Further work revealed that CXCR3 consists of 368 amino acids which assemble in a typical seven-transmembrane α-helical architecture. Chapter 2 has described in detail the structural characteristics of the chemokine receptor family and CXCR3 fits this general picture. CXCR3 exhibits typical structural motifs for GPCRs, such as the conserved DRY motif, the $NPxxYx_{5,6}F$ motif and cysteine residues in the first and second extracellular loops [2]. Also, like most chemokine receptors, CXCR3 has additional cysteine residues in the N-terminus and third extracellular loop. The threonine and serine residues in the intracellular C-terminal tail are potential sites for phosphorylation by receptor- or second messenger-regulated kinases [1, 3].

CXCR3 receptors are found on activated Th1 lymphocytes, blood T cells and on a small proportion of B cells and natural killer cells [1, 4, 5]. CXCR3 signaling occurs preferably through pertussis toxin-sensitive $G\alpha_i$ proteins and involves several downstream processes such as mediation of chemotaxis, induction of calcium flux and activation of kinases such as p44/p42 MAPK and Akt [1, 3, 6]. The endogenous agonists for CXCR3 are CXC chemokines CXCL9, CXCL10 and CXCL11, which were traditionally called Mig, IP-10 and I-TAC/IP-9, respectively [1, 7–10]. Of these, CXCL11 has the highest potency and efficacy on CXCR3 [1, 7–11]. Several reports exist on the interaction of other CXCL chemokines with CXCR3, most notably CXCL13 [12] and CXCL4 [13]. Generally, a chemokine is considered to bind to the N-terminus and extracellular loops of a chemokine receptor. Activation is thought to occur through interaction of the N-terminus of the chemokine with the transmembrane domains of the receptor [14, 15]. Indeed, manipulations at the N-terminal amino acids of CXCL10 or CXCL11 can change it from a CXCR3 agonist into a CXCR3 antagonist [16–19].

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

13.2
CXCR3 as a Potential Drug Target

Key roles for CXCR3 in disease models are emerging [20]. More specifically, based on the upregulated expression of CXCR3 and/or its ligands, CXCR3 has been implicated in a variety of diseases including multiple sclerosis [21], rheumatoid arthritis [4], atherosclerosis [22], rejection of transplanted organs [23, 24], inflammatory bowel disease [25], inflammatory skin diseases [9, 26] (e.g., psoriasis [27]) and sarcoidosis [28]. Notably, expression studies have also indicated a role of CXCR3 in various infectious diseases such as SARS [29, 30], 5HN1 bird flu [31] and malaria [32, 33]. Last, CXCR3 is suggested to play an important role in metastasis of melanoma and colon cancer cells to the lymph nodes and in metastasis of breast cancer cells to the lung [34–36].

Several preclinical approaches have been used to evaluate the therapeutic potential of the CXCR3 system: (i) the generation of CXCR3 knockout (KO) mice, (ii) targeting CXCR3 or its endogenous ligands by antibodies, (iii) inhibiting CXCR3 by means of protein-based antagonists and (iv) targeting CXCR3 by small molecule-antagonists. Approaches (i) and (ii) are briefly highlighted in Sections 13.2.1 and 13.2.2 and serve to provide a framework for the various therapeutic indications where CXCR3 is of most interest. Approaches (iii) and especially (iv) are at the heart of this chapter. Section 13.3 discusses the development of antagonists, whereas Section 13.4 discusses antagonists in the context of the mentioned therapeutic framework.

13.2.1
Use of CXCR3-KO Mice

CXCR3-KO ($CXCR3^{-/-}$) mice appear phenotypically normal in the unchallenged host, although deficiencies in NK cells in the lung and peripheral blood as well as a reduction of NK and NK T cells in the liver have been reported [23, 37–39]. Interestingly, $CXCR3^{-/-}$ mice showed delayed acute or chronic rejection of cardiac allografts [23] or pancreatic island allografts [40]. In some cases, allografts are even maintained chronically in $CXCR3^{-/-}$ mice [23]. However, this promising role of CXCR3 has recently been challenged through several $CXCR3^{-/-}$ models revealing that CXCR3 does not play an essential role in cardiac allograft rejection [41, 42]. Findings with CXCR3 antibodies and small molecules have further fueled this controversy (vide intra).

The use of $CXCR3^{-/-}$ mice has also revealed other interesting effects. For example, CXCR3 plays a role in skin wound healing [37], in the positioning of effector T cells at sites of viral inflammation in the brain [38] and in limiting lung fibrosis following lung injury [39]. Use of $ApoE^{-/-}/CXCR3^{-/-}$ mice reveals that deletion of CXCR3 reduces the early steps of atherogenesis but does not influence advanced lesion formation [43]. $CXCR3^{-/-}$ mice exhibit exaggerated severity in a model for multiple sclerosis [44] and are more susceptible to the deadly effects of West Nile virus [45]. Intriguingly, CXCR3 seems to exert a Janus-faced role in prion infections, prolonging survival times in mice but possibly inducing increased

accumulation of misfolded prion proteins [46]. Interestingly, three recent papers have shown that CXCR3-KO mice are protected against the development of cerebral malaria (CM) [32, 33, 47]. In all three instances, this protection is associated with reduced migration of T cells, such as $CD8^+$ T cells, to the infected brain.

13.2.2
Targeting of CXCR3 or its Endogenous Ligands by Antibodies

CXCL10-antibodies reduce chronic experimental colitis by blocking cellular trafficking and protecting intestinal epithelial cells [48]. A Phase II clinical trial is being conducted with a CXCL10-antibody (MDX1100) in treatment of ulcerative colitis, which in itself is an interesting example of a chemokine-binding protein as therapeutic (see also Chapter 16) [49]. In addition, the use of a CXCR3- or CXCL10-antibody was reported to significantly prolong allograft survival [23, 40, 50–53]. However, also here the controversial role of CXCR3 in allograft rejection reveals itself by the recent finding that a CXCR3-antibody had only moderate effect on graft survival [42]. In a mouse arthritis model, a CXCR3-antibody reduced T cell recruitment to inflamed arthritic joints, prevented weight loss and decreased the general severity of arthritis [54]. Notably, the CXCL10 antibody MDX1100 (vide supra) will also be investigated in a Phase II trial for rheumatoid arthritis [49]. Last, a CXCL10-antibody suppressed metastasis of melanoma cells to the lymph nodes in mice [34].

13.3
The Development of CXCR3 Antagonists

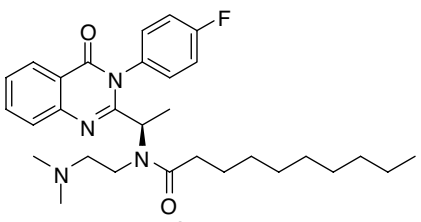
Synthetic CXCR3 ligands are expected to shed detailed light on the physiological roles of CXCR3 and may provide entry into therapeutic intervention. Virtually all development efforts so far have focused on small, low molecular weight compounds [20, 55]. Except for two publications on the same type of small CXCR3 agonists [56, 57], all disclosures deal with antagonists as current wisdom holds that CXCR3 blockage, rather than activation, holds therapeutic promise.

Section 13.3 discusses all known classes of CXCR3 antagonists, with a focus on medicinal chemistry and pharmacokinetic data. Reports from the public domain are discussed first (Section 13.3.1), followed by a concise overview of classes that have only been disclosed in patents (Section 13.3.2). For completeness, Section 13.3.3 shows a handful of protein-based CXCR3 antagonists. In all cases, affinity or activity data are accompanied by the reference chemokine and/or the type of assay used (if disclosed) and are reported for human CXCR3, unless specified otherwise. Although not discussed specifically here, it is emphasized that many if not all of the small antagonists in Sections 13.3.1 and 13.3.2 are expected to bind allosterically [58].

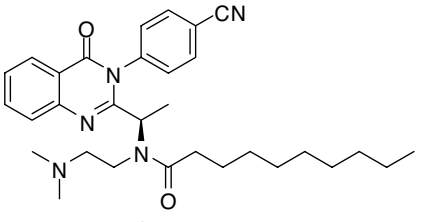
Section 13.4 adds perspective to the experimental results obtained so far with these CXCR3 antagonists in disease models and in clinical trials. As such, it provides a useful overview of how synthetic antagonists have helped unraveling the therapeutic potential of CXCR3.

13.3.1
Publications on Small CXCR3 Antagonists

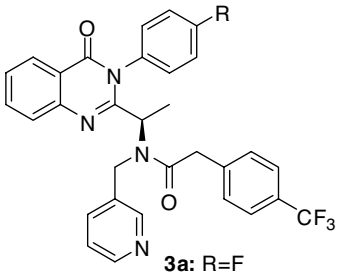
(Aza)quinazolinones. The extensive class of (aza)quinazolinones has been developed by Amgen. The first patent appeared in 2001 [59]. Compound **1** was retrieved as a moderate HTS hit ($IC_{50} = 250$ nM, ^{125}I-CXCL10). Several SAR studies on hit **1** have been reported [60, 61]. Exploration of the anilino-substituent afforded VUF5834 (**2**), which has a threefold increased affinity with respect to **1** and efficiently blocked CXCR3-mediated calcium release [61]. Other strategies for (metabolic) optimization focused on the decanoyl and dimethylamino units, leading to for example, compound **3a** ($IC_{50} = 13$ nM, ^{125}I-CXCL10) [60]. Substituting the 4-F atom by a propargyl or ethoxy group increased affinity of **3a** [60, 62]. This exercise afforded for example, **3b** (AMG1237845) which boosts an IC_{50} of 6 and 7 nM (^{125}I-CXCL10 and ^{125}I-CXCL11, respectively) and displays good functional activity in a cell migration assay against all three chemokines [63]. Eventually, the pharmacokinetically favored 4-ethoxy substituent in conjunction with a CF_3O-substituted phenylacetamide and an additional ring-nitrogen gave azaquinazolinone **4**, dubbed AMG487 ($IC_{50} = 8$ nM, ^{125}I-CXCL10) [60, 62]. The (R)-stereomer of AMG487 has the highest affinity [64]. A more active 4-F,3-CF_3 analog (**5**, NBI-74330) from the same patent [62] was the topic of studies by others ($K_i = 1.5$ nM, ^{125}I-CXCL10) [65, 66]. NBI-74330 inhibits CXCL11 in a [^{35}S]-GTPγS binding assay ($IC_{50} = 10.8$ nM), Ca^{2+} mobilization ($IC_{50} = 7$ nM) and chemotaxis ($IC_{50} = 3.9$ nM) [65]. Several reports have shown that the antagonism of NBI-74330 is noncompetitive, both in human and in mouse CXCR3 [58, 65, 66].



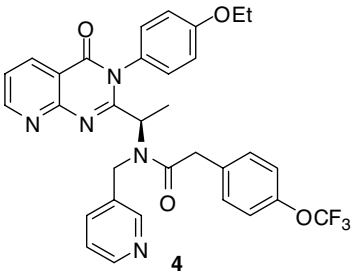
1



2
(VUF5834)

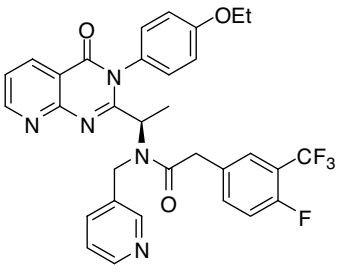


3a: R=F
3b: R=OEt

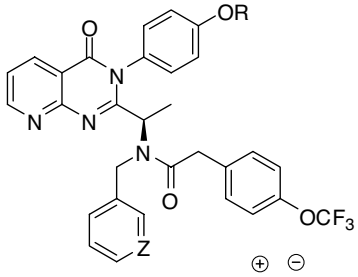


4
(AMG487)

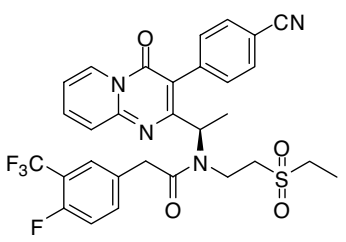
AMG487 also displays noncompetitive antagonism [58]. It potently inhibits CXCR3-mediated cell migration ($IC_{50} = 15$ nM, CXCL11) as well as Ca^{2+} mobilization ($IC_{50} = 5$ nM, CXCL11) [60] and exhibits >1000-fold selectivity over a panel of other receptors [67]. Compared to initial HTS hit **1**, AMG487 displays lower clearance (1.6 and 1.1 l h^{-1} kg^{-1}, 0.5–1.0 mg kg^{-1} i.v. in rats and dogs, respectively) and an improved bioavailability (12–57 and 85%, 2.0–2.5 mg kg^{-1} orally in rats and dogs, respectively) [60]. AMG487 passed various genotoxicity and cardiotoxicity assays [64]. The two main metabolic pathways for AMG487 were identified to be oxidation to N-oxide **6** and de-ethylation to phenol **7** [68]. These metabolic pathways, and in particular compound **7**, are suspected to play a key role in clinical failure of AMG487 (vide intra). Perhaps not surprisingly, the latest series of AMG487 analogs involve changes in the pyridine ring, ethoxy group and azaquinazolinone core [69–73]. N-oxidation can be blocked through replacement of the pyridine ring by a sulfone group, while metabolic de-ethylation is reduced by replacing the ethoxy group with a cyano group [70–72]. Certain substitutions on the 7-position of the azaquinazolinone led to improvement of potency while retaining good *in vivo* PK properties [72]. Replacement of the azaquinazolinone core by other bicyclic heteroaromatics was markedly tolerated and afforded for example, **8** which had similar affinity but reduced clearance (0.24 l h^{-1} kg^{-1}, 0.5 mg kg^{-1} i.v. in rat) compared to AMG487 [71]. However, these pyrido[1,2-a]pyrimidin-4-ones suffer from CYP induction mediated by the pregnane X receptor. This can be counteracted by incorporation of a cyclic sulfone [69]. Another replacement of the azaquinazolinone core is an imidazole group [70]. However, glutathione conjugation to the imidazole ring had to be counteracted by installing electron-withdrawing groups on the 5-position of the imidazole, giving for example, **9** ($IC_{50} = 18$ nM, ^{125}I-CXCL10). Glutathione addition was also a problem with derivatives possessing an imidazole-pyridine core [71, 73]. Here, glutathione addition could be reduced by incorporation of N in the fused ring to give pyrazine-imidazole **10** ($IC_{50} = 0.9$ nM, ^{125}I-CXCL10) which displays a good bioavailability of 40% (5.0 mg kg^{-1} p.o. in mice) [73].



5
(NBI-74330)



6: R=Et, Z=N-O
7: R=H, Z=N



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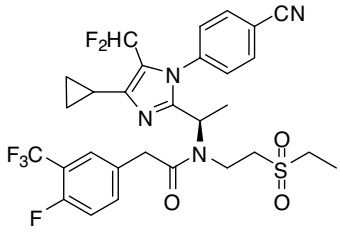
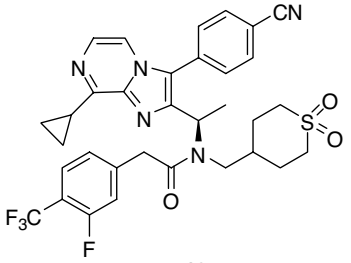
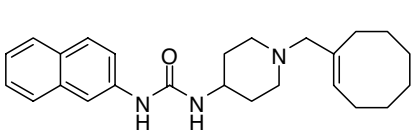


9

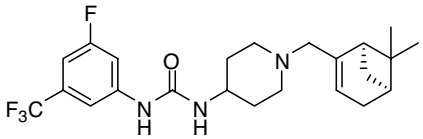


10

1-Aryl-3-piperidin-4-yl-ureas. Researchers from UCB conducted a HTS campaign using a FLIPR-based calcium flux assay, yielding cyclooctenyl hit **11** ($K_i = 110$ nM, [^{35}S]-GTPγS solubility = 0.1 μg ml^{-1}) [74]. A SAR study aiming for a cyclooctenyl-replacement was initiated, affording the naturally occurring (−)-myrtenyl group (as in **12**) as a reasonable substitute. A subsequent SAR study on the aromatic portion yielded several compounds with better affinities and improved physicochemical properties compared to hit **11** (e.g., **12**: $K_i = 16$ nM, solubility 23 μg ml^{-1}). Further optimization of *in vivo* pharmacokinetic properties was achieved by a twofold approach. One approach involved replacement of the urea linker by a hydantoin, imidazolinone, benzazole or arylazole group [75]. Increased microsomal stability and low CYP inhibition were achieved (e.g., **13**: clearance = 2.8 ml min^{-1} kg^{-1}). The second approach focused on the piperidine spacer and concomitantly revisited the (−)-myrtenyl group in **12** [76]. Certain amides, most notably a homotropenyl amide, could replace the (−)-myrtenyl group, with the clear bonus of having improved pharmacokinetic properties. Moreover, bridging of the piperidine spacer by means of an *exo*-tropanyl unit was found to reduce *in vivo* oxidation. Together, these findings resulted in for example, **14**, which has $K_i = 7$ nM ([^{35}S]-GTPγS) and good pharmacokinetic properties (clearance = 7 μl min^{-1} mg^{-1}; solubility = 40 μg ml^{-1}; bioavailability = 70%). In a subsequent effort, the urea group was again investigated [77], resulting in the discovery of quinoline-based antagonists. A noteworthy member of this class is isopropoxy-compound **15** (K_i= 5 nM, [^{35}S]-GTPγS). It exhibited a good halflife ($t_{0.5} = 7.6$ h, 30 mg kg^{-1} p.o in mice) and dose-related inhibition of CXCR3 internalization [66].



11



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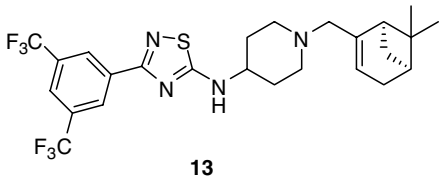
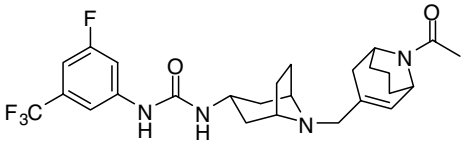
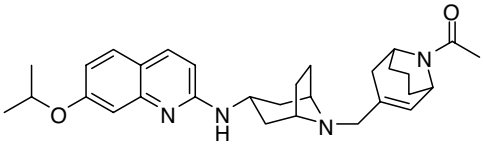


13

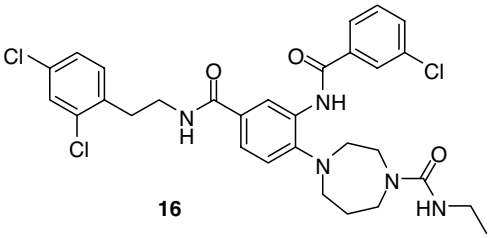


14



15

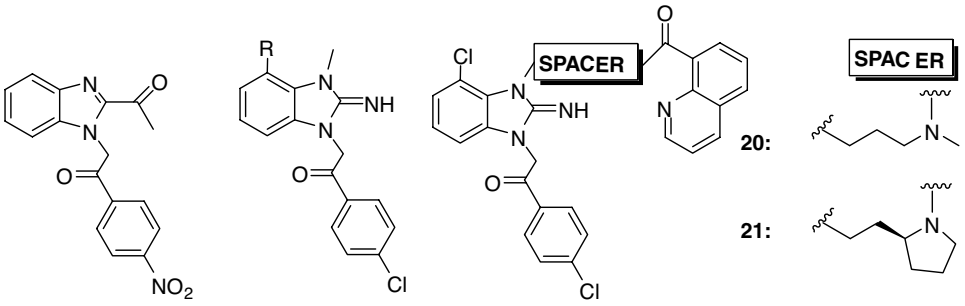
4-N-aryl-[1,4]diazepane-ureas. A screen by Pharmacopeia of over four million compounds did not only lead to the identification of various antagonist scaffolds [78] but, interestingly, also of several small CXCR3 agonists (not discussed here) [56]. One antagonist chemotype consists of 4-N-aryl-[1,4]diazepane-ureas, exemplified by **16** [78]. SAR studies revealed that 3,5-dichloro-substitution was preferred for the phenethyl group and that a 3-chloro or 3-fluoro group was beneficial on the benzamide unit, while the azepane spacer and the urea unit could only be replaced at considerable expense of affinity. Compound **16** exhibited an IC_{50} of 60 nM (CXCL11, Ca^{2+}), inhibited chemotaxis ($IC_{50} \sim 100$ nM, CXCL11) and was selective over 14 other GPCRs. An independent QSAR study on this class of compounds suggests several key descriptors to explain the affinity of the compounds [79].



16

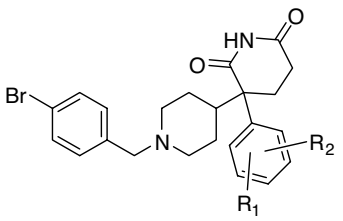
2-Imino-benzimidazoles. Researchers from Abbott Laboratories found **17** as a HTS hit ($IC_{50} = 3\ \mu M/^{125}$I-CXCL10) [80]. Solubility problems led them to replace the 2-acetyl-benzimidazole core by a 3-methyl-2-imino-benzimidazole moiety. Equipment of the

acetophenone moiety with 4-halo substituents and inclusion of small apolar substituents at the C4 position of the benzimidazole core proved beneficial. This resulted in for example, **18** and **19** ($IC_{50} = 100$ and 30 nM, respectively/^{125}I-CXCL10). Both compounds proved potent antagonists (**18**: $IC_{50} = 80$ nM; **19**: $IC_{50} = 70$ nM/CXCL10, Ca^{2+}). Compound **18** exhibited a $t_{0.5}$ of 4.9 h and a bioavailability of 57% (10 mg kg^{-1} oral in mice). Follow-up studies involved the investigation of the N-Me moiety on the benzimidazole [81]. Distal amide groups proved attractive and in conjunction with a quinolynyl group good affinities at both hCXCR3 and muCXCR3 were obtained (**20**). Constraining the linker gave further improved affinities (**21**: $IC_{50} = 8$ and 40 nM at hCXCR3 and muCXCR3, respectively/^{125}I-CXCL10).

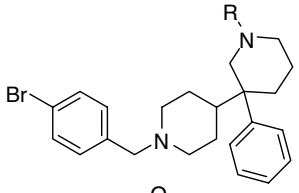


17 **18**: R=Me **19**: R=Et

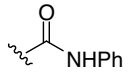
Benzetimides. Researchers from Janssen discovered benzetimide **22** as a HTS-hit ($IC_{50} = 0.78$ μM, [^{35}S]-GTPγS) [82]. SAR studies around the 4-bromophenyl group revealed no superior substituent, while the same exercise on the other phenyl group afforded 2,4-di-F substitution as a beneficial manipulation (**23**: $IC_{50} = 0.12$ μM). Removal of the two carbonyl groups from the glutarimide group in **22** abolished affinity, but other nearby carbonyl-containing groups could restore and, in fact, improve potency as illustrated by urea **24** ($IC_{50} = 60$ nM) and amide **25** ($IC_{50} =$ 80 nM). For benzetimides **22** and **23**, both enantiomers were comparably active on hCXCR3. However, for nonbenzetimides **24** and **25**, discrimination of the enantiomers by hCXCR3 emerged [**(−)24** and **(+)24**: $IC_{50} = 0.03$ and 6.31 μM, respectively].



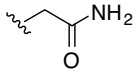
22: R_1=R_2=H
23: R_1=2-F, R_2=4-F



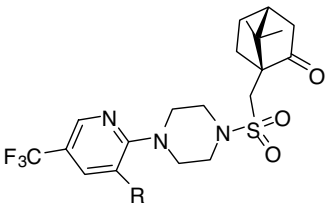
24: R=



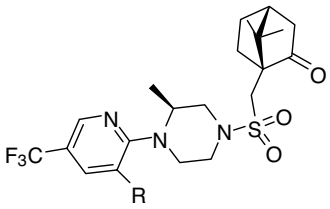
25: R=



Camphor-sulfonamides. GlaxoSmithKline researchers disclosed (S)-camphor-containing antagonists based on the HTS hit **26** ($IC_{50} = 251$ nM/CXCL10, Ca^{2+}) [83]. Neither pyridine replacement nor relocation or replacement of the CF_3 group led to improvement in hCXC3 affinity. However, with the 5-CF_3-pyridine nucleus retained, additional substitution was tolerated and afforded for example, **27** ($IC_{50} = 159$ nM). The same 0.2 log unit increase was observed for Me substitution on the spacer [**(S)-28**]. Suitable replacements for the camphor-carbonyl were identified, including alcohol and dioxalane groups. Compound **29** ($IC_{50} = 79$ nM) was selective in a panel of 50 protein targets including CXCR1, CXCR2 and CXCR4, and was devoid of hERG liability. *In vivo* PK assays on **29** in rat (2.0 mg kg^{-1}, p.o) revealed a high clearance of 108 ml min^{-1} kg^{-1}, a halflife of 0.5 h and a bioavailability of 8% [83].



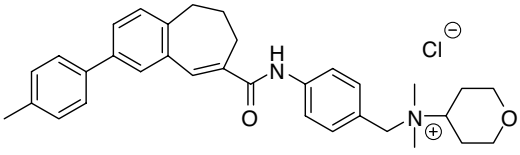
26: R=H
27: R=F



28: R=H
29: R=F

Novartis Antagonists. Novartis has reported *in vitro* and *in vivo* properties of two selective CXCR3 antagonists coded NIBR2130 and NIBR1748 [41, 84]. Their structures, which are reported to be distinct [84], are undisclosed but the structure of NIBR2130 is the topic of a manuscript in preparation [41]. NIBR2130 boosts excellent affinity for hCXCR3 ($IC_{50} = 2.2$ nM on CXCL11) and blocks Ca^{2+} mobilization by all three endogenous chemokines ($IC_{50} = 1.4$, 2.4 and 1.4 nM for CXCL9, CXCL10 and CXCL11, respectively) [41]. No or negligible species difference is observed in terms of affinity ($IC_{50} = 2.2$ nM on mCXCR3/CXCL11) and activity ($IC_{50} = 2.4$ nM on mCXCR3/Ca^{2+}, CXCL11). Similar results were obtained for compound NIBR1748. It exhibits good affinities on human and mouse CXCR3 ($IC_{50} = 2.6$ and 6.0 nM on hCXCR3 and mCXCR3/CXCL11) and blocks Ca^{2+} mobilization ($IC_{50} = 1.4$ nM/CXCL10) [84].

TAK-779. CCR5-ligand TAK-779 (**30**) has to some extent functioned as a CXCR3-tool as it proved to have moderate affinity for mouse CXCR3 ($IC_{50} = 369$ nM, ^{125}I-CXCL10) [85]. However, its poor CXCR3/CCR5 selectivity profile has left TAK-779 only of limited value.

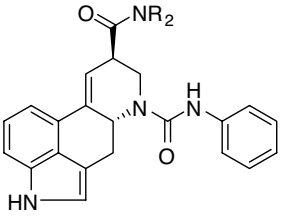


30
(TAK-779)

Antagonists from Natural Sources. Merck performed a screen (^{125}I-CXCL10) on a library consisting of extracts from microbial, plant and marine sources [86]. A diverse series of hits was found, including sugar-derivatized steroids and dipyridinium salts.

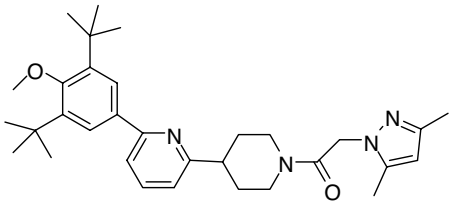
13.3.2 Patents on Small CXCR3 Antagonists

Ergolines. Novartis has patented antagonists with structures resembling LSD (i.e., ergolines) [87]. Exemplary compounds **31** and **32** both exert good binding ($IC_{50} = 54$ and 23 nM, respectively, ^{125}I-CXCL11). Compound **31** blocks Ca^{2+} mobilization ($IC_{50} = 18$ nM, CXCL11) as well as chemotaxis ($IC_{50} = 74$ nM, CXCL11).



31: NR_2=NEt_2
32: NR_2=morpholino

Piperidinyl-Amides. Merck has described a compound class broadly centered around a piperidinylamide linked to a heteroaromatic spacer [88–90]. Affinities (^{125}I-CXCL10) and functional activities (chemotaxis, CXCL10) are reported to be as low as 1 nM. Arbitrarily selected members are pyridine **33** [90], thiazole **34** [89] and thiazole **35** [88]. One publicly described member of the thiazole class is coded MRL-957 with, however, undisclosed structure. In peripheral blood mononuclear cells expressing hCXCR3, MRL-957 efficiently blocks chemotaxis by CXCL10 ($IC_{50} = 6.9$ nM) and CXCL11 ($IC_{50} = 57.4$ nM) [42].



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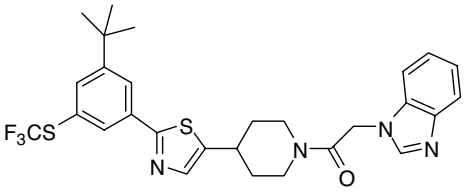

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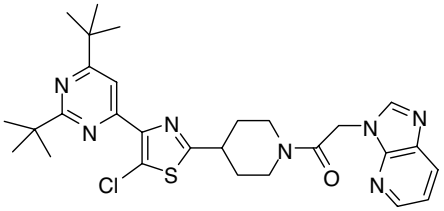
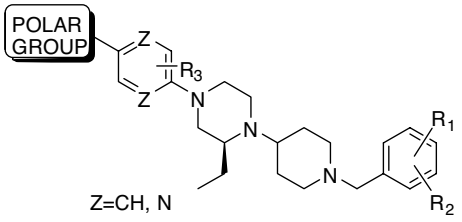


35

Piperazinyl-Piperidines. Schering–Plough has filed many patents describing a piperazinyl-piperidine scaffold, a very general structure of which is shown as **36**. It is of interest to point out that a number of compounds within this class has subnanomolar affinities and as such this class appears to be one of the most active CXCR3 series known. For example, **37–39** all have $K_i = 0.2$ nM (^{125}I-CXCL10) [91–93].



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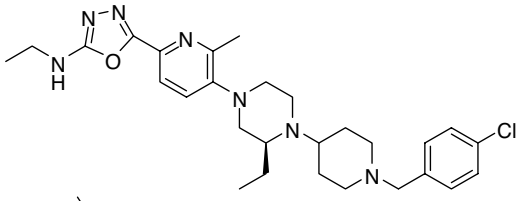

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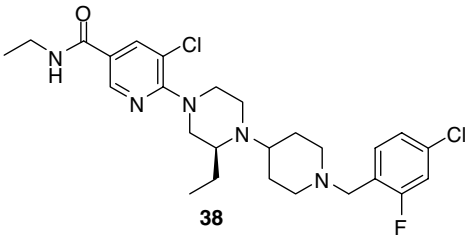

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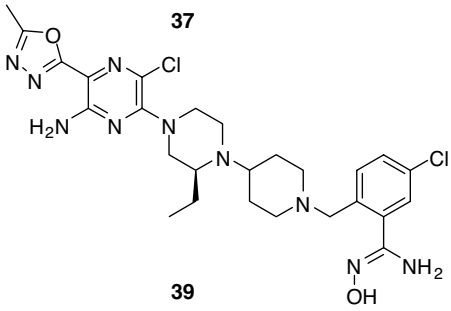
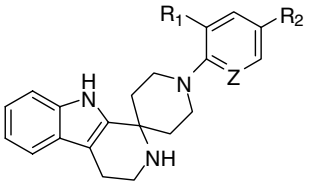
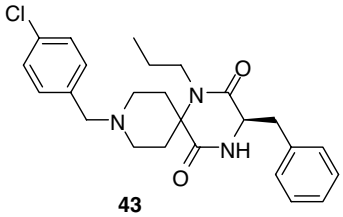


39

Spiro-Compounds. Antagonists having a spiro-bipiperidine were reported by Amgen [94]. The compounds were decorated with a fused indole and halogenated aromatic rings. Members **40–42** all had IC_{50} values <500 nM as reported in the patent [95]. Ono Pharmaceuticals has disclosed spiro-compounds such as **43** ($IC_{50} = 140$ nM/Ca^{2+}, CXCL10) [96].

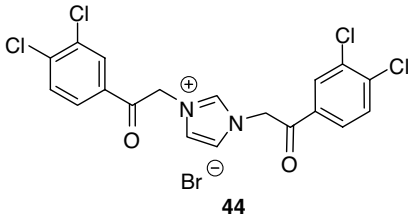


40: Z=C-NO_2, R_1=Cl, R_2=CF_3
41: Z=CH, R_1=Cl, R_2=CF_3
42: Z=N, R_1=Cl, R_2=CF_3



43

Imidazolium Salts. SmithKlineBeecham has patented permanently charged imidazolium salts as antagonists [97]. In independent studies, one member of this class (**44**) was investigated in detail [58]. It displays a K_i value of 251 nM (^{125}I-CXCL10) and exhibits noncompetitive antagonistic behavior on CXCR3.



44

13.3.3
Protein-Based CXCR3 Antagonists

Some research has focused on protein-based antagonists rather than on small-molecule antagonists. Various N-terminally truncated CXCL11 analogs exhibit antagonistic activity. For example, analogs called I-TAC (4–73) (**45**) and I-TAC (5–73) (**46**) lack agonistic activity and display K_d values of 8.5 and 135 nM, respectively (^{125}I-CXCL11) [16]. Mouse CXCL10 analogs truncated by five or more amino acid residues from the N-terminal fail to exhibit functional activity. Of these analogs, the most potent antagonist ($K_d = 24$ nM) is a mutant (IP-10-AT, **47**) with methionine added after removal of the five N-terminal amino acids [17]. A patented mutant called CXCL11-3B3 (**48**) contains three alanine substitutions eliminating a basic cluster surrounding residue 60 of CXCL11 wild type (Lys57, Lys59, Arg62) [18]. This manipulation creates an antagonist although it reduces the affinity for CXCR3 to submicromolar levels. Last, a series of truncation mutants of CXCL11 afforded antagonist CXCL11(4–79) (**49**), which strongly inhibited the migration of activated mouse T cells in response to CXCL9–CXCL11 [19].

13.4
CXCR3 Antagonists in Disease Models and in the Clinic

As explained earlier, the combination of expression studies, antibody experiments and KO models has provided indications for therapeutic applications of CXCR3 blockage. The development of CXCR3 antagonists promised to deliver the tools to interrogate these indications in a complementary fashion. The results described in Section 13.3 underscore the maturation of this field as several different structural classes of antagonist have now been developed. In the current section, the effect of various CXCR3 antagonists in relevant disease models and in the clinic are discussed. A distinction between the different therapeutic indices is used.

Recruitment of Inflammatory Cells. Azaquinazolinone AMG487 (**4**) inhibits inflammatory cell migration in a mouse model of bleomycin-induced cellular recruitment. Here, AMG487 significantly reduces infiltration of macrophages and lymphocytes into the lungs with infiltration levels being as low as in CXCR3-KO mice (3 mg kg^{-1} s.c.) [64]. In the same model, analog **10** proved very efficacious with

blockage of leukocyte migration occurring at doses as low as 1.0 mg ml^{-1} [73]. In hCXCR3 knock-in mice, MRL-957 (vide supra) efficiently blocks chemotaxis of splenocytes ($IC_{50} = 9$ and 20 nM against CXCL10 and CXCL11, respectively) [42].

Skin Inflammation. Interaction of CXCR3 with its endogenous ligands is known to play a crucial role in skin inflammation [9, 26]. Accordingly, CXCL11-based antagonist **48** (for definition see Section 13.3.3) reduces swelling of mouse skin in response to the sensitizer 2,4-dinitrofluorobenzene [18]. Likewise, small CXCR3 antagonists prove useful in skin inflammations and the field of the skin inflammatory disease psoriasis has in fact seen the biggest clinical advance of a CXCR3 antagonist (AMG487) known to date. In 2003, results of a Phase I trial on AMG487 were disclosed. The compound was assessed for safety and pharmacokinetics in 30 healthy males in a randomized, double blind, placebo-controlled dose-escalation study. Generally, the compound was well tolerated and adverse events were mild to moderate (25–1100 mg doses) [98]. In a subsequent Phase IIa trial, patients suffering from moderate to severe psoriasis received 50 or 200 mg of AMG487 or placebo orally once daily for 28 days. However, no significant differences in psoriasis severity index or physician global assessment scores were seen between patient groups [99]. It was speculated that this lack of clinical efficacy may have resulted from high variability in drug exposure [99]. Recently disclosed follow-up studies suggest a probable cause for this variability [68, 100]. Metabolism of AMG487 to **6** and **7** depends on cytochromes CYP3A4 and 3A5. Likely, mechanism-based inhibition of CYP3A by metabolite **7** leads to reduced CYP3A metabolism, in turn affording reduced intrinsic clearance of AMG487 [68]. It is evident, though, that such metabolism-based clinical problems do not necessarily disqualify skin inflammation as a therapeutic area for CXCR3.

Autoimmune Diseases. CXCR3 antagonists may have the potential to counteract unwanted immune responses in autoimmune diseases. Here, three examples are highlighted: rheumatoid arthritis, multiple sclerosis and Sjögren's syndrome.

Reductions in inflammation, pannus formation and cartilage damage were observed upon administering AMG487 at doses up to 50 mg kg^{-1} subcutaneous (s.c.) in mouse collagen-induced arthritis models [101]. Phase II trials with AMG487 on patients with rheumatoid arthritis were announced in 2004 [102], but no results have been disclosed since and complex AMG487 metabolism may also be a major hurdle here [68]. The clinical relevance of the CXCR3/CXCL10 system in treating rheumatoid arthritis is further underlined by the announcement that a CXCL10 antibody (MDX1100) is investigated in a Phase II trial for this disease [49].

Simultaneous treatment with CXCL11-based antagonist **49** (for definition see Section 13.3.3) and a CXCR4 antagonist blocked experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Additional investigations revealed that **49** inhibited the effector phase of the immune response, while the CXCR4 antagonist inhibited the sensitization phase [19].

Last, administration of CXCL10-based antagonist **47** (for definition see Section 13.3.3) to mice reduces the progression of autoimmune sialadenitis, which relates to the inflammation of the salivary glands as observed in Sjögren's syndrome [17].

Transplant Rejection. For quite some time, CXCR3 was assumed to play a key role in rejection of donor organs through its recruitment of inflammatory cells to the allograft. Two examples have been presented in which CXCR3 antagonists had beneficial effects in transplantation. In a mouse model for idiopathic pneumonia syndrome (IPS), AMG487 reduced recruitment of donor T cells to the lung after allogeneic stem cell transplantation, leading to improved survival rates [103]. Antagonist TLRK-A, likely a member of the (aza)quinazolinone class, in combination with subtherapeutic cyclosporine prevents acute rejection in a rat heart transplant model [104]. It is noted, though, that the cyclosporine co-treatment was essential.

In recent years, reports have emerged that challenge both the efficacy of CXCR3 in allograft rejection as well as the hypothesized underlying mechanism of action. As outlined earlier, part of the evidence involves work on antibodies and CXCR3-KO models. Additional fueling of the debate was delivered by *in vivo* findings with four different classes of CXCR3 antagonists. In a rat heart allograft transplantation model, s.c. treatment with NIBR2130 (vide supra) did not prolong survival (6 days versus 7 days control) despite high blockade (>90%) of CXCR3 *in vivo* [41, 84]. Structurally unrelated NIBR1748 (vide supra) had no effect either in the same model (7 days versus 7 days control), although *in vivo* CXCR3 blockage appears somewhat lower (>75%) [84]. A similar result was found in tests with MRL-957 (vide supra) on cardiac allograft survival [42]. Here, MRL-957 displayed only a very moderate increase in graft survival (10.5 days versus 8.7 days control). However, recent studies on **3b** (AMG1237845) show that treatment with this compound (32 mg kg^{-1} s.c.) gives rise to a significant but modest delay in allograft rejection (9–16 days versus 5–9 days control) [63]. Surprisingly, this was not accompanied by a difference in CD8 or CD4 T cell infiltration to the allograft. The protective effect of AMG1237845 was even larger in combination with a subtherapeutic dose of immunosuppressive antiCD154 antibody.

It is fair to say that the current controversy has led to some tempering of therapeutic hopes for CXCR3 in transplant rejection [105].

Atherosclerosis. CXCR3 antagonist NBI-74330 (**5**) gave rise to reduction in lesion formation in atherosclerosis models by the inhibition of effector cell migration to the plaque and by regulating the local immune response [106].

Infectious Diseases. Recently, AMG487 has been shown to exert several beneficial effects in the pathogenesis of ferrets infected with H5N1 (bird flu). The effects all resulted from blockage of CXCR3–CXCL10 interaction and included reduction of pulmonary viral load, improvement of respiratory function and modest delay in mortality [31].

Cancer. As outlined earlier, metastasis of breast cancer was identified as a possible therapeutic area. This was substantiated by the inhibiting effect of AMG487 on lung metastasis in a mouse model for metastatic breast cancer [35]. In contrast, AMG487 did not change the local tumor growth, indicating a metastasis-specific effect of CXCR3 antagonism.

13.5 Conclusion and Outlook

The majority of this chapter has described the medicinal chemistry efforts on CXCR3 antagonists. It is evident that many different classes of antagonists have emerged over the last years, including a handful of protein-based antagonists. The current diversity among small antagonists is remarkably high. The highest reported affinities are found for the piperazinyl-piperidines from Schering–Plough. In contrast, the two series best described in the scientific literature are the UCB compounds and most notably the Amgen class represented by AMG487 and NBI-74330. Species differences may occur between hCXCR3 and mCXCR3, but this has not prevented successful pharmacodynamic and pharmacokinetic optimalization of various CXCR3 antagonist classes.

The second part of this chapter reveals how these optimized small antagonists as well as some protein-based antagonists have shed light on the therapeutic roles of CXCR3. Encouragingly, CXCR3 antagonists have proven beneficial in preclinical models for skin inflammations, autoimmune diseases, atherosclerosis and cancer. However, questions have risen about the anticipated role of CXCR3 in transplant rejection, since several distinct antagonist classes showed no or little efficacy in rejection models. Moreover, clinical progress with AMG487 has been hampered by unacceptable variability in drug exposure. However, this failure is not a falsification of CXCR3 as a drug target per se.

In all, unambiguous CXCR3 target validation in humans is still a significant challenge. Ongoing medicinal chemistry efforts have shed hopeful as well as critical lights on hypothesized therapeutic areas of CXCR3. In the mean time, new potential CXCR3-related therapeutic areas such as H5N1 (bird flu) and perhaps malaria are emerging.

Acknowledgments

We thank Danny Scholten for useful comments. This chapter was (in part) written within the framework of Top Institute Pharma project number D1-105.

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14
Targeting CCR1

Richard Horuk

14.1
Introduction

Chemokine receptors like CCR1 are all members of the rhodopsin-like subfamily of G protein-coupled receptors (GPCRs) [1, 2]. Although CCR1 was initially described as a receptor for the chemokines CCL3 and CCL5, a number of other chemokine ligands have since been identified, including CCL6, CCL7, CCL9, CCL14, CCL15, CCL16 and CCL23. In addition, an N-terminal truncated form of CCL4 (3–69) generated by proteolytic cleavage of the full-length CCL4 (which has poor affinity for CCR1) has potent CCR1 activity [21].

CCR1 is abundantly expressed on immune cells, including monocytes, memory T cells, basophils, dendritic cells [1–3, 22] and is induced by GM-CSF in neutrophils [23]. CCR1 expression has been observed in airway smooth muscle cells in the lung suggesting a possible role in asthma [24], in both normal neurons [25] and in dystrophic neurons from patients with Alzheimer's dementia [26], in astrocytes [27], which suggests a role in inflammation in the CNS, in endothelial cells which can be induced to chemotax in response to CCL23 and may play a role in angiogenesis [28] and in multiple myeloma cells and osteoclasts [29, 30], hinting at a role for CCR1 in bone cancer.

CCRI plays an important role in host defense and is involved in regulating the chemotaxis of immune cells, a feature it shares in common with all chemokine receptors. Dysregulation of this response leads to autoimmunity and CCR1 has been associated with the pathophysiology of a number of diseases including rheumatoid arthritis [7–9], multiple sclerosis [10–12], transplant rejection [13, 14] and allergic inflammation [15–17].

The expression of CCR1 is regulated by a number of factors including cytokines, lipopolysaccharide, statins, substance P and estrogen among others. Interestingly recent work shows that proinflammatory signals can also deactivate normally functional chemokine receptors and turn them into decoy receptors [31]. Treatment of dendritic cells with LPS + IL-10 showed high expression of CCR1 and other CC chemokine receptors. However, these receptors were unable to elicit migration in

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

Table 14.1 Therapies targeting CCR1 and its ligands that have progressed to human clinical trials (small molecules unless otherwise noted).

Target	Company	Clinical phase	Compound	Indication	Status
CCR1	Schering AG (Berlex)	II	BX 471	MS, psoriasis, endometriosis	No efficacy
CCR1	Millennium	II	MLN 3701	RA	MLN 3897 no efficacy in RA
			MLN 3897	MS	
CCR1	Pfizer	II	CP-481715	RA	No efficacy
CCR1	Chemocentryx	I	CCX354	RA	Ongoing
CCL3	British Biotech	II	BB-10010 (protein)	Cancer	No efficacy

response to their ligands. Similar results were obtained for monocytes exposed to activating signals and IL-10. Thus, in an inflammatory environment, IL-10 generates functional decoy receptors on DC and monocytes, which act as molecular sinks and scavengers for inflammatory chemokines.

CCR1-deficient mice generated by targeted gene disruption have revealed that CCR1 has a number of nonredundant functions in host defense and inflammation [5, 6, 32–36]. In an animal model of inflammation CCR1 knockout mice were protected from pulmonary inflammation secondary to acute pancreatitis [6]. The protection from lung injury was associated with decreased levels of cytokines such as TNF-alpha, suggesting that the activation of the CCR1 receptor is an early event in the systemic inflammatory response syndrome. CCR1 also appears to be involved in remodeling after myocardial infarction [35], suggesting a role for this receptor in cardiac disease. A role for CCR1 in renal ischemia–reperfusion injury was recently provided by gene deletion studies that revealed a reduction in macrophage and neutrophil infiltrates into the kidneys of CCR1 deleted mice compared to wildtype mice in a 7-day model of disease [36]. Although CCR1 knockouts hinted at a role for CCR1 in diseases like multiple sclerosis [12], the fact that there is not a total abrogation of the disease in CCR1-deficient animals suggests that there is some redundancy in the system and this might partly account for the failure of a number of CCR1 antagonists in clinical trials [20] (Table 14.1) and see below for a further discussion.

14.2 CCR1 as a Drug Target

Based on its potential involvement in the pathogenesis of a number of diseases, discussed above, there has been considerable interest in CCR1 as a drug target. Although this has been mainly confined to small molecule CCR1 antagonists (see below) CCR1 ligands as potential therapeutics have also garnered interest. For example CCL3, which is a CC chemokine ligand for CCR1 and CCR5, has been

shown to act as a hematopoietic stem cell proliferation inhibitor [37]. It enhances stem cell recovery after treatment with cytotoxic agents, based not only on its cytoprotectant ability, but also because of its effects on stem cell self-renewal [38]. Since increased hematopoietic toxicity is a common feature of most chemotherapies for the treatment of cancer then these potential hematopoietic sparing properties of CCL3 seemed attractive as a therapeutic approach. BB-10010 is an active, nonaggregating variant of human CCL3, initially developed by scientists at British Biotech [39], and shown to be effective in an animal model by reducing the degree of accumulated hematopoietic damage after repeated sublethal irradiations [40]. Based on these positive animal data BB-10010 entered clinical trials and was shown to be extremely well tolerated administered either subcutaneously or intravenously in phase I studies [41]. Unfortunately in phase II clinical trials in patients with advanced breast cancer, receiving 5-fluorouracil, adriamycin and cyclophosphamide chemotherapy, BB-10010 failed to demonstrate any additional myelosuppression and was not developed any further.

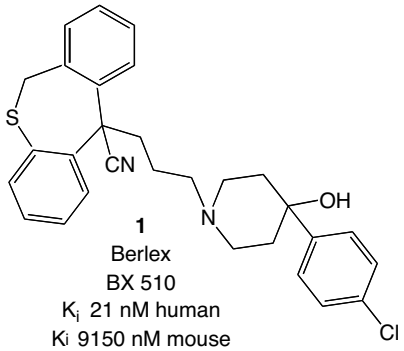
Millennium disclosed a neutralizing monoclonal antibody to CCR1 known as 2D4 [42]. The antibody was shown to inhibit CCL3, CCL5 and CCL7 binding to CCR1 with an IC_{50} of around 0.7 μg/ml. Furthermore it blocked CCR1 function and inhibited chemokine-mediated chemotaxis with an IC_{50} of less than 10.0 μg/ml. No further information regarding the potential development of this antibody is available.

14.3 CCR1 Antagonists

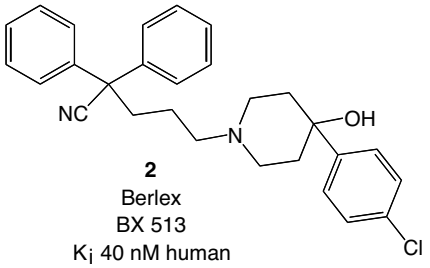
Several companies have disclosed small molecule CCR1 antagonists and a standard feature of many of these compounds is their lack of crossreactivity with nonhuman CCR1. This is a common problem in drug discovery for GPCRs and numerous examples abound in the literature [43–46]. Drug substances that are limited in specificity to human target proteins can be problematic during drug development because they are difficult to test in surrogate animal efficacy models. Without efficacy data it can become very difficult to justify further development of the drug given the considerable risks and costs involved. This issue is further discussed below, within the context of the examples given, and approaches to circumvent it are indicated.

Berlex has disclosed a number of CCR1 compounds. The first were a novel series of 4-hydroxypiperidines that were discovered by high-throughput screening assays and which potently inhibited the binding of CCL3 and CCL5 to recombinant human CCR1 [47–49]. The initial HTS lead were optimized synthetically to yield a series of highly potent receptor antagonists exemplified by BX 510 and BX 513 [47–49] (compounds **1** and **2**) that had $K_i$values of 21 nM and 40 nM for CCR1 respectively, but unfortunately crossreacted with a number of other GPCRs, including adenosine A_3, adrenergic-α_{2A}, dopamine D_2, and muscarinic M_1 and M_2 (at K_i values between 2 and 8 μM) thus precluding any further development [47]. The crossreactivity of BX 513 with biogenic amine neurotransmitter receptors [47], was not surprising for a

structure reminiscent of a typical neuroleptic or antidepressant structural motif [50]. Recently this compound which had a measured K_i of 1 μM for rat CCR1 was shown to be efficacious in a rat MOG model of multiple sclerosis [51]. Given the crossreactivity of the compound with other GPCRs, as discussed above, it is possible that the beneficial effects observed, at the high micromolar concentrations of the drug, could be mediated through other GPCRs.

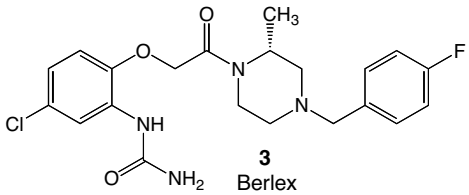


1
Berlex
BX 510
K_i 21 nM human
K_i 9150 nM mouse



2
Berlex
BX 513
K_i 40 nM human
K_i 1000 nM rat
K_i 3500 nM mouse

Following the abandonment of the tricyclic 4-hydroxy piperidine series both for their poor crossreactivity with nonhuman CCR1 and their lack of specificity, Berlex pursued a second class of CCR1 antagonists that had additional properties necessary for preclinical evaluation. The optimized lead compound from this series, BX 471 (compound **3**) is a potent diacyl piperazine more than 1000-fold selective for CCR1 [52]. The antagonist has a reported K_D of 1.0 nM for human CCR1 calculated from radiolabeled binding studies. The compounds from this series also had issues with crossreactivity with rodent CCR1 (compound **3**) but they had sufficient affinity that they could be tested in animal models. BX 471 was efficacious in a number of mouse and rat models of disease including an acute rat EAE model of multiple sclerosis [52] a rat heart transplant model in the rat [53] and mouse models of renal fibrosis [54], multiple myeloma [55] and renal ischemia–reperfusion injury [36]. Based on these data and positive phase I safety studies [18] the CCR1 antagonist entered phase II clinical trials for multiple sclerosis in early 2004. Although the drug was well tolerated and showed no safety concerns its development was stopped after the clinical Phase II study failed to show a reduction in the number of new inflammatory CNS lesions (detected by magnetic resonance imaging, MRI) [18].

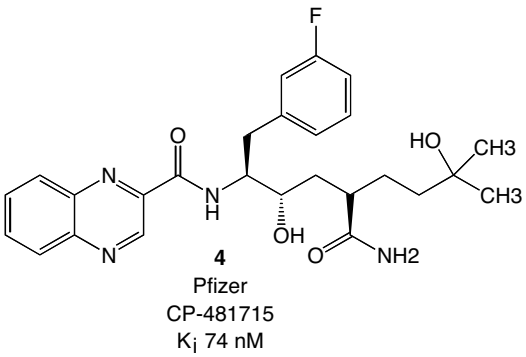


3
Berlex
BX 471
K_i 1 nM human
K_i 121 nM rat
K_i 215 nM mouse

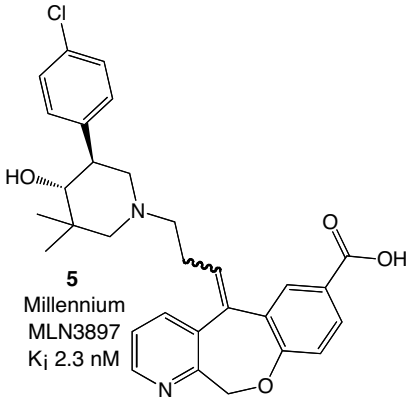
Pfizer has been very active in the CCR1 field and has disclosed a number of CCR1 compounds in peer-reviewed journals [56–61] and in patents [62–67]. Screening their compound library for CCR1 binding inhibitors Pfizer researchers discovered that a quinoline carboxamide containing a hydroxyethylene peptide isostere was a weak 2.3 μM inhibitor [59]. Optimization of this weak hit included replacing the cyclohexyl ring with a phenyl ring [59] and adding an extra nitrogen to the quinoline ring yielding a 34-fold improvement in potency to 64 nM [58]. This template underwent further optimization to increase its metabolic stability and pharmacokinetic properties finally yielding CP-481,715, (compound **4**) a quinoxaline-2-carboxylic acid derivative [56, 57]. The antagonist has a reported K_i of 74 nM for human CCR1 calculated from displacement of radiolabeled CCL3 binding studies, and 71 nM from inhibition of Ca^{2+} transients [56]. The CCR1 antagonist is a competitive and reversible antagonist and is more than 100-fold selective for CCR1 as compared to a panel of G protein-coupled receptors, including related chemokine receptors [56]. Unfortunately, the compound does not crossreact with CCR1 in mouse, rat, guinea pig, dog, rabbit, or monkey at concentrations of up to 25 μM preventing its evaluation in classical animal models of disease. To circumvent this problem Pfizer researchers generated transgenic mice expressing human CCR1 and demonstrated efficacy in models of inflammation in these animals [60]. The compound successfully completed phase I safety studies [68] and was successful in a 16-patient phase Ib clinical trial [69]. In this trial patients with active rheumatoid arthritis responded to the antagonist as demonstrated by a significant reduction in the number of macrophages and CCR1 positive cells in the synovium compared with a placebo group. A trend but no significant clinical improvements were seen in treated patients [69]. Based on these data CP-481715 entered phase II studies for rheumatoid arthritis in February 2004, however the trial was stopped after 6 weeks since, although the compound was well tolerated, it did not demonstrate any efficacy [19, 61].

Millennium has disclosed a number of CCR1 antagonists [70–75] the most advanced of which is MLN3897 (developed in association with Sanofi–Aventis) [73, 74, 76]. This antagonist is a substituted pyridylbenzoxepine of a series previously described by this group (compound **5**) [73, 74] and optimized from a tricyclic nonspecific CCR1 antagonist originally described by Berlex researchers (compound **1**) [47, 48]. It demonstrated high affinity binding for CCR1 (K_i 1.2 nM) and had an IC_{50} of 3.4 nM for the inhibition of chemotaxis induced by CCL3. The compound was effective *in vivo* and demonstrated an EC_{50} of 0.03 mg/kg when inhibiting CCL3-induced immune cell recruitment in a guinea pig skin sensitization model [76] (Pharmacokinetic studies revealed that it had a halflife of 3 h in rat and had oral bioavailability of 35% in rat and 100% in dog. Recently MLN3897 was shown to be able to impair osteoclastogenesis and to inhibit the interaction of multiple myeloma cells with osteoclasts thus demonstrating a potential utility in treating multiple myeloma [77]. In 2004 Millennium announced that they were in phase I clinical trials with MLN3897 [76] and the major indications appeared to be rheumatoid arthritis, multiple sclerosis and psoriasis [76]. In November 2007 Millennium announced that they were terminating the development of MLN3897

for rheumatoid arthritis because it failed to reach its clinical endpoint in a phase II trial [78]. Millennium, along with partner Sanofi–Aventis is assessing the next steps for the program [78].

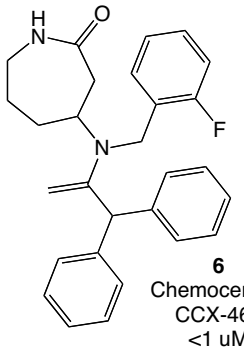


4
Pfizer
CP-481715
K_i 74 nM

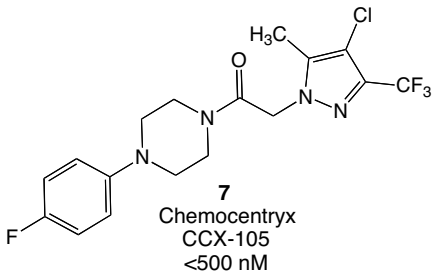


5
Millennium
MLN3897
K_i 2.3 nM

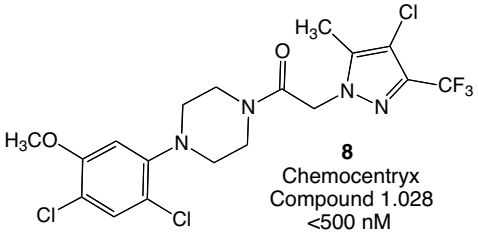
Chemocentryx has disclosed CCR1 inhibitors in a number of patent applications [79–86]. In one patent application [79], the authors claim a family of azaheterocylclic derivates with IC_{50} values ranging from 0.36 to 45.0 μM. Of the 16 compounds that were tested for their inhibition of CCR1, only one had an IC_{50} below 1 μM [79]. CCX-469 (compound **6**) is the only compound claimed with *in vitro* inhibitory activity in the binding assay and had IC_{50} values of 0.6 and 2.0 μM in NSO cells (a mouse myeloma cell line) and HEK-293 CCR1-expressing cells, respectively, and in calcium flux assays [79]. The piperazine derivates claimed in another patent application [81] have a broad range of *in vitro* receptor binding activity, 12.5 μM $< IC_{50} <$ 500 nM, measured by the ability of the compounds to compete with ^{125}I-CCL3 to THP-1 cells. The *in vivo* effects of two compounds, CCX-105 (compound **7**) and compound 1.028 (compound **8**), both with IC_{50} values below 500 nM, have been studied [80]. In a rabbit model of destructive joint inflammation CCX-105 was given intra-articularly at two doses (50 and 100 μM). Histophatologic evaluation of the synovial inflammation showed a dose dependent improvement of the inflammation score. Compound 1.028 was evaluated in a 17-day rat model of collagen-induced arthritis, where the compound was administered subcutaneously daily at a dose of 25 mg/kg from day 0 to day 17. The authors report a significant reduction in ankle joint diameter, 9.1% compared with 15.7% in the vehicle treated group [80]. There is however, no data available for specificity towards other GPCRs and no pharmacodynamic and/or pharmacokinetic studies have been reported. In March 2004 Chemocentryx reported an agreement with Forest Laboratories for the development of CCR1 small-molecule modulators for the treatment of autoimmune and inflammatory diseases such as rheumatoid arthritis and multiple sclerosis [87]. Presumably CCX354, which Chemocentryx recently announced on their company Web site has entered phase I clinical trials for rheumatoid arthritis (http://www.chemocentryx.com/product/CCR1.html), is a member of this class of compounds.



6
Chemocentryx
CCX-469
<1 uM

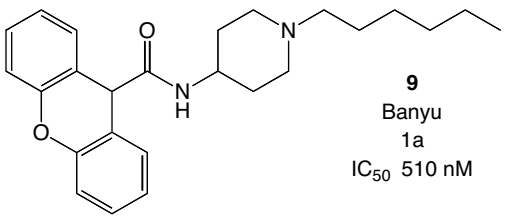


7
Chemocentryx
CCX-105
<500 nM

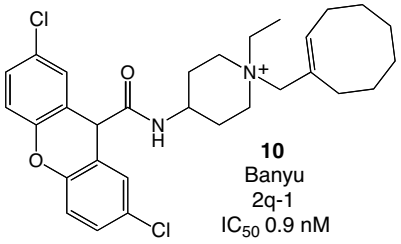


8
Chemocentryx
Compound 1.028
<500 nM

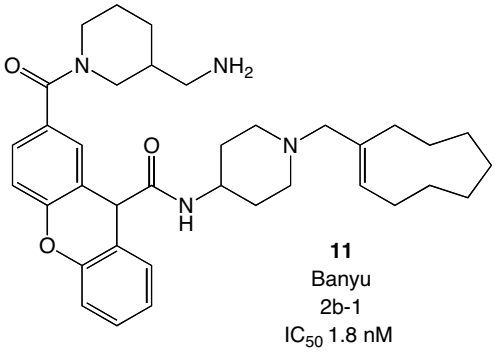
Banyu identified a tricyclic CCR1 antagonist, the tricyclic xanthenecarboxamide 1a (compound **9**), with an IC_{50} of 510 nM from high-throughput screening assays based on inhibition of CCL3 binding to human CCR1 receptors [88, 89]. Derivatization of the screening hit by substitution on the piperidine nitrogen and placement of substituents into the xanthene group resulted in the discovery of compond 2q-1(compound **10**) which was a potent antagonist for human and mouse CCR1 (IC_{50} 0.9 and 5.8 nM, respectively) and also showed potent human CCR3 activity (IC_{50} 0.58 nM) similar to their previously described compounds [90]. Limited *in vivo* data shows that the compound is able to significantly inhibit neutrophil infiltration and levels of TNFα and MCP-1 in the mouse air pouch model at 10 mg/kg i.p. with good PK characteristics [91]. In a collagen induced arthritis model in DBA/1 mice the compound showed dose-dependent inhibition of disease progression [91]. Unfortunately the compound had poor oral activity due to its quaternarized structure and attempts were made to address this in subsequent work by the same group [92] and a compound was identified that was a potent inhibitor of human CCR1 both in receptor binding and functional assays with IC_{50} values of 1.8 and 13 nM respectively (compound **11**). This compound retained its crossreactivity to rodent CCR1 but no *in vivo* data was provided.



9
Banyu
1a
IC_{50} 510 nM



10
Banyu
2q-1
IC_{50} 0.9 nM

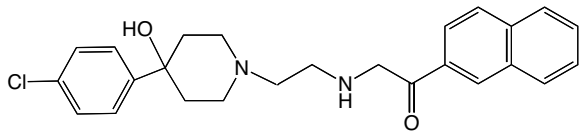


11
Banyu
2b-1
IC_{50} 1.8 nM

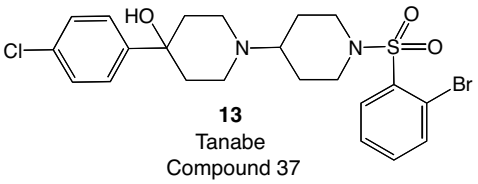
Merck entered Phase II studies with two CCR1 antagonists, C-6448 for multiple sclerosis [93] and C-4462 for rheumatoid arthritis [94]. Both programs were listed in the Merck annual report for 2004 as being in phase II (http://www.merck.com/finance/annualreport/ar2004/research_pipeline/) however, neither compound was listed in the Merck annual report for 2005 (http://www.merck.com/finance/annual-report/ar2005/research_pipeline.html). The implication from this is that the phase II trials did not yield sufficiently strong data for efficacy. Although little has been reported about these programs, it is possible that both compounds are from the CCR1 antagonist program developed by Banyu (Merck's subsidiary in Japan).

In a recent publication, researchers from Tanabe identified a hydroxypiperidine compound (compound **12**) with a modest IC_{50} of $< 6\,\mu M$ as the best hit when they screened a library of 4277 compounds using inhibition of Ca^{2+} transients in a FLIPR assay [95]. Hit to lead modification strategies were developed around this compound to increase its potency. The molecule was divided into four regions: hydrophobe 1 (right aromatic), amide functionality, aliphatic linker, and hydrophobe 2 (4-hydroxy-4-phenylpiperidine). Systematic structure–activity studies were conducted on each portion to improve the potency yielding a series of substituted sulfonamides. The best of these was a 2-Br substituted1,4-bipiperidine (compound **13**) with an IC_{50} of 90 nM based on inhibition of binding and activity assays. The molecule was specific for CCR1 however no *in vivo* data or pharmacokinetic properties were presented. In a second approach, Tanabe described the design of a novel series of CCR1 antagonists based on the structure of BX 471 [96]. Their approach was to replace the urea moeity of the compound, which is dispensable, with diaminocyclobutenedione. This approach yielded a series of potent and orally available CCR1 antagonists exemplified

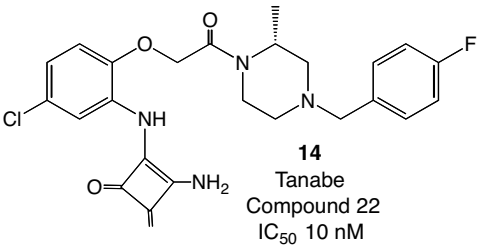
by compound 22 (compound **14**). This compound inhibits CCL3 binding to human CCR1 (IC_{50} 10 nM), and is a full antagonist inhibiting Ca^{2+} transients (IC_{50} 4 nM) and chemotaxis (IC_{50} 7 nM). In addition, the antagonist exhibits an enhanced ability to bind to rodent CCR1 allowing for testing in animal models. Indeed the compound showed significant reduction of paw inflammation and histopathologic damage in the mouse collagen-induced arthritis model. Finally, the antagonist had improved pharmacokinetic properties over BX 471 and was almost 100% orally available in the mouse with a high C_{max} in both rats and mice.



12
Tanabe
HTS hit
IC_{50} <6 uM

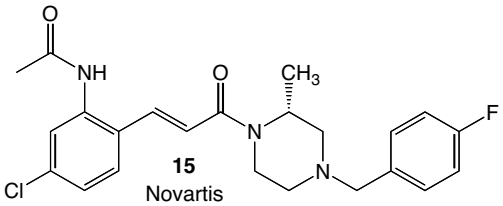


13
Tanabe
Compound 37
IC_{50} 90 nM

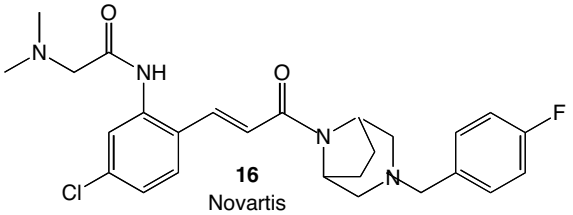


14
Tanabe
Compound 22
IC_{50} 10 nM

The Berlex CCR1 antagonist BX 471 was also used as a starting point by Novartis scientists who were interested in generating more potent rodent CCR1 antagonists to show proof of efficacy in animal models of disease [97, 98]. The *p*-fluorobenzyl substituent of BX 471(compound **3**) was left unchanged, while the aryloxy acetic acid moiety and the piperazine ring were substituted by a number of isoteres. This approach yielded a number of cinnamide derivatives, as exemplified by compounds A1B1 and A4B7, that had much better species crossreactivity than the parent compound (compounds **3**, **15**, **16**). The ether functionality in BX 471 could be favorably replaced by the bioisosteric double bond and showed a much higher affinity for mouse and rat CCR1 (compounds **3**, **15**, **16**). The urea functionality of BX 471 was replaced without any loss of affinity by the acetyl group (compound **15**) and was kept through the whole series, since it was pharmacokinetically far superior to the urea functionality. The ethylene-bridged piperazine fragment, as in A4B7, (compound **16**) was able to substantially increase the potency of the compounds for rodent receptors. The compounds were specific for CCR1 against a panel of other GPCRs including chemokine receptors. Both compounds demonstrated *in vivo* oral efficacy in a mouse collagen induced arthritis model of rheumatoid arthritis.

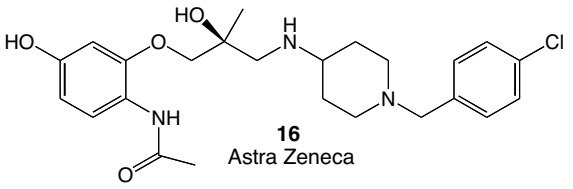


15
Novartis
A1B1
K_i 30 nM human
K_i 320 nM rat
K_i 580 nM mouse

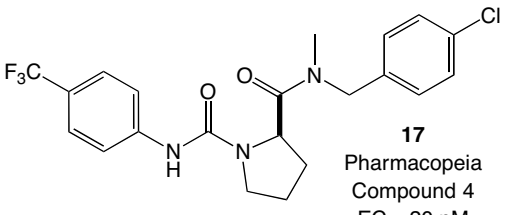


16
Novartis
A4B7
K_i 30 nM human
K_i 10 nM rat
K_i 40 nM mouse

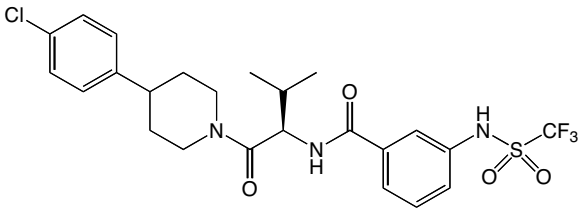
A number of other companies, including Astra Zeneca [99], Pharmacopeia [100] and Bristol Myers Squibb [101], have also disclosed CCR1 inhibitors but no detailed SAR information has been published so far except for the Pharmacopeia Compound [100].



16
Astra Zeneca



17
Pharmacopeia
Compound 4
EC_{50} 20 nM



18
Bristol Myers Squibb

14.4 Conclusions

As this chapter amply demonstrates, the pharmaceutical industry has expended an enormous amount of effort in identifying potent CCR1 antagonists and many of these have progressed to human clinical trials. Unfortunately none of these programs has been successful so far and the roadway of drug discovery has been littered with the corpses of multiple clinical failures. In the light of this, the early optimism that CCR1 will turn out to be an excellent drug target needs to be tempered somewhat. To be fair it is possible that part of the reason that success has been so hard to come by is perhaps reflected in the fact that autoimmune diseases are extremely heterogenous and this coupled with the potential redundancy of chemokine receptors might make it difficult to achieve clinical success. Thus to achieve clinical success with CCR1 antagonists will probably require more effort in developing clinical markers that more accurately delineate different subtypes of disease. Failure to do so will lead not only to poorly designed clinical trials in which potentially useful therapeutic approaches continue to fail, but also to a continuing major unmet medical need.

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15
Targeting CCR3

James Edward Pease

15.1
Introduction

Allergic responses are a result of an undesirable response to an otherwise innocuous antigen or allergen and are characterized by an increase in the number of eosinophils, Th2 lymphocytes, basophils and mast cells within the inflammed tissue. The specific recruitment of these leukocytes to the site of inflammation is principally mediated by a chemokine receptor known as CCR3, which signals in response to the eotaxin family of chemokines. Since the incidence of allergic diseases such as asthma and atopic dermatitis are approaching epidemic proportions in the Western World, much effort has been focused upon the specific targeting of CCR3 as a potential therapeutic avenue. This chapter reviews our understanding of the biology underpinning this ligand–receptor axis and discusses recent developments in antagonizing the interactions of CCR3 with its ligands.

15.2
CCR3 and the Eotaxin Family of Chemokines

The discovery of CCR3 and its chemokine ligands followed intensive efforts by several groups. The principal working hypothesis was that the selective recruitment of eosinophils oberved during the late response to allergen in sensitized individuals was mediated by endogenous selective eosinophil chemoattractant(s) and complimentary cell surface receptors on the eosinophil. The first CCR3 ligand to be discovered was named eotaxin and was identified following the fractionation of bronchoalveolar lavage fluid from allergen-sensitized guinea pigs [1]. Protein sequencing suggested that it was a member of the recently discovered CC family of chemokines including RANTES/CCL5, a chemokine which had previously been shown to be chemotactic for eosinophils but was

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

nonselective, being chemotactic for other subsets of leukocytes. The cDNA encoding eotaxin was subsequently cloned [2], followed by the identification of human [3], mouse [4] and rat orthologs [5]. Following an internationally agreed change to the nomenclature of chemokines in 1999, eotaxin is now also known as CCL11 [6].

Eotaxin/CCL11 binds with nanomolar affinity to its specific receptor CCR3, which was independently identified and characterized in 1996 by several laboratories [7–9]. CCR3 is a class A G protein-coupled receptor (GPCR) and is highly expressed by human eosinophils [7] (around 50 000 receptors/cell) and also by basophils [10], mast cells [11] and a subpopulation of Th2 lymphocytes [12], supportive of the notion that antagonism of CCR3 function may be beneficial in treatment of allergic disease, where these cell types predominate (Figure 15.1). Two additional CCR3-specific chemokines named eotaxin-2/CCL24 and eotaxin-3/CCL26 were subsequently discovered by groups using molecular biological methods [13–17], with the original eotaxin being renamed eotaxin-1. CCL24 and CCL26 both induce eosinophil chemotaxis *in vitro*, although CCL26 is an order of magnitude less potent than CCL11 [16, 18]. It is noteworthy that although CCL24 and CCL26 are known as eotaxins upon the basis of their specificty for CCR3, they bear little homology at the amino acid level with CCL11. CCL24 and CCL26 are encoded in close proximity to each other in a different chromosomal location, suggesting that they arose by gene duplication, independently of CCL11.

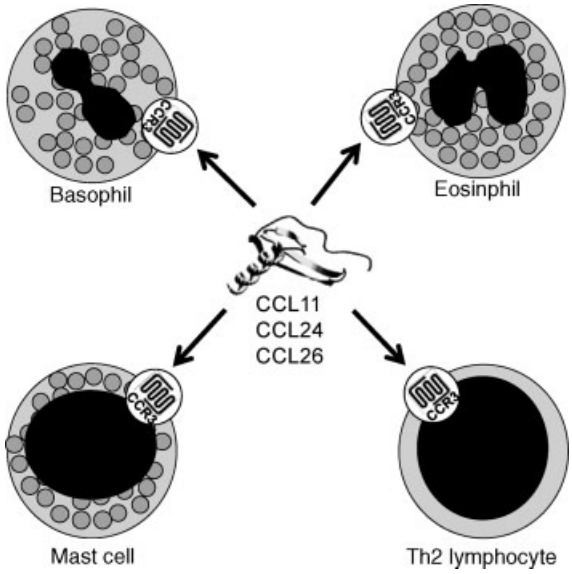


Figure 15.1 CCR3 is expressed on the surface of eosinophils, basophils, mast cells and Th2 lymphocytes, allowing them to respond to the eotaxin family of chemokines (CCL11, CCL24, CCL26) which are implicated in the pathogenesis of allergic inflammation.

15.3
Structure–Function Studies of CCR3 and its Ligands

CCR3 is the most promiscuous of all chemokine receptors, binding upwards of a dozen chemokine ligands, including CCL8/MCP-2, CCL7/MCP-3, CCL13/MCP-4 and CCL5/RANTES [19]. As is the case with CCR2, the receptor for the MCP family of chemokines [20], binding of CCL11 and subsequent activation of CCR3 is thought to occur via a two-step model in which a high affinity interaction between the core residues of the chemokine and the N-terminus of the receptor initially tethers CCL11 to CCR3 [21]. This facilitates subsequent interactions between the chemokine and the remainder of the receptor leading to signaling. Site-directed mutagenesis of the extracellular loops of CCR3 suggests several charged residues are important in this process, which is supported by an earlier study showing CCR3 function is highly dependent upon local pH and salt concentrations [18, 22]. Studies of the related receptor CCR5 have further refined the two-step model of chemokine receptor activation, with the suggestion that the chemokine N-terminus inserts into a transmembrane intrahelical bundle, disrupting hydrophobic interactions between helical sidechains and inducing the conformational changes necessary for receptor activation and G protein recruitment to the intracellular face of the receptor [23, 24]. Supportive of such a model for CCR3 activation, a small molecule agonist of CCR3 has recently been shown to bind within an intrahelical binding pocket, mimicking the N-terminus of CCL11 [25]. This correlates with studies regarding the N-terminal truncation of CCL11 by the action of CD26/dipeptidyl-peptidase IV (CD26/DPP IV) which generates the species CCL11 (3–74) with much reduced chemotactic activity [26] and is thought to act as brake to limit excessive CCL11-mediated inflammatory responses as inhibition of CD26/DPP IV *in vivo*, enhances the CCL11-mediated recruitment of eosinophils [27].

Highly conserved between chemokine receptors and class A GPCRs in general, is an aspartate/arginine/tyrosine [27] motif at the cytoplasmic end of the third transmembrane helix, which by analogy with rhodopsin was originally thought to hold class A GPCRs in an inactive conformation via an "ionic lock" [28]. Mutation of this domain within CCR3 is poorly tolerated, suggestive of an important function [29], although the recent elucidation of the crystal structure of the β_2 adrenergic receptor suggests that the conserved motif is unlikely to function as an ionic lock in all class A GPCRs, which may explain the structural instability and consequent high basal activity of the β_2 adrenergic receptor [30]. This phenomenon may also be true of CCR3 as the receptor has been previously shown to spontaneously couple to G proteins in CCR3 transfectant systems, as demonstrated by changes in basal and CCL11-induced [^{35}S]-GTPγS binding [31].

15.4
CCR3 and its Ligands in the Pathogenesis of Allergic Disease

The importance of both CCL11 and CCR3 in both homeostasis and allergic inflammation has been supported by numerous publications reporting the results

of both clinical studies and those from animal models of disease. The next few pages briefly review the literature.

15.4.1 Supporting Evidence from Animal Models

A mentioned in the introduction to this chapter, CCL11 was originally identified from the BAL obtained from a guinea pig model of allergic inflammation [1, 2] and early studies following on from this work showed that CCL11 levels in airway tissue and BAL correlated with eosinophil recruitment [32]. In the same guinea pig model, CCL11 was also shown to act in concert with the important eosinophil survival factor IL-5, promoting both eosinophil recruitment from the microcirculation [33] and eosinophil mobilization from the bone marrow [34, 35]. The generation of mice deficient in CCL11 allowed further investigations into the role of CCL11 in the inflammatory setting. An initial study describing BALB/c mice deficient in CCL11 showed a reduction in ovalbumin-induced lung eosinophilia, supportive of a role for the chemokine in allergic inflammation [36]. This concurred with another report describing the effects of a CCL11 neutralizing antibody in reducing in lung eosinophilia and associated bronchial hyperresponsiveness following the challenge of BALB/c mice with ovalbumin [37]. Interestingly, neutralization of CCL5, CCL12 and CCL2 also have similar inhibitory effects upon eosinophil trafficking to the lung, suggestive of a coordinated effort involving several chemokines and leukocyte subsets. A subsequent study by the same group using an adoptive transfer approach to examine the role of CCL11 in Th2 cell recruitment found that CCL11 was involved in the early phase of Th2 cell recruitment, with the CCR4 ligand CCL22 the principle chemokine responsible for late phase recruitment [38]. It is noteworthy that the genetic background of the mice employed in studies of allergic inflammation favor the inbred BALB/c background; indeed, when CCL11-deficient mice were generated on the outbred ICR strain, no reduction in allergen-driven recruitment of eosinophils to the lung was observed compared with littermate controls [39].

The generation of mice deficient in CCR3 has enabled the precise role of the receptor in eosinophil migration to be addressed. Humbles and colleagues reported reduced numbers of eosinophils in the airways of CCR3 deficient mice, sensitized and challenged with aerosolized ovalbumin [40]. Microscopical analysis determined that this phenomenon was not due to a global inability to recruit eosinophils (the vasculature of the being full of eosinophils in challenged mice) but was due to a defect in migration from the vasculature into the lung parenchyma. Intriguingly, CCR3-deficient mice exhibited elevated airway hyperresponsiveness to methacholine following allergen challenge, which was attributed to the increased numbers of intraepithelial mast cells within the trachea. In contrast, a later study by the same group of allergic skin inflammation revealed CCR3 to be critical for eosinophil migration into the skin and lungs and in contrast to the earlier study, found CCR3-deficient mice to have reduced AHR responses following methacholine and allergen challenge [41]. The precise differences between the AHR observed in both models are

unclear but were attributed to the different routes of immunization used for allergen sensitization, namely the intraperitoneal route in the initial study and the intradermal route in the second study, this latter mode of sensitization being thought to favor a Th2-type response [41]. A recent study using *Aspergillus* as the allergen challenge, reported reduced numbers of perivascular and peribronchial eosinophils in both CCR3-deficient mice and double-knockout CCL11/CCL24-deficient mice [42]. Deletion of the genes for CCL11 and the Th2 cytokine IL-5 in double-knockout mice appears to be very effective in suppressing airway eosinophilia and hyperresponsiveness [43]; interestingly, IL-13 levels were markedly reduced in these animals.

Of relevance to clinical studies of eosinophil esophagatis, which are discussed in the next section, both CCR3 and CCL11 appear to be important in the constitutive homing of mouse eosinophils to the gut and the subsequent homeostasis of this organ. Mice deficient in either ligand or receptor exhibit reduced numbers of eosinophils in the gut [36, 40]. This is in contrast to CCL24-deficient mice, which have been reported to have comparable numbers of eosinophils within in the gastrointestinal tract to their wildtype littermates [44].

15.4.2
Supporting Evidence from Clinical Studies

A comprehensive body of data from clinical studies supports the notion that as is the case with rodents, CCR3 and its principal ligands play a prominent role in the pathogenesis of human allergic disease. Several cells have been described as sources of CCL11 including alveolar macrophages [45, 46], eosinophils [46], epithelial cells [47], fibroblasts [48], lymphocytes [46] and smooth muscle cells [49]. Increases in the numbers of $CCR3^+$ cells have been detected in the sputum of asthmatics 24 h following the inhalation of allergen [50] and cells expressing both CCL11 and CCL24 have been identified in the submucosa and epithelium of bronchial biopsies in asthmatic patients [45, 47, 51]. The specific roles of CCL11, CCL24 and CCL26 in cell recruitment appear to be distinct, the chemokines being expressed at different time points during the inflammatory process. In a study of atopic subjects, expression of CCL11 at both mRNA and protein levels was shown to peak at 6 h following intradermal allergen challenge, which correlated with early eosinophil infiltration [51]. In contrast, CCL24 expression was maximal at 24 h and correlated with eosinophils infiltrating at later time points. Although the precise role of CCL26 in eosinophil recruitment *in vivo* remains to be elucidated, CCL26 mRNA has been reported to be upregulated in bronchial biopsies from asthmatics 24 h after challenge, supportive of a role in later eosinophil recruitment [52].

A microarray study of patients with eosinophilic oesophagitis by the group of Rothenberg revealed the CCL26 transcript to be the most highly induced mRNA in esophageal samples from sufferers compared with healthy controls, suggesting that the CCR3/CCL26 axis plays a prominent role in the pathology of this condition [53]. More recent studies by the same group found children with eosinophilic oesophagitis to exhibit enhanced CCL26 expression and elevated levels of peripheral blood

eosinophils compared to healthy controls and to suggest that blood levels of CCL26 may be a potentially useful biomarker for the diagnosis of disease [54, 55].

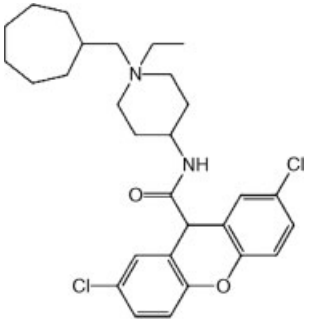
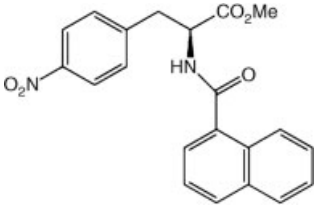
15.5 Targeting CCR3 Function

Initial proof of concept studies targeted at CCR3 used monoclonal antibodies raised against human, mouse, guinea pig and macaque orthologs of the receptor. These studies were able to offer both *in vitro* and *in vivo* proof that CCR3 blockade was feasible [19, 56–59]. Among these studies, it is notable that *in vivo* administration of an anti-CCR3 mAb to eosinophilic mice resulted in depletion of eosinophils from the circulation and a subsequent reduction in tissue eosinophilia [58]. In parallel to these studies, initial efforts by the pharmaceutical industry employed high-throughput screens of CCL11 binding assays to identify potent small molecule antagonists of CCR3 from existing compound libraries with activities in the low nanomolar range (Table 15.1).

One of the first CCR3 antagonists to be revealed in the patent literature was a molecule named UCB35625 [60], a trans-isomer of a molecule originally identified by scientists at Banyu Pharmaceutical Company [61]. UCB 35625 is bispecific and also possesses nanomolar inhibitory activity at the closely related chemokine receptor, CCR1. This is not surprising, since both receptors share high homology in the transmembrane domains where subsequent mutagenesis studies revealed the compound is likely to bind [24, 62]. The bispecificity of such a molecule may be clinically useful as work from our laboratory [63, 64] and supported by that of others [65] has suggested that although donors from all individuals appear to express CCR3 on the cell surface of their eosinophils, around 15–20% of individuals also have CCR1 expressed at comparatively high levels. This makes the eosinophils of such individuals highly responsive to CCL3, suggesting that the CCR1:CCL3 axis has the potential to recruit eosinophils in allergic disorders affecting a significant proportion of the population. Supportive of this postulate, expression of CCL3 in the lungs of human asthmatics has been reported in several studies [66–69] and increased CCL3 levels have also been observed in the serum of atopic dermatitis patients [70].

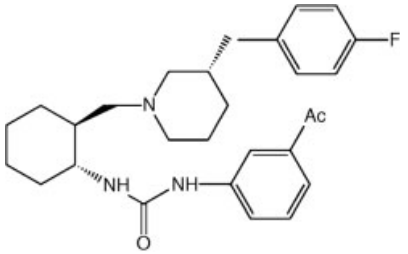
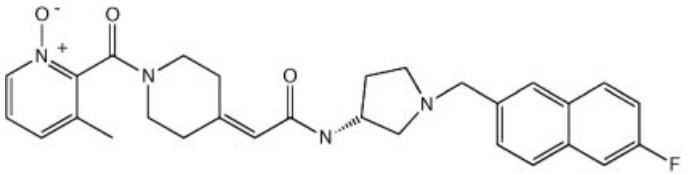
Based on the UCB35625 structure, scientists at UCB Pharma recently described pyrazolone methylamino piperidine derivatives following the synthesis of a library with a central aminopiperidine core as a privileged structure required for CCR3 antagonism [71]. In a converse approach directed at obtaining a CCR3-specific molecule, the original bispecific CCR1/3 antagonist was used as template for the generation of a focused library by scientists at Banyu [72]. The resulting screen lead to the identification of a compound with low nanomolar activity at CCR3 and 3600-fold selectivity over CCR1. It is of interest that the CCR3 binding site occupied by the antagonist UCB35625 appears to overlap that of a structurally related small molecule agonist and that a single mutation in the receptor binding site CCR3 can confer agonist activity to UCB35625 [25]. This suggests that there is a fine line between agonist and antagonist activity at CCR3; and CCR3 antagonist programs from other

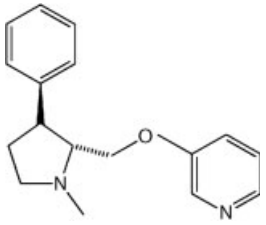
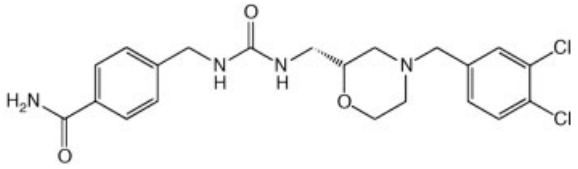
Table 15.1 A selection of the CCR3 antagonists described in both the patent and scientific literature, together with their chemical structures and reported efficacies *in vitro* and *in vivo*.

Compound	Structure	Activity *in vitro*	Activity *in vivo*	Reference(s)
UCB35625 UCB Pharma/Banyu		Yes	No	[24, 60, 61]
SB-328437 SmithKlineBeecham		Yes	Yes	[78–80, 97]

(*Continued*)

Table 15.1 (*Continued*)

Compound	Structure	Activity *in vitro*	Activity *in vivo*	Reference(s)
DPC-168 Bristol Myers Squibb		Yes	Yes	[81]
YM-344031 Yamanouchi Pharmaceutical Company		Yes	Yes	[98]

A-122058 Abbott Laboratories		Yes	Yes	[100]
GSK766994 GlaxoSmithKline		Yes	Yes	[101]

groups have also reported the identification of small molecule agonists of the receptor following the optimization of the structure–activity relationships of lead compounds [73, 74]. This may be a common feature of chemokine receptors as recent reports have also described small molecule agonists of CCR5, CCR8 and CXCR3 arising from other drug discovery programs [75–77].

A group at SmithKlineBeecham described several small molecule CCR3 antagonists based upon phenylalanine, the most potent of which was SB-328437 [78–80]. These compounds also had low-nanomolar activity at CCR3 but unlike UCB 35625 had no measurable activity at CCR1. Scientists from Bristol Myers Squibb described piperidine-based compounds with antagonist activity in both calcium mobilization and chemotaxis assays, notably DPC168 with picomolar inhibtory activity in CCL11-induced chemotaxis assays [81]. Unfortunately, although DPC168 had modest oral bioavailability in mice, further development was discontinued due to off-target effects at cytochrome P450 (CYP) 2D6 and hERG. Subequent refinement led to the compound BMS-570520 with improved selectivity against CYP2D6 without a significant loss of potency against CCR3 [82]. Similar problems with unwanted CYP2D6 and hERG activity from piperidine-based compounds have also been reported by several groups, although subsequent SAR studies were often sucessful in dialing out the unwanted activity [73, 83–85].

15.6
Small-Molecule CCR3 Antagonists with *In Vivo* Activity

A major obstacle that has had to be overcome is that finding that in general, there is often limited homology between human and rodent orthologs of chemokine receptors. CCR3 is no exception to this rule and consequently, many of the small molecules originally identifed for their activity against human receptors have proved ineffective in rodent models of allergic inflammation, making target validation difficult. Moreover, many CCR3-specific small-molecule programs were mothballed following the publication of data from clinical trials with monoclonal antibodies directed against the cytokine IL-5 [86, 87]. In these studies, neutralization of IL-5 resulted in a dramatic decrease in blood eosinophils levels, presumably by inhibiting IL-5 mediated release from the bone marrow, although little improvement of lung function was observed. The subsequent questioning of a direct role for eosinophils in asthma pathogenesis resulted in many groups within the pharmaceutical industry losing interest in developing CCR3 antagonists, as recently reviewed by Wells and colleagues [88].

Subsequent critiques of these studies have challenged various parameters of the study design employed [89–91] and more recent studies elucidating the role of the eosinophil in airway remodeling have rekindled interest in CCR3 antagonists. Deletion of the mouse IL-5 gene was observed to suppress both lung eosinophilia and tissue remodeling, with a decrease in TGF-β_1 [92]. This correlated with earlier clinical data in which administration of an antiIL-5 mAb was reported to result in a reduction in both the numbers of TGF-$\beta_1{}^+$ airway eosinophils and the deposition of

extracellular matrix proteins in the reticular basement membrane of bronchial biopsies [93]. The subsequent generation of mice deficient in eosinophils allowed the role of the cell to be probed in further detail and the ensuing data from allergen challenge models with these mice implicated the eosinophil in the processes of airways remodeling and airway hyperresponsiveness [42, 94, 95] although it should be emphasized that the process role of the eosinophil in both processes is still hotly debated [96].

These *in vivo* studies have served to renew interest in CCR3 as a therapeutic target, and several compounds with activity at the rodent orthologs of CCR3 have been subjected to *in vivo* analysis. The SmithKlineBeecham compounds SB-297006 and SB-328437 have been reported to suppress antigen-induced accumulation of Th2 cells and eosinophils in the lungs in an adoptive transfer model of allergic airways disease [97], while oral administration of the Bristol Myers Squibb compound DPC168 has been reported to reduce eosinophil recruitment into the lungs in a dose-dependent manner following allergen challenge [81]. The Yamanouchi Pharmaceutical Company reported their compound YM-344031 to be a potent CCR3 inhibitor with low nanomolar activity in *in vitro* assays of calcium flux assay and good efficacy *in vivo*. Oral administration of doses up to 100 mg/kg of YM-344031 significantly inhibited both the immediate and late-phase allergic skin reactions in a mouse allergy model [98]. Similarly, YM-355179, also from the Yamanouchi Pharmaceutical Company has been reported to have low nanomolar potency in *in vitro* assays of chemotaxis and calcium flux and to inhibit eosinophil infiltration into airways of cynomolgus monkeys after segmental bronchoprovocation with CCL11 [99]. Abbot Laboratories have also described the compound A-122058 which was reported to be effective in reducing the number of eosinophils following *i.p.* injection of CCL11 into mice [100].

At the time of writing, the only small-molecule antagonist of CCR3 to reported in clinical trials is the compound GSK766994 generated by scientists at GlaxoSmithKline. In preclinical studies this compound showed good pharmacokinetics and was orally active in a Brown Norway rat model of asthma. Unfortunately, in a phase III clinical trial for the treatment of allergic rhinitis, the compound did not exhibit efficacy [101]. Other CCR3 antagonists from the same company have been reported to be efficacious in vagally mediated bronchoconstriction in antigen-challenged guinea pigs and a mouse model of allergic conjunctivitis, although no data from clinical studies of these compounds has been reported [102, 103].

15.7 Summary

Although great progress has been made in the past decade regarding our understanding of the biology of CCR3, it is disappointing to report that, in common with several other programs directed at antagonizing chemokine receptors, no specific CCR3 antagonist has shown efficacy in phase II/III trials. To date, the sole success story in the entire field is the CCR5 antagonist from Pfizer named miraviroc which was recently licensed for the treatment of HIV-1 infection [104]. One reason that

might be behind such a record of failure is the fact that, while HIV-1 entry is reliant upon a single receptor (CCR5), inflammatory processes typically involve the generation of multiple chemokines and the ensuing recruitment of leukocytes is not mediated by a single species of receptor. It might therefore be envisaged that the targeting of multiple chemokine receptors with small-molecule antagonists may provide a more efficacious therapy for the future treatment of allergic disease. Indeed, several currently prescribed GCPR antagonists rely upon broad spectrum activity for their efficacy such as Zyprexa, a tricyclic benzodiazepine which is used in the treatment of schizophria and bipolar disorder [105]. A dual antagonist of CCR3 chemokine and the histamine H1 receptor has already been described which might be envisaged to inhibit both the early and late phase parts of a typical allergic response [106]. Likewise, UCB 35625 which antagonizes both CCR1 and CCR3 might be envisaged to have greater *in vitro* efficacy than a CCR3-specific drug [60]. Molecules such as these may reveal new therapeutic opportunities for the treatment of allergic disease and offer alternatives to the standard steroid-based therapies currently employed with their well documented side effects [107].

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16
Chemokine Binding Proteins as Therapeutics

Ali Alejo and Antonio Alcami

16.1
Immune Modulation by Secreted Chemokine Binding Proteins (CKBPs)

The primary function of the immune system is to protect the host from invading pathogens. Infection triggers signals that initiate the immune and inflammatory responses and the recruitment of immune cells to sites of infection. Leukocyte migration is largely controlled by the function of chemokines, a family of chemoattractant cytokines that share structural similarities and can be divided into four classes: CC, CXC, C and CX3C chemokines [1–3]. The production of a particular set of chemokines, together with the differential expression of specific seven-transmembrane domain G protein-coupled chemokine receptors by leukocyte, determines the immune cells that will infiltrate the infected tissues in the animal host. It is believed that under physiological conditions chemokines do not act in solution but are presented to leukocytes as ligands immobilized to a solid phase via interaction with glycosaminglycans (GAGs) [4, 5]. Disruption of the interaction of chemokines with specific receptors or the formation of chemokine-GAG complexes might therefore inhibit the migration of immune cells in response to chemokines [6, 7].

Pathogens such as viruses and parasites have evolved a variety of strategies to control the immune response in order to survive in the presence of potent host defense mechanisms [8–10]. One of the immune evasion strategies employed by large DNA viruses, such as poxviruses and herpesviruses, is the molecular mimicry of cytokines and their receptors to intercept the cytokine networks that control the immune response to infection [11]. Viral mechanisms that modulate the activity of chemokines include the expression of chemokine homologs, seven-transmembrane domain chemokine receptor homologs and secreted CKBPs. More recently, some examples of CKBPs have been found in pathogens other than viruses (Figure 16.1) [12].

Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6

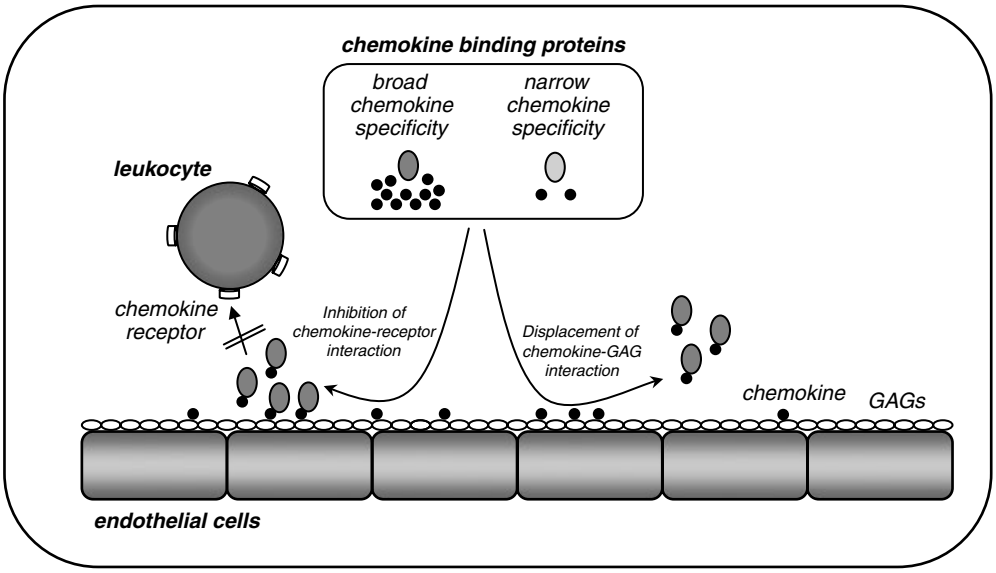

Figure 16.1 Mechanisms of chemokine inhibition by the CKBPs encoded by pathogens. Chemokines interact with GAGs present at the surface of endothelial cells and are presented to the leukocytes. The chemokine receptors expressed at the surface of leukocytes interact with chemokines and intracellular signaling is triggered to induce cell migration. The CKBPs may have broad chemokine binding specificity, such as the poxvirus 35-kDa protein, the gammaherpesvirus M3 protein or the MYXV M-T7 protein. By constrast, the evasin family of CKBPs and the poxvirus-encoded SECRET domain show narrow binding specificity. CKBPs may interfere with chemokine-GAG interactions, disrupting the presentation of chemokines on the surface of endothelial cells and the formation of chemokine gradients, or may sequester chemokines and block the interaction of chemokines to specific receptors and the induction of cell migration.

The neutralization of cytokine activity through the expression of soluble versions of the extracellular binding domain of cytokine receptors is a strategy used by the immune system to limit the extent of the response. For example, the type 2 intereukin-1 (IL-1) receptor and the tumor necrosis factor (TNF) receptor are cleaved at the cell surface and secreted into the extracellular medium as a mechanism to neutralize IL-1 and TNF, respectively [13, 14]. Poxviruses employ a similar strategy by encoding soluble versions IL-1 and TNF receptor homologs that bind the cognate ligands with high affinity and block their interaction with cell-surface receptors. Several viruses utilize a similar strategy to neutralize cytokine activity [11]. However, due to the structural nature of the seven-transmembrane domain chemokine receptors, the production of secreted versions of these receptors is not feasible and pathogens have evolved proteins structurally different to the host chemokine receptors but with the ability to bind chemokines with high affinity [12]. Here we review our current knowledge of known CKBPs and their unique properties, and we discuss the potential therapeutic application of CKBPs as inhibitors of chemokine activity.

16.2
Viral CKBPs (vCKBPs)

vCKBPs are the most abundant group of CKBPs and were the first to be identified. So far, vCKBPs have been only identified in pox- and herpesviruses, two distantly related families of DNA viruses including important human pathogens such as variola virus, the causative agent of smallpox, and human cytomegalovirus. These secreted proteins bind to different chemokines with high affinity. The inhibition of chemokine-mediated cell migration is achieved directly by blockade of the interaction site of the chemokine with its cognate receptors and/or by binding of the vCKBPs to the GAG binding site of the chemokine. The latter process would disrupt the interaction of the chemokine with the cell surface, impairing the establishment of a chemotactic gradient. Although several vCKBPs with differential binding and inhibitory profiles have been described, only a few have been tested for their therapeutic potential *in vivo*.

16.2.1
vCKBPs as Therapeutics

16.2.1.1 The Poxvirus 35-kDa Protein

The 35-kDa protein or viral CC chemokine inhibitory protein (vCCI) is a secreted protein found in vaccinia virus (VACV) and other poxviruses that binds with high affinity almost all human and mouse CC chemokines [15–17]. The mechanism of action of the 35-kDa protein is competitive inhibition of CC chemokine binding to cellular receptors. The ortholog of myxoma virus (MYXV), named M-T1, has the unique ability to interact with GAGs via a domain at its C-terminus that is not present in other 35-kDa family members [18]. This unique property of M-T1 would retain the protein in the vicinity of infected cells and may enhance its ability to protect the sites of infection from chemokine-mediated antiviral responses *in vivo*. Orf virus, a parapoxvirus that causes infections in sheep and cattle, and sporadically in humans, encodes a 35-kDa protein related vCKBP but with a broader chemokine binding specificity that includes the C chemokine lymphotactin [19].

The experimental evidence in mouse and rabbit models of infection indicates that the 35-kDa protein encoded by VACV, rabbitpox virus and MYXV inhibit the chemokine-mediated infiltration of immune cells into primary sites of infection but have little influence on the progression of disease [17, 20, 21]. The expression of the 35-kDa protein from VACV Western Reserve (WR), a strain that does not encode the vCKBP, causes a slight attenuation of the virus associated with reduced inflammatory pathology in the lungs [22].

The first illustration of the potential of 35-kDa protein as a therapeutic inhibitor of inflammatory disease *in vivo* was produced using a guinea pig skin inflammation model. Purified recombinant vCKBP completely inhibited local eosinophil infiltration induced by eotaxin, a feature of allergic inflammatory reactions [16]. Similarly, the related vCKBP from orf virus has been recently shown to inhibit migration of monocytes in a mouse acute skin inflammation model induced by LPS [23].

Intranasal administration of purified 35-kDa protein significantly reduced inflammation of the airway and lung parenchyma and improved the physiological function of the lungs during airway hyperreactivity in a mouse model of allergen-induced asthma without compromising systemic immunity or chemoattraction at extrapulmonary sites [24].

Delivery of the 35-kDa protein has also been suggested as a useful approach to prevent vein graft stenosis, a common cause for reoperation after coronary artery bypass grafting. Administration of the MYXV M-T1 protein inhibited intimal hyperplasia after aortic allograft transplant in rats by blocking CC chemokine mediated macrophage and T cell invasion [25]. Additionally, adenovirus-mediated *ex vivo* gene therapy showed that the presence of the 35-kDa protein alone or in combination with superoxide dismutase efficiently reduced neointimal formation in vein grafts in New Zealand white rabbits [26, 27].

In a series of articles, the possibility of preventing atherosclerosis by virally mediated delivery of the 35-kDa vCKBP has been explored. Atherosclerosis is a chronic inflammatory disease and the underlying cause for heart attack and stroke, one of the leading casues of death in the Western world. Using the atherosclerosis prone apolipoprotein E knockout mice, adenoviral delivery of the 35-kDa protein *in vivo* prevented the development of native atherosclerosis in these mice through inhibition of CC chemokine activity [28, 29]. Additionally, the 35-kDa protein was shown to prevent accelerated atherosclerosis after vein graft in the same mouse model, suggesting this may be an interesting approach to ensure the success of long-term coronary or peripheral artery bypass grafts [30]. More recently, lentivirally mediated long-term delivery of the 35-kDa protein has been suggested as a promising strategy to prevent the development of atherosclerosis [31]. A further approach has been the development of a membrane-bound version of the 35-kDa protein, which confers localized inhibition of CC chemokine mediated inflammation *in vivo*. This strategy was shown to decrease leukocyte recruitment into the peritoneal cavity in a sterile peritonitis model in mice. Additionally, intravenous delivery of the adenovirus expressing the membrane bound 35-kDa protein allowed localized expression of this protein to hepatocytes, which significantly protected against liver damage in a Con A-induced hepatitis model [32].

16.2.1.2 **The Gammaherpesvirus M3 Protein**

The M3 protein is a secreted broad spectrum vCKBP encoded by the mouse gammaherpesvirus 68 (MHV-68) [33, 34]. An ortholog with similar properties encoded by MHV-72 has recently been described [35]. M3 binds human and mouse CC, CXC, C and CX3C chemokines with high affinity. Similar to the 35-kDa protein, M3 can prevent binding of chemokines to their cognate receptors. Additionally, M3 has the ability to block the interaction of chemokines with GAGs *in vitro*, suggesting it may be able to act by disrupting chemokine gradients *in vivo* [36, 37]. During infection with MHV-68, M3 has been suggested to block CD8 T cell recruitment into lymphoid tissue, allowing the establishment of latent infections [38], although these findings have been disputed. Using an intracranial infection model, M3 has been shown to control inflammatory responses in the brain. An MHV-68 mutant lacking M3 was

100-fold less virulent [39] and the M3 protein was suggested to act by altering the nature of the associated inflammation infiltrate from a lymphocyte- and monocyte-rich infiltrate to one composed mainly of neutophils.

As described before, chemokine inhibition may be a useful approach for the prevention of restenosis after coronary surgery or atherosclerosis. This concept has been further substantiated *in vivo* through the use of the broad spectrum vCKBP M3. Thus, systemic M3 expression from a transgene, or delivery of recombinant M3 protein, have been shown to reduce intimal hyperplasia after femoral artery injury [40] and aortic allograft transplantation [25], respectively.

To explore the role of chemokines in the development of diabetes, a series of studies have taken advantage of transgenic mice expressing the M3 protein in pancreatic islets. Using double transgenic mice expressing both the M3 protein as well as CCL21 ectopically in the pancreas, M3 was shown to block chemokine-induced mononuclear cell recruitment to this organ [41]. Later, blockade of CCL2- and CXCL13-induced leukocyte migration by M3 was demonstrated using the same approach [42]. Further, transgenic mice expressing M3 in the pancreas have been shown to be remarkably resistant to diabetes induced by multiple injections of low-dose streptozotocin [43]. The reduced inflammation observed by the authors was postulated to be due to local inhibition of CCL2, CXCL10 and CCL21 chemokine activity by M3 in the pancreas. Additionally, M3 expression in pancreatic islet β cells in NOD mice was found to prevent the development of insulitis and diabetes by disrupting both the influx and generation of diabetogenic cells [44]. This demonstrated that broad-spectrum chemokine blockers might be useful for the prevention of autoimmune diabetes.

Recombinant M3 protein inhibited FGF2-induced angiogeneisis *in vivo* and has thus been proposed as a model for the development of novel angiogenesis inhibitors for the therapy of pathological conditions, including tumor growth and metastasis [45]. The developing notion of cancer as an inflammatory disease and the role of chemokines in cancer progression suggest that chemokine activity blockade might help prevent the development of this pathology [46]. An interesting example is the use of a modified oncolytic vesicular stomatitis virus (VSV) vector expressing the M3 vCKBP for the treatment of cancer [47]. A safe oncolytic VSV was proposed as a novel therapeutic agent for cancer treatment but was found to induce a potent inflammatory reaction *in vivo* which severely attenuated its oncolytic potency. Expression of the MHV-68 M3 protein from this modified VSV was shown to circumvent this problem in a rat hepatocellular carcinoma model. Thus, neutrophil and natural killer cell accumulation at the site of lesions was significantly reduced, intratumoral viral titers were increased by two log units, tumor necrosis was increased and animal survival rates prolonged when the animals were infected with the M3-expressing VSV as compared to the parental VSV. Importantly, no systemic toxicity was observed in the treated animals.

16.2.1.3 **The MYXV M-T7 Protein**

MYXV M-T7 is a secreted 37-kDa glycoprotein with sequence similarity to the interferon γ receptor (IFN-γR) that binds to and inhibits the biological activity of

rabbit IFN-γ in a species-specific manner [48, 49]. M-T7 was also found to bind a broad range of C, CXC and CC chemokines and studies with CXCL8 mutants suggested that chemokine binding to M-T7 is via the conserved C-terminal GAG binding domain found in a variety of chemokines [50]. This is a unique property of the IFN-γR encoded by MYXV since the VACV IFN-γR does not bind chemokines [16, 50]. The interaction of M-T7 with the chemokine GAG binding domains led to the suggestion that M-T7 might prevent the correct localization of chemokines and the formation of a chemokine gradient, rather than blocking chemokine binding to specific receptors [50].

Infection of rabbits with a MYXV mutant with an inactivated *M-T7* gene demonstrated the contribution of M-T7 to MYXV pathogenesis in European rabbits [51]. Marked differences were seen in the size and progression of skin lesions, the onset and severity of secondary bacterial infections and clearance of the virus. M-T7 was implicated in the control of migration of inflammatory cells to sites of infection. However, these results are difficult to interpret because M-T7 targets IFN-γ and chemokines, both having relevant roles in inflammatory responses.

The fact that M-T7 binds not only to IFN-γ from rabbit but also to chemokines from various species has allowed its use as a vCKBP in mouse models. The ability of low amounts of recombinant M-T7 protein to attenuate restenosis after balloon angioplasty in rats has been described to be similar to that of the M3 and 35-kDa proteins, as mentioned before. Whether this effect is due to direct inhibition of chemokine activity is not clear at present [25]. Using a rat renal allograft model, chronic rejection was attenuated by M-T7 protein, with significant reduction in tubular and glomerular atrophy, scarring and lymphocyte infiltration [52].

Also, the effect of M-T7 on the response to implanted materials has been analyzed in a rat model. In this case, M-T7 was expressed from intramuscularly injected plasmid DNA encoding the corresponding gene one week before implantation of crosslinked dermal sheep collagen discs. M-T7 reduced significantly the progression of the foreign body reaction (rejection of implanted material), reducing both the characteristic macrophage influx as well as the formation of new blood vessels. This effect is thought to be mediated by disruption of: (i) chemokine-GAG interaction and (ii) the interaction of the proangiogenic cytokine vascular endothelial growth factor with GAGs [53].

16.2.2
Other vCKBPs

To date, only the mentioned vCKBPs have been studied as potential therapeutic agents. However, a whole set of other vCKBPs from different origins and with different properties and modes of action have been described. Possibly, each of them has evolved to control specific aspects of the activation of the immune response. Therefore, a detailed description of their roles *in vivo* in their own biological context may help define their potential clinical applications (Table 16.1). Indeed, the examination of the chemokine spectrum bound by each of them in comparison

Table 16.1 Main properties of CKBPs.

CKBP	Origin	CKs known to bind	Mode of action
35-kDa/M-T1	Poxvirus	Broad specificity: CC chemokines	Block receptor interaction site
A41	Poxvirus	CCL21, CCL24, CCL25, CCL26, CCL27, CCL28, CXCL12α, CXCL12β, CXCL14	Block GAG binding site
SCPs	Poxvirus	CCL25, CCL28, CCL27, CCL26, CXCL12, CXCL13, CXCL14	Not determined
M-T7	Poxvirus	Broad specificity: CC, CXC, C chemokines	Block GAG-binding site
M3	γ-Herpesvirus	Broad specificity: CC, CXC, C, CX3C chemokines	Block receptor interaction and GAG-binding site
gG	α-Herpesvirus	Broad specificity: CC, CXC, C chemokines	Block receptor interaction and GAG-binding site
pUL21.5	β-Herpesvirus	CCL5	Block receptor interaction site
smCKBP	*Schistosoma mansoni*	CCL2, CCL3, CCL5, CXCL8, CX3CL1	Block receptor interaction site
Evasin-1	Tick	CCL3, CCL4, CCL18	Block receptor interaction site
Evasin-3	Tick	CXCL8, CXCL1	Block receptor interaction site
Evasin-4	Tick	CCL5, CCL11	Block receptor interaction site

with the known roles of different chemokines in the context of disease may suggest the use of specific vCKBPs for the treatment of different conditions.

16.2.2.1 The Smallpox Virus-Encoded Chemokine Receptor (SECRET) Domain from Poxviruses

The SECRET domain was first described as a chemokine binding domain present in the variola virus secreted TNF receptor homolog CrmB [54]. This domain binds to a limited number of both CC and CXC chemokines with high affinity, inhibiting their biological activity. Five different SECRET domain-containing proteins (SCPs) have been described from various poxviruses. However, only the role of the CrmB protein has been analyzed *in vivo*. CPXV CrmB was suggested to have an anti-inflammatory role in an intracranial model of mouse infection with a CPXV deletion mutant [55]. No information as to whether this is due to TNF or chemokine blockade is available. Additionally, the recombinant CrmB protein protects BALB/c mice from LPS-induced septic shock [56], although this is probably due to its effect on TNF and not on chemokines. The nature of chemokines bound by the SCPs suggest they may be useful in the treatment of inflammatory diseases of the skin and mucosal surfaces.

16.2.2.2 The Poxvirus A41 Protein

The A41 protein from VACV has sequence similarity to the VACV 35-kDa protein described above. This protein as well as its ECTV ortholog have been recently found to interact with a set of CC and CXC chemokines [57, 58], although the recombinant protein was not able to block chemokine-induced cell migration in cell culture assays. However, the A41 protein can interact with the GAG-binding domain of chemokines, suggesting that it may inhibit leukocyte migration *in vivo* by blocking chemokine-GAG interactions, which are required for appropriate chemokine function *in vivo* [58]. Accordingly, deletion of the *A41L* gene from VACV strain WR enhanced virulence slightly and showed an altered inflammatory response to infection in a dermal model [59].

16.2.2.3 The Alphaherpesvirus Glycoprotein G (gG)

The alphaherpesvirus gG is a component of the viral particle and is expressed as a membrane-anchored protein at the plasma membrane of infected cells, with a secreted version of the gG protein being generated after proteolytic cleavage of the membrane form. The horse herpesvirus 1 and cow herpesvirus 1 and 2 gG have been characterized in more detail and found to bind a variety of CC and CXC chemokines [60]. These proteins block chemokine activity by interfering with chemokine–receptor interactions and the subsequent activation of cell migration, although the interaction of chemokines with GAGs is also prevented by gG. Interestingly, both the transmembrane and secreted forms retain chemokine-binding activity. Moreover, the felid herpesvirus 1 gG present at the surface of the virion was also shown to bind chemokines [61].

16.2.2.4 The Human Cytomegalovirus pUL21.5 Protein

The pUL21.5 protein encoded by human cytomegalovirus is a small secreted glycoprotein that has been demonstrated to bind CCL5 with high affinity and to block the interaction of CCL5 with its specific cellular receptor [62]. This CKBP was reported to be highly specific for one chemokine, in contrast to the broad binding specificity of previously described vCKBPs, but only three chemokines were tested and the possibility that pUL21.5 binds other chemokines of this complex family of chemoattractant cytokines cannot be ruled out. The role of pUL21.5 during infection is not known at present.

16.3 The *Schistosoma mansoni* CKBP

Schistosoma mansoni is a trematode parasite that causes schistisomiasis, a common disease that affects millions of people in the developing world and that is severe in 10% of the infected individuals [63]. Schistosomes establishes chronic infections that cause high morbility. Schistosomes are well prepared to modulate the immune system to the benefit of the parasite. For example, a major cause of pathology is the granulomatous inflammation induced around the parasite eggs trapped in various organs, which has been shown to facilitate the excretion of eggs from the host [64, 65].

A CKBP secreted by *S. mansoni*, named smCKBP, was the first CKBP identified in a human pathogen and is the only one identified in a parasite to date [66]. smCKB was found to be expressed only in schistosome egg secretions but not in the other life cycle stages (cercariae, schistosomulae, worms) of *S. mansoni* and is also produced by eggs from the two other major schistosome species that infect man, *S. haematobium* and *S. japonicum*. The gene encoding smCKBP was identified following a proteomic approach and has amino acid sequence unrelated to other CKBPs or mammalian proteins [66]. Binding and functional studies have shown that smCKBP interacts with several chemokines, prevents the interaction of chemokines with their cellular receptors and inhibits chemokine-mediated cell migration.

It has been shown that, in an experimental granulomatous inflammation model, secretion of smCKBP by live eggs modulated the differential recruitment of cells and the size of the egg granuloma, and it was proposed that smCKBP may facilitate granuloma formation and the propagation of the *S. mansoni* eggs. Recombinant smCKBP suppressed inflammation induced in a mouse contact hypersensitivity model and inhibited CXCL8-induced pulmonary inflammation [66].

16.4
Evasins, a Family of CKBPs from Ticks

The evasin family comprises four small proteins of 7–12 kDa that are produced in the tick saliva [67, 68]. Ticks are blood-sucking parasites that feed on their hosts for several days. Ticks do not cause an inflammatory response, and it was suggested that the role of evasins is to inhibit innate responses mediated by chemokines that may protect the host. Evasins show a restricted chemokine binding specificity: evasin-1 binds CCL3, CCL4 and CCL18, evasin-3 binds CXCL8 and CXCL1, and evasin-4 binds CCL5 and CCL11 [67, 68]. Interestingly, it appears that ticks have evolved a family of CKBPs with narrow binding specificity as an alternative to a single CKBP with broad binding activity, such as some vCKBPs (M-T7, 35-kDa, M3 proteins). Evasins block the interaction of chemokines with their cellular receptors and inhibit in this way chemokine-induced recruitment of leukocytes *in vitro* and *in vivo*.

The anti-inflammatory activity and therapeutic potential of evasins have been demonstrated in animal models [67]. Evasin-1 caused a significant reduction in skin inflammation in $D6^{-/-}$ mice sensitized with TPA and reduced lethality, decreased leukocyte infiltration and preserved the lung architecture in a lung injury model. Evasin-3 was tested in a mouse model of antigen-induced arthritis and it was found to reduce leukocyte infiltration in the synovial cavity and inflammation. Both evasin-1 and evasin-3 reduced lethality in an intestinal ischemia model.

16.5
Advantages and Limitations of the Use of CKBPs as Therapeutics

Because of the role that chemokines play in controlling the infiltration of leukocytes into inflamed tissues, the chemokine network is considered a major target to develop

new drugs to control inflammatory diseases [6, 7, 69]. The structural constraints of the seven-transmembrane domain chemokine receptors limit their use as secreted versions that bind chemokines with high affinity and neutralize their activity, and thus alternative mechanisms have evolved to limit chemokine activity. A number of decoy receptors, such as the Duffy antigen receptor for chemokines (DARC), D6 or CCX-CKR, that bind chemokines but do not transduce signals and function as chemokine scavengers have been described in the host immune system [70, 71]. An alternative mechanism is the expression of the anti-inflammatory cytokine IL-10 that decouples chemokine receptors from intracellular signaling processes, thereby modifying the function of chemokine receptor that turn into decoy receptors that sequester chemokines without inducing biological responses [71]. The identification of secreted proteins encoded by pathogens that have unique structure allowing them to bind chemokines with high affinity and to block their activity represent an attractive alternative to control chemokine function [11, 12] and have great potential as new medicaments [72, 73].

The complexity and potential redundancy of the chemokine family, with more than 40 chemokine ligands [3], may limit the use of single-chemokine antagonists to block cell migration. The finding that some CKBPs, such as the gammaherpesvirus M3 or the poxvirus 35-kDa proteins [33, 34, 74], interact with a large variety of chemokines may reflect the need to block simultaneously several chemokines to control the immune response. The availability of broad-specificity chemokine inhibitors from pathogens offers a unique opportunity to test the advantage of using proteins that neutralize many chemokines, in contrast to monoclonal antibodies specific for one chemokine, as inhibitors of cell migration in disease. A potential problem of such broad-range inhibitors is that they may cause an undesirable general downregulation of the chemokine system, including chemokines needed for the normal trafficking of immune cells through different tissues. Other pathogen-encoded CKBPs, such as the evasins from ticks and the poxvirus-encoded SECRET domain, recognize a more restricted set of chemokines and offer an alternative to the broad inhibitors [54, 67].

An interesting property of some CKBPs is that they have evolved either to inhibit the binding of chemokines to specific receptors, blocking intracellular signaling, or to interfere with chemokine-GAG interactions. It is likely that the ability of gammaherpesvirus M3 and alphaherpesvirus gG to block chemokine interaction with both receptors and GAGs may enhance the antichemokine activity of these CKBPs *in vivo* [36, 37, 60]. Also, the finding that the MYXV M-T7 [50, 51] and the A41 protein from VACV and ECTV [57–59] interact with the GAG-binding domain of chemokines and inhibit cell infiltration into infected tissues, but do not block chemokine-induced cell migration, would support the idea that the disruption of chemokine localization mediated by GAGs may be a target for therapeutics [4, 5].

Considering that CKBPs have amino acid sequence unrelated to chemokine receptors and lack transmembrane domains, determination of their three-dimensional structure will be of high relevance to understand the molecular basis of chemokine recognition by CKBPs. This information will allow the future design of

variant forms of CKBPs with reduced immunogenicity and different chemokine specificity. So far, the crystal structure of a CKBP-chemokine complex has been determined only for the gammaherpesvirus M3 protein bound to CCL2 or XCL1 [36, 75]. The crystal structure of the poxvirus 35-kDa and A41 CKBPs, and the tick evasins 1 and 3 have been solved [57, 67, 76, 77], but these structures do not provide information on the regions involved in chemokine binding because the complex with chemokines was not crystallized. In the case of the poxvirus 35-kDa CKBP, complementary studies by nuclear magnetic resonance of a complex with CCL2 identified the interface of the interaction with chemokines [78]. These studies have uncovered the diversity of CKBP structures, which are generally unrelated to each other.

A consideration for the use of pathogen-encoded CKBPs as therapeutics is their potential immunogenicity, which may limit their use in chronic diseases that require repeated administration of the chemokine inhibitor. As foreign proteins CKBPs are likely to induce an antibody response after repeated inoculation that may neutralize their ability to inhibit chemokines or compromise their halflife. The blockade of the chemokine binding activity in the presence of antibodies has been formally demonstrated for the VACV 35-kDa protein [16]. However, it is possible that some CKBPs may have evolved as low immunogenic proteins in their natural host to avoid their neutralization by the host antibody response, as it has been suggested for evasins [67]. The immunogenicity of therapeutic proteins is a problem even found with human proteins currently used as therapeutics [79]. Protocols to reduce immunogenicity, such as the attachment of polyethylene glycol (PEGylation), removal of T cell epitopes, humanization or tolerization, may be applied to pathogen-encoded CKBPs to take advantage of their immunomodulatory activities.

Pathogens have evolved with their hosts for millions of years and have optimized their immune evasion strategies. Further studies in animal models of infection will define the immune modulatory activity of the different CKBPs and their contribution to pathogenesis. Moreover, a better knowledge of the mechanisms used by pathogens to control chemokine activity will help us to understand the function of chemokines *in vivo* and to identify new strategies to modulate chemokine activity. For example, the binding properties of the CKBPs identify specific immune functions mediated by particular sets of chemokines, and point at critical chemokines that need to be targeted. Also, the broad binding specificity of some CKBPs and their ability to interfere with chemokine-GAG interactions suggest strategies to control chemokine activity *in vivo*.

The CKBPs identified in the genome of pathogens were not predicted from sequence analysis to bind chemokines, and it is possible that the secretion of CKBPs of unrelated structure is a strategy used by other pathogens. The genome of pathogens can be considered as repositories of information on immune modulation and can help us to uncover the physiological role of chemokines. Several studies have demonstrated the therapeutic value of CKBPs in different animal disease models. It is interesting that products derived from pathogenic organisms hold substantial promise for the treatment of human inflammatory diseases [72].

Acknowledgments

We acknowledge the support from the Wellcome Trust, the Spanish Ministry of Science and Innovation, the Comunidad de Madrid and the European Union.

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Chemokine Receptors as Drug Targets. Edited by Martine J. Smit, Sergio A. Lira, and Rob Leurs

ISBN: 978-3-527-32118-6