Review

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Bias in chemokine receptor signalling

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Chemokine receptors are widely expressed on a variety of immune cells and play a crucial role in normal physiology as well as in inflammatory and infectious diseases. The existence of 23 chemokine receptors and 48 chemokine ligands guarantees a tight control and fine-tuning of the immune system. Here, we discuss the multiple regulatory mechanisms of chemokine signalling at a systemic, cellular, and molecular level. In particular, we focus on the impact of biased signalling at the receptor level; an emerging concept in molecular pharmacology. An improved understanding of these mechanisms may provide a framework for more effective drug discovery and development at a target class that is so relevant for immune function.

Regulation of the chemokine system

Chemokines are the most important regulators of leukocyte trafficking and play a central role in the immune system [1]. They act via abundantly expressed chemokine receptors, which belong to the family of G protein-coupled receptors (GPCRs) (Box 1), on a wide variety of immune cells. Activation of these chemokine receptors induces migration and differentiation of immune cells, which both are essential processes during innate and adaptive immune responses [2].

The chemokine-directed immune response involves a complex network of reactions that are carefully fine-tuned at multiple levels throughout the body (Figure 1). At the systems level this involves spatiotemporal and tissue-specific expression of chemokine receptors and their ligands. At the cellular level the chemokine receptor signal can be modulated by coexpression of many differentially expressed proteins on immune cells. Finally, there is growing evidence of biased signalling at the molecular level for chemokine receptors, which implies that different chemokine ligands activate different intracellular pathways although binding to the same receptor.

With regard to this bias at the receptor level, novel mechanistic insights have been attained lately due to the advances in X-ray crystallography and NMR methods to resolve the structure of membrane proteins, such as GPCRs. Several structures of chemokine receptors have been elucidated now, among which are chemokine CXC receptor (CXCR)1, CXCR4 and chemokine CC receptor (CCR)5 [3–5]. In addition, for the serotonin 5-hydroxytryptamine $(HT)_{1B/2B}$ and the β_2-adrenergic receptors a structural basis for biased signalling was reported [6,7]. Similar mechanisms for ligand bias are likely to be present for the family of chemokine receptors, because these are particularly prone to biased signalling due to the presence of multiple endogenous chemokine ligands.

So far there has only been limited success in clinical trials targeting chemokine receptors. We propose therefore to consider chemokine regulation and bias at multiple levels in order to better understand their intricacies. Thus, in this review we present a summary of chemokine receptor signalling at a systems, cellular, and molecular level. Immunologists should be aware of the bias that can be introduced at a molecular level, whereas pharmacologists need to keep in mind that their target molecule could be modulated or expressed differently at a systems level.

Regulation of chemokine expression and receptor activation

The human chemokine system consists of ~23 receptors and 48 ligands [IUPHAR/BPS Guide to Pharmacology, http://www.guidetopharmacology.org, accessed on 07-02-2014], of which the classically signalling chemokine receptors are presented in Figure 2. Most chemokine receptors can be activated by multiple chemokines, and one chemokine often has the ability to activate multiple receptors. Although previously regarded as redundant, the unique expression patterns of the various chemokines suggest that they form the basis for a specific and fine-tuned functioning of the immune system [1]. This is not only important in normal physiology, but also during certain immunopathological disease states, as illustrated by the CCR2 receptor and its ligands. CCR2 can be activated by the chemokine ligands chemokine CC ligand (CCL)2/monocyte chemotactic protein (MCP)-1, CCL7/MCP-3, CCL8/MCP-2, CCL11/eotaxin, CCL13/MCP-4, and CCL16/human CC chemokine (HCC)-4. Most studies have been focused on the CCL2–CCR2 interaction because CCL2 is the endogenous ligand with the highest affinity for CCR2. Nevertheless, in infectious diseases, CCL7 has been found to be crucial for monocyte recruitment to inflammatory sites mediated through CCR2 [8]. An example of distinct expression patterns observed in immunopathology is the regulation of the CCR4 ligands CCL17/thymus- and activation-regulated chemokine (TARC) and CCL22/macrophage-derived chemokine (MDC), which are not expressed in healthy skin tissue [9]. However, in inflamed skin lesions, CCL17 is detected on endothelial cells, whereas CCL22 is only presented by dendritic cells [9]. This distinct chemokine

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Box 1. Chemokine receptors as GPCRs

GPCRs

- With >800 members, GPCRs are the largest family and most diverse group of cell surface receptors and the most common target for therapeutic drugs [79].
- The GPCR structure consists of an extracellular N terminus, an intracellular C terminus, and seven transmembrane (TM) helices, connected by three cytoplasmic and three extracellular loops [80].
- Ligand binding mostly takes place in a pocket formed by the seven helices close to the extracellular side of the receptor; it induces a conformational change at the intracellular side of the receptor that results in receptor activation and subsequent signalling [81].
- At the intracellular side different effector proteins can bind and transduce signals, among which are G proteins and β-arrestins [82].

Chemokine receptors

- Chemokine receptors belong to the class A rhodopsin-like family of GPCRs.
- 23 different chemokine receptors have been identified that can be activated by ~48 chemokine ligands [IUPHAR/BPS Guide to Pharmacology, http://www.guidetopharmacology.org, accessed on 07-02-2014].
- Four subclasses of chemokine ligands have been identified on the basis of the pattern of conserved cysteine residues (C, CC, CXC, and CX_3C) [83].
- Chemokine receptors have been classified as C, CC, CXC, and CX_3C receptors based on the chemokine subclass ligand that they bind.
- Most chemokine receptors bind multiple chemokines, and most chemokines can bind to and activate multiple chemokine receptors.
- The chemokine receptors ACKR1 (DARC), ACKR2 (D6), ACKR3 (CXCR7), and ACKR4 (CCX-CKR) are so-called decoy receptors that predominantly scavenge chemokine ligands from the extracellular environment, although some of these also couple to β-arrestins [16].

expression pattern has been demonstrated in diseases ranging from psoriasis to atopic dermatitis, therefore, this could be a general feature underlying the disease state. In general the balance, timing, and pattern of chemokine expression appears to regulate the generation of immune-cell-specific responses in health and disease [10].

In addition to the difference in release and production of chemokines among various tissues, their *in vivo* availability also depends on the interaction of chemokines with specific glycosaminoglycan (GAG) chains that are presented at the cell surface as part of membrane proteoglycans. The binding of chemokines to GAGs allows immobilization, accumulation, and retention of chemokines on cell surfaces near their sites of production in order to provide directional signals to migrating cells [11]. In addition, GAG interactions are involved in the transport of chemokines across cell surfaces. GAGs may selectively bind chemokines and therefore fine-tune the immune response, because they display varying affinities for specific chemokines and are differentially expressed in time and location on specific cell types and tissues [12]. Furthermore, cells and tissues can alter the expression of GAGs in pathophysiology. This has been observed upon inflammatory stimuli in diseases of the gastrointestinal tract as well as in multiple different tumours [13,14]. GAGs might even be directly involved in signalling, because their attached core proteins that span the membrane can undergo tyrosine phosphorylation and thereby contribute to signal transduction, as reported for CXCL12/SDF-1 and the proteoglycan syndecan-4 [15]. Although they are a crucial factor for chemokine signalling, the exact functional consequences of chemokine–GAG interactions and the level of specificity are still largely speculative.

Not only GAGs can alter the availability of chemokines, but also chemokine receptors themselves. A certain group of chemokine receptors, known as atypical chemokine receptors (ACKRs) [16], have been proposed to act mainly as chemokine ligand scavengers [17,18]. Furthermore, under certain circumstances the G protein-coupled chemokine receptors have been demonstrated to become uncoupled from G protein signalling. For example, dendritic cells and monocytes treated with anti-inflammatory interleukin (IL)-10 express 'uncoupled' or 'nonsignalling' CCR1, CCR2, and CCR5, which can scavenge their corresponding inflammatory chemokines *in vitro* as well as in mice [19]. Another study demonstrated both *in vitro* and *in vivo* that apoptotic leukocytes express 'silent' CCR5 receptors, scavenging CCR5 ligands, and thereby contributing to the resolution of inflammation in a mouse model of peritonitis [20]. Therefore, expression of a certain chemokine receptor does not always imply a contribution to the disease state. In fact, one might speculate that a pharmacological blockade of these receptors can increase free chemokine levels and therefore result in enhanced pathology.

Altogether, the examples above illustrate that the expression of chemokines and their receptors varies over time and between different conditions, and studies of mechanisms and outcomes associated with this differential expression in several disease states have been reviewed previously [21,22]. As noted above, it is clear that expression of chemokines and their receptors does not necessarily imply a role as stimulator or enhancer of a pathophysiological state, which is an important factor to consider while developing antagonists targeting the chemokine system.

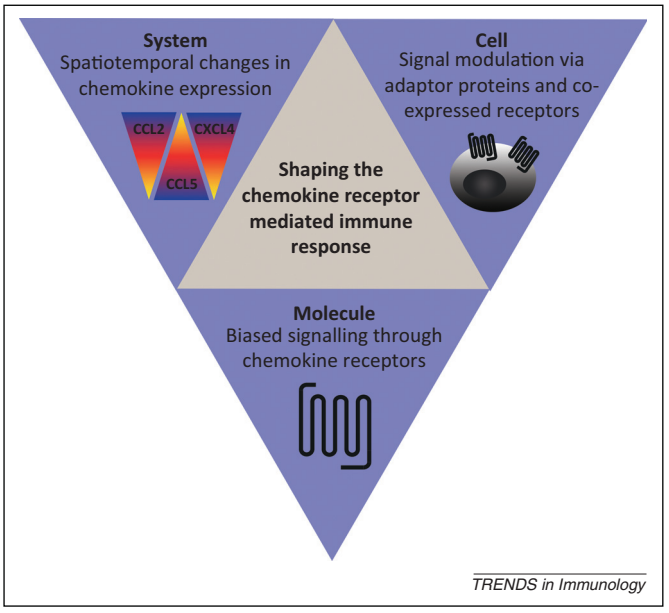

Figure 1. Schematic representation of the structure of this review. The chemokine receptor-mediated immune response is discussed at a systems, cellular, and molecular level.

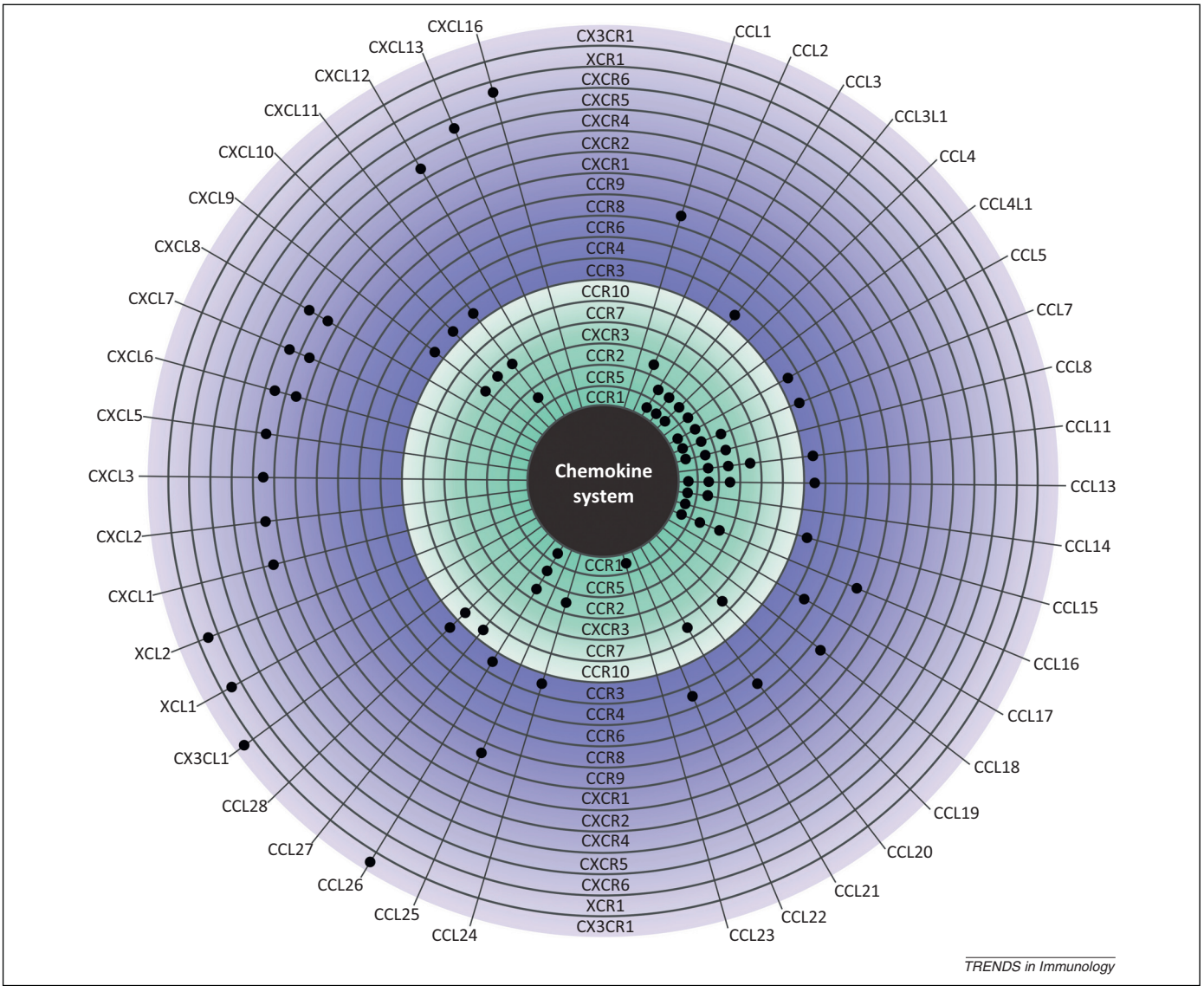

Figure 2. Overview of the family of chemokines and chemokine receptors. The green inner circle represents those chemokine receptors for which some form of biased signalling has been documented. This is not (yet) the case for the chemokine receptors in the blue outer circle. The black dots represent the chemokine ligands that have been shown to bind to a given chemokine receptor. The group of atypical chemokine receptors (ACKRs) is not depicted in this figure.

Besides the regulation of chemokines and their receptors throughout the body, there is substantial evidence that chemokine receptors modulate each other within a particular immune cell. This is discussed in the following section.

Regulation of chemokine receptor signalling in immune cells

Chemokine receptors are expressed by immune cells in both the innate and adaptive compartments, including B and T lymphocytes, monocytes, and neutrophils [23]. Distinct expression profiles characterize the different leukocyte subtypes. For example, in T helper (Th) cells, several chemokine receptors are associated with the Th1 phenotype (including CXCR3 and CCR5), whereas others are associated with the Th2 phenotype (including CCR4 and CCR8). This phenomenon is likely related to their discriminate functions in response to viral and bacterial pathogens or during allergic reactions [24,25]. In the case of monocytes, a different repertoire of chemokine receptors can be expressed depending on environmental factors and stimuli. Lipopolysaccharide (LPS) downregulates CCR1, CCR2, and CCR5 expression in monocytes, whereas IL-2 stimulates CCR2 expression [26]. In addition, CCR7 is upregulated upon immunogenic stimulation, possibly to facilitate lymph-node homing [27,28]. Tight regulation of the different chemokine receptors on immune cells therefore shapes the immune cell response.

The majority of immune cells express multiple chemokine receptors simultaneously. At a cellular level, chemokines can counteract each other or display synergy, thereby reducing the inflammatory response or increasing the selectivity of cell recruitment [29,30]. For example, via heterologous receptor desensitization or internalization one chemokine can lower the responsiveness of a cell to other chemokines binding to a distinct chemokine receptor [31]. This phenomenon has been studied in human peripheral blood T cells, which express CCR5 and CXCR4 [32]. Upon simultaneous addition of their chemokines CCL4/macrophage inflammatory protein (MIP)-1β, CCL5/regulated on activation, normal T cell expressed and

secreted (RANTES), and CXCL12, the capacity of CXCL12 to induce chemotaxis *in vitro* is suppressed. This crosstalk does not involve the internalization of the receptor, but rather a cross-desensitization via a decrease in phosphorylation of downstream signalling proteins. The rich chemokine environment surrounding the leukocytes during inflammatory conditions can therefore induce different cellular responses than determined in assays that only reflect the behaviour of one particular chemokine receptor and ligand [33].

Chemokines can also modulate signalling responses through other chemokine receptors due to the presence of heterodimeric or hetero-oligomeric receptor complexes [34]. This has been demonstrated for several chemokine receptors, among which CCR2, CCR5, and CXCR4 [35–37]. In CCR2–CCR5 heterodimers, the CCR5 ligands CCL3/MIP-1α, CCL4, and CCL5 were able to displace CCL2 from CCR2 [37]. This so-called negative cooperativity was further analysed in different *in vitro* assays to confirm the allosteric nature of this displacement via heterodimers [36]. The relevance for immune cell functioning has been demonstrated as well, because negative binding cooperativity takes place in hetero-oligomeric complexes between the binding pockets of CCR2, CCR5, and CXCR4 in T cells and monocytes that endogenously express these receptors [35]. As a result, the recruitment of these cells mediated by the CXCR4 agonist CXCL12 in mice could be inhibited by antagonists of CCR2 and CCR5.

Within immune cells, the magnitude and duration of the signal depends on the exposed chemokine concentration and on (subsequent) chemokine receptor desensitization, phosphorylation, and internalization. These processes are regulated via G protein-coupled receptor kinases (GRKs) and β-arrestins [38]. On the intracellular side of the cell, the different repertoires of these adaptor proteins regulate the eventual cellular effects. In RBL-2H3 cells stably expressing both receptors, it has been shown that CXCR1 and CXCR2 couple to distinct GRK isoforms [39]. CXCR1 predominantly couples to GRK2, whereas CXCR2 interacts with GRK6 to negatively regulate receptor sensitization and trafficking, eventually affecting cell signalling [39,40]. The role of GRK6 in neutrophil recruitment was further demonstrated in studies using wild type and GRK6$^{-/-}$ knockout mice [39]. In addition, different types of immune cells express different types of GRKs and β-arrestins; the levels of which may also vary, adding another layer of bias and fine-tuning of the response of chemokines and their receptors [41,42].

Thus, there are multiple co-receptors and adaptor proteins that define the eventual chemokine receptor signal. In order to study the effect of a chemokine or potential drug candidate, it is important to include cell types and tissues that reflect the *in vivo* situation more so than cell lines, devoid of physiological context, with heterologous receptor expression.

Biased signalling through chemokine receptors

At the molecular level yet another type of bias is present in the chemokine system, because chemokine receptors are capable of differentially signalling in a ligand-specific manner. This biased signalling, also called functional selectivity, refers to agonist ligands that favour the activation of a certain intracellular signalling pathway over another [43]. The following sections discuss the multiple intracellular signalling routes that can be activated by chemokines. The aim is to give a comprehensive overview of the biased signalling events that have been reported for chemokine receptors so far, illustrating that the chemokine system is extensively fine-tuned at the receptor level already.

GPCR signalling

GPCRs transduce the effects of many extracellular signals/ligands (whether those are chemokines or other hormones and neurotransmitters) to intracellular pathways and signalling routes (Figure 3). They bind to and activate heterotrimeric G proteins that consist of a G_α, G_β, and G_γ subunit, for which 21, 6, and 12 different types are present in humans, respectively [44]. Activation of these G proteins modulates the production of second messenger molecules such as cyclic AMP (cAMP), intracellular calcium (Ca^{2+}), and inositol phosphates (IPs), which control further downstream effectors such as protein kinase (PK)C and Akt. GPCR activation and consequently G protein-mediated signalling are terminated via phosphorylation of the GPCR by GRKs. The phosphorylated receptor recruits β-arrestins, of which various subtypes exist. This association often results eventually in receptor internalization to the cytosol, effectively impeding further signalling from the receptor. After receptor coupling, β-arrestins are also able to transduce signals themselves, for example, via subsequent activation of the extracellular signal-regulated kinase (ERK) pathway [45]. For the purpose of the present discussion, we focus on the major signalling pathways via G proteins and β-arrestins to illustrate the phenomenon of biased signalling through chemokine receptors in functions of the immune system.

In case of extreme signal bias through GPCRs, one ligand may mainly activate G proteins, whereas another ligand only activates β-arrestins. This results in different cellular effects ('texture') although both ligands act via the same receptor; a process that has been extensively studied and discussed for GPCRs in general, as reviewed by Kenakin and Christopoulos [46]. Biased signalling does not only comprise distinct signalling via either G proteins or β-arrestins, but also includes more subtle differences in the activation of other downstream signalling proteins. For example, ligands can discriminate between different types of G proteins, whereas others differently affect signalling events such as ERK activation or Ca^{2+} mobilization. It is important to note that pathway activation depends also on the expression level of the receptor as well as the cellular expression and availability of signalling molecules, which result in cell-specific differences (Figure 3).

Advances in structural biology have led to an accumulating understanding of the underlying mechanisms. The first structural features in a GPCR crystal structure that are responsible for biased signalling were recently revealed for the serotonin receptors 5-HT_{1B} and 5-HT_{2B} [6]. Conformational changes at the intracellular side in their helix VI and helix VII were reported to be responsible

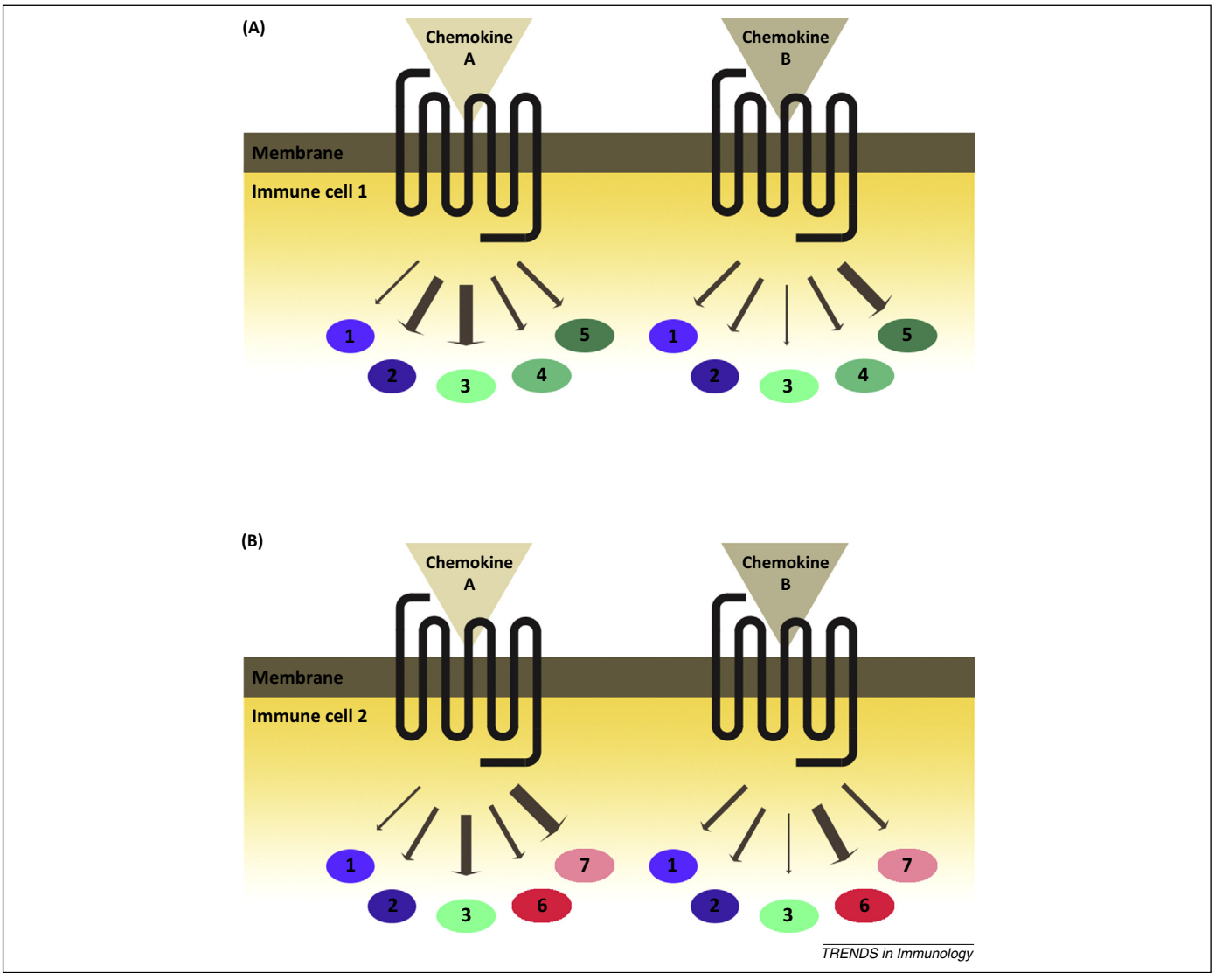

Figure 3. Schematic representation of biased signalling through chemokine receptors. The extent of signalling via signalling proteins 1–7 is represented by the thickness of the arrows. **(A)** Chemokines A and B bind to the same chemokine receptor in immune cell 1, but activate distinct signalling pathways. A signals predominantly via proteins 2 and 3, whereas B signals mainly via protein 5. **(B)** Immune cell 2 expresses signalling proteins different from immune cell 1, which results in differential signalling profiles for chemokine A and B.

for G protein signalling or β-arrestin signalling, respectively [6,47,48]. In addition to the 'snapshots' of bias in crystal structures, the emerging field of protein molecular dynamics further contributes to our understanding of ligand bias. Such studies have simulated at the atomic level how small perturbations at the more extracellularly located ligand binding site can lead to large conformational changes at the intracellular side of the receptor [49]. Importantly, not only do we start to understand the molecular features of biased signalling, we now also recognize its implications as it may lead to the development of therapeutics that have selective efficacy and fewer side effects [50].

The chemokine receptor family is prone to ligand-induced biased signalling, because most chemokine receptors can be activated by multiple chemokines. Interestingly, this is different from many other GPCRs with only one endogenous ligand, where ligand bias has largely been observed for synthetic agonists only. In several studies the activation of multiple pathways has been compared among the different chemokines, and for certain chemokine receptors, a biased signalling pattern has been discovered (Figures 2 and 3). These receptors include CCR1 [51–54], CCR2 [55–57], CCR5 [58–60], CCR7 [61–63], CCR10 [52], and CXCR3 [52,64]. With the exception of the homeostatic chemokine receptor CCR7, all are inflammatory chemokine receptors that bind multiple chemokines. The following sections discuss the biased signalling through each of these receptors individually.

Biased signalling through CC chemokine receptors

CCR1. The expression of CCR1 is known to coincide with the G protein subtypes $G_{i/o}$, G_{14} and G_{16} [65]. Four chemokines, CCL3, CCL5, CCL7, and CCL15/HCC-2, were found to differentially activate these G protein subtypes in COS-7 cells or HEK293 cells transfected with CCR1, resulting in different intracellular levels of cAMP, ERK, and IP [51]. More specifically, CCL15 was discovered to be the only chemokine ligand that did not signal via G_{14} and G_{16}. The inability of CCL15 to activate CCR1 as effectively

as the others could potentially be caused by its long N-terminal region. The N-terminal region is responsible for receptor activation, whereas the N-loop region is responsible for receptor binding. CCL15 can bind the receptor with high affinity due to its structural homology to other CC chemokines in the N-loop region, but its extended N-terminal region largely precludes subsequent receptor activation [53]. Further support comes from the finding that neither CCL14/HCC-1 nor CCL23/myeloid progenitor inhibitory factor (MPIF)-1, both having a long N-terminal region, can activate CCR1 despite sufficient binding affinity [51]. Another study on CCR1 agonism examined a total of eight different chemokine ligands, and reported that CCL8 was a poor G protein activator, whereas CCL4 was proposed to act as an *in vivo* inhibitor of CCR1 in the leukaemia cell line HL-60 because it showed only marginal receptor activation in functional assays [54]. In CCR1-transfected HEK293 cells, CCL5 and CCL23 have been identified as G protein-biased chemokines compared to CCL3, whereas CCL5, CCL15, and CCL23 display bias for internalization following β-arrestin recruitment [52]. All these different studies illustrate that CCR1 is heavily prone to biased signalling.

CCR2. The CCR2 receptor has been reported to bind eight chemokines, namely CCL2, CCL7, CCL8, CCL11, CCL13, CCL16, CCL24/eotaxin-2, and CCL26/eotaxin-3. Berchiche *et al.* studied the G_i activation and β-arrestin recruitment profiles of these different ligands in HEK293 cells transfected with CCR2 [55]. Overall, the potency and efficacy rank orders of G protein activation and β-arrestin recruitment were comparable. However, when the β-arrestin subtypes 1 and 2 were compared, the weak arrestin recruiters CCL8 and CCL13 were found to show bias towards β-arrestin 2. Interestingly, the study also reported the kinetics of the CCR2–β-arrestin complexes. CCL7 had a high potency to recruit β-arrestin, but with a short half-life of the signal, whereas CCL8 and CCL13 induced weak but stable signals over time. These differences in kinetic patterns are additional factors leading to signal bias that deserve more attention; it has been reported for other GPCRs that this can be an underlying factor for differential signalling as well as signal termination [66]. Besides the signalling properties of the different CCR2 ligands, it has been reported that CCL2, CCL7, and CCL13 induce homodimer formation of CCR2, whereas the other chemokines do not [55]. The formation of dimers, hetero- or homo-, is yet another mechanism to create bias in signalling and should therefore be taken into account when interpreting signal bias of any chemokine receptor.

CCR5. The signalling properties of CCL3, CCL4, CCL5, CCL8, and CCL13 via CCR5 have been investigated in cAMP accumulation and Ca^{2+} mobilisation assays in CHO cells stably expressing CCR5. The potency rank order of chemokine ligands was similar between the two assays, however, CCL13 was completely unable to affect cAMP concentrations [58]. The Ca^{2+} responses were G protein dependent, except for CCL3 that produced a Ca^{2+} signal in a partly G protein-independent manner. These results illustrate that the CCR5 chemokines act differently. Interestingly, other groups that used different cell systems, including RBL-2H3 and COS-7 cells expressing CCR5, reported different rank orders of signalling efficiencies [60,67]. This indicates cell-type specific effects or differential activation of the various intracellular effectors by these chemokines (Figure 3). It further emphasizes that the choice of cell lines must be an important parameter; primary cell lines and immune cells may better represent an *in vivo* relevant bias in signalling. The underlying mechanism for the differential effects of CCR5 ligands has been addressed in studies that focussed on the structural determinants at the receptor level. Biased signalling could be a result of ligand-specific induction of phosphorylation sites and thereby specific recruitment of GRKs, which differs among the CCR5 chemokine ligands [60]. A mutagenesis study further explored molecular determinants of CCR5-induced signalling. Residues in helices VI and VII were identified that are responsible for causing biased signalling [59]. In the crystal structures of the serotonin receptors it was the orientation of these same helices that was found to induce biased signalling [6]. It would be interesting to determine if these structural features can be translated to other chemokine receptors as well, which would be helpful for drug development targeting these receptors.

CCR7. CCL19/Epstein–Barr virus–induced molecule 1 ligand chemokine (ELC) and CCL21/secondary lymphoid tissue chemokine (SLC) are the endogenous agonists for CCR7. They are equally active in promoting G protein activation, Ca^{2+} mobilization, and chemotaxis [62,63]. However, in terms of β-arrestin recruitment and subsequent internalization of the receptor they behave differently. It was found that β-arrestin 3 was responsible for CCR7 internalization in the T cell lymphoma cell line HuT78 after stimulation with CCL19, which was not the case for CCL21-induced internalization [61]. Although both ligands allowed recruitment of β-arrestin 2, only CCL19 led to redistribution of β-arrestin-2 into endocytotic vesicles and classical receptor desensitization in HEK293 cells stably expressing CCR7 [62]. The underlying mechanism for this difference in β-arrestin recruitment was found to be a differential recruitment of GRKs that phosphorylate CCR7. CCL19 activates both GRK3 and GRK6, whereas CCL21 activates GRK6 only. Kohout and co-workers have reported findings that support this differential recruitment of GRKs, revealing that CCL19 induces specific phosphorylation sites on CCR7 in HEK293 cells [63].

CCR10. CCL27/cutaneous T cell-attracting chemokine (CTACK) and CCL28/mucosae-associated epithelial chemokine (MEC) are the two endogenous chemokines for CCR10. Both ligands are capable of G protein signalling measured by cAMP accumulation, but only CCL27 is capable of recruiting β-arrestin and internalizing the receptor [52]. In the same study it was assessed that CCL28 displayed a higher efficacy for migration of peripheral blood mononuclear cells (PBMCs). The biased signalling of CCL28 was speculated to be causal for this effect, explained by the lack of β-arrestin signalling and internalization that could result in prolonged and unopposed G protein activation [52].

Biased signalling through CXC chemokine receptors and ACKRs

CXCR3. CXCR1, CXCR2, and CXCR3 are the three CXC chemokine receptors that can each bind to multiple chemokines, but thus far biased signalling has only been reported for CXCR3. The activation of CXCR3 by CXCL9/monokine induced by gamma-interferon (MIG), CXCL10/IP-10 and CXCL11/interferon-inducible T cell alpha chemoattractant (I-TAC) has been investigated in a label-free impedance-based cellular assay [64]. For this technique, low-voltage currents run through microelectrode sensors at the bottom of a plate to which cells are attached. Changes in impedance upon ligand addition are continuously measured, and reflect receptor activation in the absence of a chemical or biological biosensor within the cell. Using this technique, Watts *et al.* showed that CXCL9 behaved as a biased CXCR3 agonist in HEK293 cells stably expressing CXCR3, stimulating solely G protein-dependent pathways [64]. However, another study compared cAMP accumulation, β-arrestin recruitment and internalization in classical functional assays with HEK293 cells transfected with CXCR3, and reported the opposite finding of CXCL9 being β-arrestin biased [52]. In addition CXCL11 was biased towards internalization. Apparently, CXCR3 is subject to biased signalling, of which the extent and nature may be determined by the different receptor expression levels and cellular assays used (Figure 3). Although future studies will ascertain whether biased signalling through other CXC receptors exists, we would hypothesize that this is likely, because, for example, CXCR2 binds seven different chemokine ligands.

ACKRs. It should be noted that the ACKRs [16] such as ACKR1 (Duffy antigen/receptor for chemokines (DARC)), ACKR2 (D6), ACKR3 (CXCR7), and ACKR4 (chemocentryx chemokine receptor (CCX-CKR)) can bind a wide variety of chemokine ligands, with up to 18 ligands for ACKR1. These receptors were initially regarded as nonsignalling decoy receptors because of their inability to activate typical G protein-signalling pathways, and therefore ACKRs were proposed to only scavenge ligands or function as co-receptors [17]. However, some recent studies have shown that these ACKRs can signal predominantly through β-arrestins. The chemokines CXCL11 and CXCL12 induce β-arrestin-2 recruitment to ACKR3 transfected in CHO cells, and a small molecule ACKR3 ligand has been reported to induce this β-arrestin-2 recruitment with even higher potency and efficacy [68]. Another study has also reported that CXCL12 induces β-arrestin-2 recruitment to ACKR3 in HEK293 cells transfected with ACKR3, as well as rat vascular smooth muscle cells (VSMCs), which results in downstream activation of ERK [69]. These signalling properties were discovered to be important in physiologically relevant cell lines, because the ACKR3-mediated migration of rat VSMCs was found to be a β-arrestin-mediated process [69]. Besides ACKR3, ACKR2 can also recruit β-arrestins, however, further evidence of subsequent signalling remains to be elucidated for this receptor [70]. Given these reports of β-arrestin recruitment and signalling through ACKRs, it has been proposed that these receptors should be regarded as a subfamily of β-arrestin-biased GPCRs. However, there is now one case reported of ACKR3 signalling via $G_{i/o}$ in primary rodent astrocytes and human glioma cells [71]. This finding reveals the presence of thus far hidden properties of ACKRs, and the exact role and significance of ACKR-mediated signalling remains an open question for future research. ACKRs serve homeostatic functions by clearing chemokines from circulation and tissues [72], and in addition viruses and parasites are capable of modulating the expression of ACKRs to elude chemokines [73]. These receptors are therefore important for innate and adaptive immunity, emphasizing the necessity to resolve their functioning.

Concluding remarks

Here, we have discussed the regulation and bias in chemokine expression and signalling at a systems, cellular, and molecular level. The presented evidence implies that the focus on only one natural ligand of a chemokine receptor as a prototype agonist is insufficient; neither should we focus on one single chemokine receptor *in vitro* because there might be several other receptors that determine the fate of the immune cell *in vivo*.

Over the past few years the 'drugability' of chemokine receptors has been reviewed and questioned [74]. The family of chemokine receptors is involved in a wide variety of diseases, mostly characterized by chronic inflammation.

Box 2. Chemokine signalling: implications for drug discovery in immunology

The complexity of the chemokine receptor family and its ligands should be considered during drug discovery. Most chemokine receptors are activated by multiple chemokine ligands, which should be taken into account upon developing small molecule antagonists. This can be illustrated by research on the chemokine receptor CCR1. For this receptor, it has been shown that small molecule ligands can both act as allosteric enhancers for CCL3 and at the same time as a competitive blockers of the binding of CCL5 [84]. This drug-induced bias is important, because chemokines are differentially expressed and regulated during immunopathology.

Not only small molecule ligands, but also short lipidated peptide sequences named pepducins have been found to be candidate drugs targeting chemokine receptors. The pepducin ATI-2341 selectively targets CXCR4 and is an allosteric agonist *in vitro* as well as *in vivo* [85]. In a recent study it was discovered that ATI-2341 revealed functional selectivity for G_i pathways over G_{13} and β-arrestin [86]. In comparison to the CXCR4 antagonist AMD-3100, which is used in the clinic to mobilize haematopoietic stem cells from the bone marrow for transplantation of leukaemia patients [76], the pepducin ATI-2341 does not induce the additional undesired mobilization of lymphocytes [85]. Whether CXCR4-mediated biased signalling of ATI-2341 with respect to AMD-3100 is causal for this difference remains a question for further research.

Besides the pepducins, small molecule ligands can also induce biased signalling upon binding to chemokine receptors [59,64,87,88]. For CCR5, the small molecule agonists YM-370749 and ESN-196 are able to induce G protein coupling and activation of Ca^{2+} responses [87,88]. However, they do not stimulate chemotactic activity but instead induce internalization of CCR5 from the cell surface [87,88]. These ligands are therefore functionally selective CCR5 ligands that act differently from the chemokine CCL5. This biased profile of small molecules acting via CCR5 could be of use as a novel class of anti-HIV-1 therapeutics, for which internalization of the receptor has been found to inhibit viral entry to the cell.

In conclusion, it is important to consider that drugs might differently affect one chemokine receptor depending on the chemokine that binds this receptor. In addition, these drugs can exert biased signalling via chemokine receptors themselves.

Although >50% of these receptors have been examined in clinical trials in the past decade, only two drugs targeting chemokine receptors have been approved by the regulatory authorities. The CCR5 antagonist maraviroc inhibits entry of HIV into CCR5-positive cells [75], and the CXCR4 antagonist AMD3100 is used to mobilize human haematopoietic stem cells from the bone marrow [76]. Notably, neither of these conditions is an inflammatory disease, nor is biased signalling involved. Possible reasons for the failure of other drug candidates targeting chemokine receptors are: (i) lack of efficacy due to inappropriate disease models; (ii) lack of efficacy due to poor pharmacokinetics such as binding to serum components; and (iii) off-target (side) effects. The complexity at multiple levels of the chemokine system is another factor that introduces challenges for effective intervention in disease states (Box 2). Integration of these issues in early stages of drug discovery and development programmes is necessary in order to develop clinically effective drugs with an acceptable benefit/risk profile. The clinical relevance of biased ligands is illustrated by the μ-opioid receptor agonist TRV130, which has recently been tested in Phase I studies and is entering Phase II in 2014 for the treatment of acute pain [77]. TRV130 causes G protein signalling with similar potency and efficacy as morphine, but with far less β-arrestin recruitment and receptor internalization [78]. Subsequent studies in mice have demonstrated that this bias results in higher analgesic efficacy, less gastrointestinal dysfunction, and less respiratory suppression than morphine.

In summary we have reviewed the regulation of and bias in chemokine receptor signalling that should be taken into account by immunologists as well as pharmacologists (Figure 1). The expression profile of chemokine ligands at the target tissue should be determined and considered in order to design appropriate *in vitro* studies, taking into account the differential expression profiles in health and disease. This is important given the aforementioned data that have reported crucial and different roles between chemokine ligands that bind to one chemokine receptor. In addition, the growing evidence of biased signalling through chemokine receptors shows that we cannot restrict ourselves to incorporate one chemokine ligand only, because the effect of drug candidates in the presence of other chemokine ligands might be different. Although heterologous cell systems can be used at the start of a project, the function of the chemokine receptor and impact of a drug should be studied in physiologically relevant cells as early as possible, because coexpressed proteins and receptors can modulate the behaviour and response of a chemokine receptor. It is hoped that this review will help in designing such experiments.

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