

Biased agonism at chemokine receptors: obstacles or opportunities for drug discovery?

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ABSTRACT

Chemokine receptors are typically promiscuous, binding more than one ligand, with the ligands themselves often expressed in different spatial localizations by multiple cell types. This is normally a tightly regulated process; however, in a variety of inflammatory disorders, dysregulation results in the excessive or inappropriate expression of chemokines that drives disease progression. Biased agonism, the phenomenon whereby different ligands of the same receptor are able to preferentially activate one signaling pathway over another, adds another level of complexity to an already complex system. In this minireview, we discuss the concept of biased agonism within the chemokine family and report that targeting single signaling axes downstream of chemokine receptors is not only achievable, but may well present novel opportunities to target chemokine receptors, allowing the fine tuning of receptor responses in the context of allergic inflammation and beyond. *J. Leukoc. Biol.* 99: 901–909; 2016.

Introduction

Chemokine receptors are key orchestrators of cell migration, both during development and during an immune response. More than 20 members of the chemokine receptor family have been identified to date, together with >40 chemokine ligands (1). Given the expression of chemokine receptors on a wide range of hematopoietic and nonhematopoietic cells, over- or inadvertent expression of chemokines has been implicated in the pathogenesis of numerous inflammatory diseases, including allergic asthma, atopic dermatitis, atherosclerosis, and rheumatoid arthritis (2–5). However, despite an extensive worldwide research effort, successes in translating our understandings of

chemokine biology to the clinic have been limited to the CCR5 antagonist maraviroc and the CXCR4 antagonist mozobil, neither of which has shown efficacy in an inflammatory clinical setting. In this minireview, we discuss recent discoveries surrounding the concept of biased agonism at chemokine receptors, focusing on the receptor CCR4, which we believe shows potential for exploitation to provide more specific, efficacious antagonists for the treatment of a variety of diseases.

HARNESSING BIASED AGONISM FOR DRUG DISCOVERY

Chemokines are noted for their promiscuity (i.e., multiple chemokines typically bind to the same receptor and chemokines seldom bind to a single specific receptor). The physiologic relevance of these traits has long been the subject of debate within the chemokine field. Originally thought to be a form of redundancy to ensure robust outputs in the face of microbial subversion (6), increasingly numerous examples from the field of GPCRs point to the fact that structurally different ligands acting on the same receptor can activate different signaling pathways within the cell (7, 8). The result has been termed functional selectivity or biased agonism (9), whereby an agonist may preferentially stabilize a receptor conformation over another, leading to the recruitment of a particular group of intracellular signaling molecules to the receptor and the preferential activation of one downstream signaling pathway over another (Fig. 1). Biased agonist activity has been proposed by Steen and colleagues (8) to take 3 forms: *ligand bias*, *receptor bias*, and *tissue bias*. Ligand bias is defined as the diverse response that ensues when different ligands activate the same receptor. Receptor bias describes the process by which the same ligand induces different responses from different receptors. Tissue bias reflects the situation where the same ligand elicits different responses

Abbreviations: ACKR = atypical chemokine receptor, ANG II = angiotensin type 2, ATLL = adult T-cell leukemia/lymphoma, BAL = bronchoalveolar lavage, BRET = bioluminescence resonance energy transfer, CGRP = calcitonin gene-related peptide, CHO = Chinese hamster ovary cell, CS = chondroitin sulfate, ERK = extracellular signal-regulated kinases, GRIK = G protein-coupled receptor kinase, HEK = human embryonic kidney cell,

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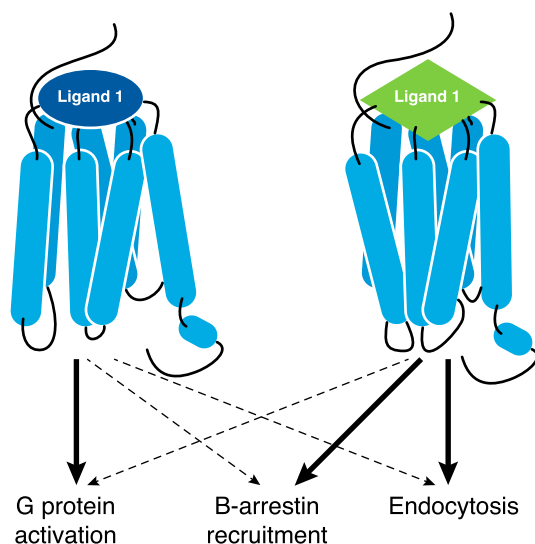


Figure 1. Ligand bias at chemokine receptors. Two different chemokines that exhibit ligand bias at the same receptor by stabilizing distinct receptor conformations and therefore preferentially activate certain intracellular pathways (bold arrows) over other pathways (dashed arrows).

depending on the cellular context in which it is expressed. A migrating cell that is under the instructions of chemokines to leave the confines of the bloodstream to explore a tissue is likely to encounter all three types of biased signaling as its microenvironment changes, exposing the cell to a variety of chemokine combinations. The physiologic consequences of biased agonism at chemokine receptors are now beginning to be appreciated and may go some way toward explaining the lack of success of chemokine receptor antagonists in the treatment of inflammatory conditions. As such, biased agonism can be viewed as an exciting opportunity for drug discovery.

As a means of fine tuning chemokine receptor signaling, agonists that are biased toward one signaling pathway but spare another may be therapeutically advantageous. For example, the antagonist TRV120027 (Ser-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH) binds to the ANG II receptor and inhibits the G-protein signaling induced by ANG II, yet leaves β -arrestin signaling intact. When examined in in vivo rat models of heart failure, TRV120027 improved both cardiac performance and cardiac stroke volume, presumably by maintaining the activation of the p42, p44, MAPK, and SRC signaling pathways (10). This finding is in contrast to conventional ANG II receptor antagonists, which have been reported to result in decreased mean arterial pressure and cardiac performance.

The concept of biased agonism adds complexity to the well-established definitions of agonists and antagonists. Synthetic small-molecule antagonists are typically allosteric, by definition, binding to a site on a receptor that is distinct from the site bound

by the orthosteric, or natural ligands of the receptor. For example, reparixin (formerly known as repertaxin), an allosteric ligand of the chemokine receptors CXCR1 and -2 with nanomolar affinity, does not displace radiolabeled CXCL8 from either receptor (11). Full agonists are usually identified as agonists that produce the maximum response for a given signaling pathway, whereas antagonists maximally inhibit this response. Partial agonists induce a submaximum response compared with that of a full agonist, even when all of the available receptors are occupied. Inverse agonists are ligands with antagonist properties that act upon constitutively active receptors to decrease signaling responses to below basal levels (12). Considering biased agonists, a ligand that may appear to be a full agonist of 1 functional read-out may also be described as an antagonist of another pathway. Such is the case for the β -blockers, propranolol, and carvedilol, which are inverse agonists of G_{α_s} -mediated cAMP production, yet act as agonists of the ERK pathway (13, 14).

EVIDENCE FOR BIASED AGONISM AT CHEMOKINE RECEPTORS

Biased signaling appears to be a common feature of the chemokine receptor family, given that most chemokine receptors can be activated by multiple chemokines, and there is growing evidence that activation of multiple receptors can result in differential functional responses (summarized in **Table 1**). This feature contrasts with GPCRs with a single endogenous ligand, where ligand bias has largely been observed for synthetic agonists only. As with other biased GPCR agonists, the relative potencies of chemokines in differing in vitro assays, such as β -arrestin recruitment, cAMP production, and calcium release, are not identical, suggestive of biased agonist activity (15, 16). However, our understanding of the molecular mechanisms by which this activity occurs is, at best, sketchy. In this section, we will discuss biased agonist activity at the receptors CCR1, CCR2, CCR5, CCR7, ACKR2/D6, and ACKR3/CXCR7, before focusing on recent data from our own and other groups pointing to biased agonist activity at CCR4.

CCR1

CCR1 was the first CC chemokine receptor to be described (17, 18). It binds more than a third of the entire CC chemokine family (19). Using a COS cell transfectant system in which CCR1 was coexpressed with a variety of G_{α} subunits, Tian et al. (20) showed the chemokines CCL3 and -15 to be able to couple to $G_{\alpha_{14}}$ and $G_{\alpha_{16}}$ to generate inositol phosphate production, whereas CCL5 and -7 were without activity. Rajagopal and coworkers (23) subsequently used assays of cAMP inhibition, β -arrestin recruitment and CCR1 endocytosis to compare a handful of CCR1 ligands in a transfectant system. CCL23 was found to be the most potent ligand for cAMP inhibition, followed by CCL3 and -5, with all 3 ligands effective at subnanomolar concentrations. However, it was notable that unlike CCL23, CCL3 and -5 failed to completely suppress cAMP signaling, with 25% of the response still intact at the highest concentration ($1 \mu\text{M}$). All 3 ligands fared well in assays of β -arrestin recruitment

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LIMK = LIM kinase, MAPK = mitogen-activated protein kinases, PAK = p21-activated kinase, RA = retinoic acid, P2 = position 2, PF = platelet factor, RBL = rat basophilic leukemia, RCC = renal cell carcinoma, SAR = structure-activity relationship, SRC = sarcoma family kinase, T_{reg} = T regulatory cell, VSMC = vascular smooth muscle cell

TABLE 1. Chemokine receptors and ligands for which biased agonism has been reported in the literature

Chemokine receptor	Chemokine ligands	Effect	Reference
CCR1	CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23	- Differential activation of G protein subtypes. - CCL5, CCL15, CCL21 display internalization bias via β-arrestin	[20] [15]
CCR2	CCL2, CCL5, CCL7, CCL8, CCL13, CCL16	- CCL8 and CCL13 are biased toward β-arrestin2 rather than β-arrestin1. - Kinetic differences in β-arrestin recruitment.	[24]
CCR5	CCL3, CCL4, CCL5, CCL7, CCL14, CCL15	Ca^{2+} mobilization by CCL3 alone is partially G protein-independent. - CCL13 has no effect on cAMP accumulation.	[30]
CCR4	CCL17, CCL22	- CCL22 is the dominant ligand in CCR4 endocytosis, calcium flux and chemotaxis assays. - CCL22 but not CCL17 couples CCR4 to β-arrestin2. - CCL17 stimulates greater αCGRP release than CCL22 in hBECs. - CCL22 activates a greater proportion of receptors than CCL17.	[53] [54] [55]
CCR7	CCL19, CCL21	CCL19 but not CCL21 induces β-arrestin-mediated CCR7 internalization.	[32]
CCR10	CCL27, CCL28	- Differential GRK recruitment. - CCL27 but not CCL28 induces β-arrestin-mediated CCR10 internalization.	[35] [23]
CXCR3	CXCL4, CXCL4L1 CXCL9, CXCL10, CXCL11	- CXCL11 shows internalization bias. - Cell-type dependent G protein/β-arrestin bias shown for CXCL9.	[82] [83]
ACKR2/D6	CCL2, CCL3 CCL22, CCL14	Rac1-PAK1-LIMK1-cofilin signaling pathway	[40]
ACKR3/CXCR7	CXCL11, CXCL12	G protein independent, β-arrestin2 biased signaling.	[10]

with nanomolar potencies and similar efficacies. Most striking were the findings when endocytosis was examined. Although 30 nM CCL23 induced complete internalization of CCR1, CCR3 and -5 were poorly potent and were efficacious with barely 20% of the receptor endocytosed at the 1 μM concentration.

Chou et al. (21) examined the effects of CCR1 ligands on endogenous CCR1 in RA-treated HL-60 cells, examining potency and efficacy in assays of GTP γ S activation, intracellular calcium flux, and chemotaxis. In contrast to transfectant studies where all the CCR1 ligands tested (except CCL2) were full agonists, the researchers observed CCL23 to be the most efficacious ligand, although CCL3 was several orders of magnitude more potent. Gilchrist et al. (22) characterized some small-molecule antagonists of CCR1 that inhibited chemotaxis in response to CCL3, but had no antagonistic activity in assays of β -arrestin recruitment. Conversely, they were also able to identify compounds that inhibited β -arrestin recruitment, but not CCR1 internalization. These data indicate that β -arrestin recruitment and receptor internalization at CCR1 are 2 separate processes, in keeping with the data from the study by Rajagopal et al. (15).

CCR2

CCR2 binds what used to be referred to as the MCP family of chemokines, comprising CCL2, -7, -8, and -13. Berchiche and colleagues (23) used an HEK293 expression system to examine the ability of CCR2 to recruit β -arrestin-1 and -2 in response to all 4 ligands. The chemokines recruited β -arrestin-2 to CCR2 with a potency rank order of CCL2 $\geq$ CCL8/CCL7 $\geq$ CCL13. A similar rank order of potency for CCL2, -7, and -8 was reported for β -arrestin-1 recruitment, although CCL13 showed little activity. CCL2 was the most efficacious ligand in both assays. Inhibition with pertussis toxin showed the process of arrestin recruitment to be largely independent of $\text{G}\alpha_i$ activation. Similar rank potencies for CCR2 endocytosis and $\text{G}\alpha_i$ activation (measured by BRET) were reported. This result suggests that, in the HEK293 cell system, CCR2 internalization is likely to be a function of arrestin recruitment. CCL8 was shown to have the least efficacy in terms of $\text{G}\alpha_i$ activation, which fits with chemotaxis data from our own group in which CCL8 was found to be largely devoid of activity (24).

More recently, Corbisier and colleagues (16) have examined CCR2 coupling to a variety of $G\alpha_i$ subtypes ($G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$), $G\alpha_o$ isoforms ($G\alpha_{oa}$ and $G\alpha_{ob}$), and $G\alpha_{12}$ and $G\alpha_{13}$ by using an HEK293-BRET-based system. Once more, CCL2 was shown to be the dominant CCR2 ligand for $G\alpha_i$ activation, exhibiting significantly more potency and efficacy than CCL8 and -13. Similar profiles were observed with $G\alpha_o$, $G\alpha_{ob}$, and $G\alpha_{12}$ activation, whereas all 4 CCR2 ligands did not activate $G\alpha_{i3}$. Notably, their relative activities in this assay did not overlay their relative binding affinity for CCR2, suggesting an element of signaling bias at CCR2.

CCR5

CCR5 is a receptor for the chemokines CCL3, -3L1, -4, and -5 and is notable for its expression on monocytes/macrophages which M-tropic strains of HIV-1 utilize as an entry factor in conjunction with CD4 (25). Consequently, CCR5 ligands have been a source of great interest: at high concentrations, they act as inhibitors of HIV entry (26). Using RBL cells stably expressing human CCR5, Oppermann et al. (27) showed that CCL5 coupled more efficaciously to intracellular Ca^{2+} release than either CCL3 or -4, which correlated with the ligands' ability to induce C-terminal phosphorylation of CCR5. This "pecking order" was corroborated in CCR5 CHO transfectants, when Mueller and colleagues (28) showed CCL5 to have greater potency than CCL3 and -4 in assays of GTP γ S binding and receptor internalization. A follow-up study by the same group extended these findings to include the use of pertussis toxin and found that, whereas intracellular Ca^{2+} release in response to CCL4 and -5 were abolished by pertussis toxin treatment, CCL3 responses were only partially affected, suggestive of biased coupling to $G\alpha_{i/o}$ -independent pathways (29). Corbisier et al. (16) recently extended these findings by means of BRET assays examining specific G protein activation. Although CCL3, -4, -5, -8, and -13 were found to significantly activate $G\alpha_i$, $G\alpha_o$, and $G\alpha_{12}$ subunits, evidence of bias was forthcoming, with CCL13 displaying significantly lower potency and efficacy than the other chemokines, showing the advantages of using an extended ligand set at such a promiscuous receptor, since an earlier study by Rajagopal et al. (15) reported little evidence of signaling bias at CCR5, with CCL3, -3L1, and -4 inducing the inhibition of cAMP production, β -arrestin recruitment, and CCR1 endocytosis, with similar potency and efficacy.

CCR7

CCR7 is expressed by dendritic, T, B, and NK cells and directs the chemotaxis of these cells toward lymphoid organs, which generate gradients of the 2 endogenous CCR7 ligands: CCL19 and -21 (30). CCL19 and -21 have been shown to couple the receptor to different GRKs, which are responsible for mediating the different downstream signaling events. Both chemokines were originally thought to display similar levels of $G\alpha_i$ coupling (31), although this idea has recently been challenged by another study (16). What is undisputed is that, although CCL21 interacts with CCR7 just as efficiently as CCL19 and leads to similar outputs in chemotaxis assays, CCL21 is considerably more efficacious in inducing CCR7 internalization (31–33). CCL19 binding to CCR7 recruits GRK3 and -6, leading to receptor phosphorylation, β -arrestin-2 recruitment, and receptor

desensitization. In contrast, CCL21 recruits GRK6 alone, which neither phosphorylates nor desensitizes CCR7, despite coupling to β -arrestin-2 (34). Differential GRK recruitment therefore introduces another level of regulation and specificity into the chemokine signaling cascade. Chemokine binding leads to the stabilization of the receptor in a conformation that favors the recruitment of specific GRKs, which phosphorylate distinct residues within the receptor's C terminus, allowing the association of the receptor with a distinct subset of intracellular signaling molecules.

ACKR2/D6

The atypical chemokine receptor ACKR2 (formerly known as D6) binds over a dozen CC chemokines associated with inflammation (35). ACKR2 is believed to function predominantly as a scavenger receptor, constitutively coupling to β -arrestins (36) and targeting chemokines for intracellular degradation. Evidence for biased signaling at ACKR2 first came from studies of a truncated form of CCL14^(9–74) which was readily endocytosed and degraded by ACKR2 in contrast to the full-length CCL14^(1–74), although both chemokines bound with identical affinity to the receptor (37). Truncation of CCL14 is believed to reveal a proline residue at P2 which is critical for activation of ACKR2, reminiscent of activation mechanisms found in more typical CC chemokine receptors such as CCR3, where one of our own modeling and mutagenesis studies implicated P2 of CCL11 in activating CCR3 via formation of a hydrogen bond with a highly conserved glutamate residue (E277) in helix VII of the receptor (38).

Subsequent studies implicated a G protein-independent, β -arrestin-1-dependent pathway in chemokine endocytosis, which was triggered by a Rac1-PAK1-LIMK1 cascade, concluding with cofilin phosphorylation and remodeling of the actin cytoskeleton. Borroni et al. (39) described ACKR2 as a biased receptor in terms of preferentially coupling to β -arrestins, presumably evolving a scavenger function as a result of mutations within ACKR that preclude coupling to the more typical downstream "machinery" of chemokine receptors and instead promote coupling to β -arrestins.

ACKR3/CXCR7

Another chemokine receptor that displays evidence of biased agonism is the ACKR known as ACKR3, previously known as CXCR7 (1). This receptor is notable for the fact that it binds both the CXCR3 ligand CXCL11 and the CXCR4 ligand CXCL12, but does not appear to activate conventional signaling pathways, such as the G protein signaling that leads to chemotaxis (40, 41). ACKR3 was initially thought to act solely as a decoy chemokine-scavenging receptor (42). This role is thought to be critical during the migration of interneurons during development where localized internalization of CXCL12 by ACKR3 prevents desensitization of CXCR4 (43). Similarly, CXCR4⁺ breast cancer cell metastasis is spatially regulated by the sequestration of CXCL12 by ACKR3 expressed on separate populations of tumor cells (44). It can now be appreciated, however, that ACKR3 in fact signals via β -arrestin-2 (45). This β -arrestin-2 bias at ACKR3 has functional consequences in rat VSMCs, which migrate in response to ACKR3 activation in a

β -arrestin-2-dependent manner (40). To further complicate matters, ACKR3 is also known to heterodimerize with the related receptor CXCR4 (46). In a breast cancer cell line, these heterodimers have been shown to recruit β -arrestin-2, leading to enhanced migration in response to CXCL12 together with impaired $G\alpha_i$ protein signaling (47). These data demonstrate that the β -arrestin-2-bias observed at ACKR3 can dramatically influence signaling via CXCR4, with consequences for cell migration.

EXPLORING BIASED AGONISM AT CCR4

Potential for the exploitation of biased agonism

Evidence of biased agonism is also emerging at the chemokine receptor CCR4 through the work of our own and other groups. CCR4 is expressed predominantly on Th2 and T_{reg} cells and has been appreciated as a potential target in the pathogenesis of allergic diseases, notably allergic asthma and atopic dermatitis (2, 3, 48). More of a surprise, perhaps, was the discovery that CCR4 is frequently up-regulated in ATL where it plays a role in skin homing, making the receptor an attractive therapeutic target (49). Mogamulizumab is a fully humanized monoclonal antibody specific for CCR4, which has proved efficacious in the treatment of ATLL. Disappointingly, however, no small-molecule chemokine receptor antagonists have been licensed for use in the allergic setting to date. A promising candidate CCR4 antagonist, GSK2239633, was discontinued after a phase I trial in healthy male subjects, because the compound did not reach the minimum target level of $\geq 90\%$ CCR4 inhibition in whole blood. At best, only 74% receptor occupancy was achieved 1 h after a 1500 mg dose of the compound was administered (50).

Evidence for biased agonism at CCR4

The 2 chemokine ligands of CCR4 are CCL17 and -22, which are produced by both dendritic and endothelial cells in the skin and also by airway epithelial cells (51, 52). Evidence suggests that the spatial distribution of these chemokines is essential for disease pathogenesis. For example, in inflamed but not in healthy, skin tissue, CCL17 is localized to endothelial cells, whereas CCL22 is produced by dendritic cells (52). In addition to their differing spatial distributions, CCL17 and -22 also behave differently in in vitro assays of CCR4 functionality. CCL22 is the dominant chemokine of the pair in inducing CCR4 endocytosis, as well as calcium flux and migratory responses (53). Compellingly, CCL22, but not -17, induces concentration-dependent coupling of CCR4 to β -arrestin-2 (54), defining both ligands as biased at CCR4 (Fig. 2A). Ligand bias at CCR4 is also evident from our own studies of bronchial epithelial cells. Stimulation of primary human bronchial epithelial cells with CCL17 induced 20,000-fold greater expression of the vasodilator α -CGRP than did stimulation with CCL22 (55). Whether this expression level is also an example of tissue bias is unclear at present, because we do not know whether T cells are also induced to produce α -CGRP in response to CCL17. Ligand and tissue biases need not be mutually exclusive. α -CGRP has been implicated in asthma pathogenesis (56), and CCL17 levels have been reported to be elevated in the BAL of patients with asthma (3); thus, the link

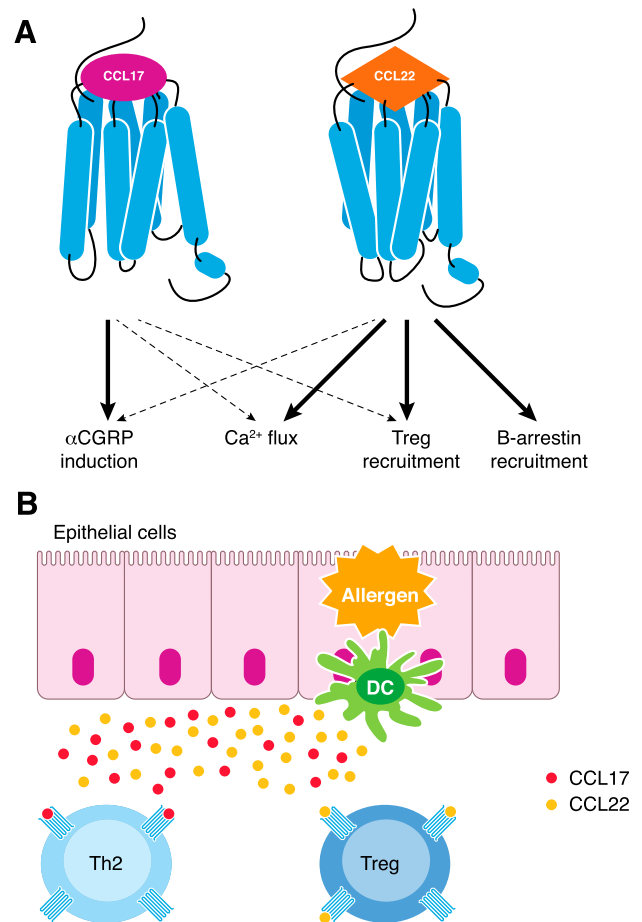


Figure 2. Ligand bias at CCR4. (A) Differently biased agonist activates at CCR4 ascribed to CCL17 and -22. (B) A suggestion of how the biased agonism may play out in allergic dermatitis based on in vitro findings. CCL17 is produced by sensitized epithelial cells and serves to recruit Th2 cells, whereas CCL22 is produced by dendritic cells and preferentially recruits T_{reg}s, leading to suppression of the Th2 response. Targeting CCL17 activation but sparing CCL22 may thus be advantageous.

between CCL17 expression and CCR4-dependent α -CGRP transcription could have consequences for therapeutic intervention at CCR4. In this situation, targeting CCL17 signaling rather than CCL22-driven signaling, to specifically inhibit α -CGRP production, could be an advantageous approach. CCR4 is expressed on T_{reg} cells, and in in vitro assays, CCL22 appears to be the dominant recruiting factor produced by dendritic cells after stimulation (57). One can therefore see that indiscriminate inhibition of CCR4 signaling could result in a loss of the natural immunosuppression provided by T_{reg}s, which may be a cause for concern. Such is the case for mogamulizumab, which depletes memory T_{reg} cells, in addition to inducing antibody-dependent cellular cytotoxicity against CCR4⁺ tumor cells (58). Although T_{reg} cell depletion typically leads to the promotion of antitumor immune responses, there are examples of autoimmune reactions associated with mogamulizumab treatment, such as Stevens-Johnson syndrome (59). Fine tuning the inhibition of CCR4 signaling through the use of biased drugs may reduce the risk of

potentially harmful side effects while promoting a more targeted anti-inflammatory effect (Fig. 2B). Recent work from the Balkwill lab studying human RCC by means of a tissue microarray revealed expression of CCR4 in 153 of 173 malignant tumor cores from 57 patients with advanced RCC. This correlated with the expression of the ligands CCL17 and -22 and the presence of infiltrating CCR4 CD4⁺ T cells. A comparative study of plasma samples from RCC patients and age-matched control samples produced the intriguing finding that circulating CCL17 levels were 2-fold higher in RCC patients, whereas circulating CCL22 levels were almost 4-fold higher in healthy controls. Determination of the CCL17 to -22 ratio was found to be a statistically significant indicator of tumor burden and survival, with healthy control subjects having a very low plasma CCL17:CCL22 ratio, whereas conversely, the most patients with renal cancer had a high plasma CCL17:CCL22 ratio. This result suggests that CCL17 and -22 have discrete functions in the pathogenesis of RCC, which could be appropriately targeted with selective CCR4 antagonists (60).

Mechanistic explanations for biased agonism at CCR4

Structurally, CCL17 and -22 share only 32% amino acid homology (61) and were originally proposed to interact with a common epitope on CCR4 via a conserved binding domain present in both chemokines. However, recent work has suggested that this simplistic view of CCR4 activation does not explain experimental observations. Using 2 distinct recombinant monoclonal antibodies against CCL17, each composed of a chimeric molecule with rat V_L and V_H domains fused with mouse IgG1 Fc, Santulli-Marotto et al. (62) found that, whereas both antibodies inhibited CCL17 function, only 1 of the 2 was effective in blocking CCL22 activity. Furthermore, in competitive binding assays, CCL17, but not -22, competed for CCL17 binding to either antibody, indicating that the 2 antibodies bind to nonoverlapping sites on CCL17. These differences suggest that CCL17 binds to a site on CCR4 distinct from that occupied by CCL22, which may account for their different responses in signaling assays.

Data from our own group have provided further insight into the modes of ligand binding to CCR4 (63). In support of the data by Santulli-Marotto et al. (62), there appears to be a distinct difference in the molecular mechanisms by which CCL17 and -22 activate CCR4. Whereas mutation of the C-terminal residue K310 had little effect on migration in response to CCL22, it abolished the chemotactic activity of CCL17. Molecular modeling of CCR4 suggests that K310 forms a salt bridge with an aspartic acid residue in the cytoplasmic region of the helix, thereby influencing interactions with intracellular G proteins (63). Following up this observation with competitive binding assays, Viney and colleagues noted that, whereas a 1000-fold excess of CCL17 was able to displace radiolabeled CCL17 from CCR4, CCL17 was unable to displace a significant proportion of [¹²⁵I]-CCL22 from CCR4, indicating that CCL17 and -22 recognize conformationally distinct populations of CCR4. In addition, the modes of action of 2 monoclonal antibodies specific for CCR4 were found to be different. The 10E4 antibody recognized a significantly greater proportion of receptors than did the 1G1 antibody and was more sensitive in CCR4 endocytosis assays. In

the competition assays, a 1000-fold excess of unlabeled CCL22 displaced a greater proportion of radiolabeled CCL22 than the 10E4 antibody, demonstrating the presence of a 10E4-insensitive population of CCR4 that remains sensitive to CCL22. Furthermore, although 10E4 significantly inhibited 1G1 binding to CCR4, 1G1 did not block 10E4 binding. These data are suggestive of a model in which 2 distinct conformations of CCR4 exist: a major population of receptors that is activated by both ligands and recognized by both antibodies and a minor population that is activated by CCL22 alone.

Toward the discovery of biased antagonists of CCR4

The above example demonstrates ably the proof of principle that inhibition of CCL17 binding and signaling (using the 10E4 antibody) leaves a proportion of CCL22 signaling intact. As such, it is conceivable that a small-molecule antagonist with similar properties could be developed to target CCR4 and block CCL17-signaling while sparing CCL22 signaling and therefore T_{reg} recruitment. CCR1-specific small molecules have already been described that inhibit chemotactic responses to CCL3, but have no effect on β -arrestin recruitment (22). Peptide-based chemokine receptor agonists and antagonists have been described in the literature—several of which target CXCR4 including the 17mer CXCR4 agonists RSVM and ASLW (64), peptide fragments that were derived from the CXCL12 sequence and ALX40-4C, a CXCR4 antagonist polypeptide of 9 Arg residues stabilized by terminal protection and inclusion of D-amino acids (65). However, problems with low potency (RSVM and ASLW peptides) and oral formulations (ALX40-4C) suggest that nonpeptide-based molecules are likely to fare better *in vivo*.

In terms of identifying biased antagonists of CCR4, AstraZeneca (Loughborough, UK) identified several small-molecule antagonists targeting CCR4 by using a high-throughput recombinant cell-based assay that measured CCL22-induced responses at CCR4. Among these antagonists was a series of pyrazinylsulfonamides that were reported to bind to an intracellular binding site on the receptor (66). This intracellular allosteric binding site (subsequently dubbed site 2) is distinct from both the orthosteric binding site and the site bound by another antagonist, BMS-397, termed site 1 (54). Unpublished mutagenesis analyses from our group indicated that site 1 encompasses hydrophobic residues in transmembrane helix III, whereas site 2 is likely situated in an intracellular location close to the lipid bilayer and involves residues within helix VIII.

In addition to displacing the endogenous CCR4 ligands, several small-molecule antagonists of CCR4 induce receptor internalization, with differing responses observed for site 1 and 2 antagonists. K777, a pyrimidine derivative, induced CCR4 internalization while also inhibiting CCL17-mediated chemotaxis (67). Likewise, Ajram et al. (54) found that compounds belonging to a class of arylsulfonamides bind intracellularly to site 2 and do not induce receptor internalization in contrast to antagonists belonging to a class of lipophilic amines that bind extracellularly to site 1.

In our own laboratory, we have observed that mutation of the cytoplasmic-most area of helix VII results in the dissection of a site 2 antagonist profile, with a site 2-specific compound that still abolishes CCL22 function but without inhibitory effects on

CCL17 signaling (unpublished). This result supports our previous data showing different molecular mechanisms for CCL22 and -17 activation of CCR4 (63) and suggests that the identification or design of CCR4 antagonists with selective activity at one CCR4 ligand over another is a distinct possibility.

CONCLUDING REMARKS

There is ample evidence to support the notion that biased agonism is a general feature of the chemokine network. A major challenge ahead lies with the biologists and physicians who have to decipher which pathways contribute to disease and which are necessary for immune homeostasis. Armed with this information, it is feasible that pharmacologists will identify the small molecules that spare desirable signaling pathways while targeting those that are pathogenic. The current reductionist approach of drug discovery favored by high-throughput screening methods may be insufficiently sophisticated to identify biased agonists when only one signaling outcome is measured (68). In the same vein, current screening regimens to deorphanize GPCRs are becoming increasingly reliant on complementation-based assays involving β -arrestin or components of the endocytosis pathway. If these are the sole step in a primary ligand screen, then it is likely that several ligand–receptor pairings will be missed. Simple adaptations to existing screening methodologies, ensuring that lead compounds and agonists are screened against several signaling outputs would circumvent this possibility. The current wealth of GPCR crystal and NMR structures (including the chemokine receptors CXCR1, CXCR4, and CCR5) is highly informative with respect to the molecular modeling of chemokine receptors and, coupled with the use of artificial agonists such as metal chelators (69,70) and SAR work around such molecules, much can be deduced about different active receptor conformations. This information could aid the rational design of selective antagonists (i.e., molecules that show bias and preferentially target a single pathway). Providing generic drug development obstacles can be overcome, we predict that selective antagonists are likely to have increased efficacy in the clinical setting.

Biased agonism is likely to be a product of another level of complexity in the chemokine field—namely, the many post-translational modifications of chemokines that are now appreciated, including truncation, sulfation, citrullination, and glycosylation (71). It is not inconceivable that such modifications fine tune chemokines to preferentially activate one pathway over another. Moreover, the ability of chemokines to form heterodimers and other higher order structures adds yet another dimension to the puzzle. The chemokine CXCL4/PF-4 can heterodimerize with several CC and CXC chemokines *in vitro*, notably CCL5 (72). Given the high serum levels of CXCL4 compared to other chemokines, coupled with the greater stability of the heterodimer (73) it is likely that CXCL4:CCL5 heterodimers prevail *in vivo*. One can speculate that this affects the biased signaling exhibited by CCL5 at both CCR1 and -5, although experimental data to support this idea is currently thin on the ground. Work from the group of Christian Weber has shown that inhibition of CXCL4–CCL5 heterodimer formation

in vivo is therapeutically beneficial in models of atherosclerosis where CCL5 drives disease, suggesting that heterodimerization is not only a concept, but a biologic reality (74). No doubt tied up in this thought are the roles of GAGs in sequestering higher order chemokine species on the surface of cells and presenting them to receptors (75). The activity of CXCL4 on monocytes and neutrophils has an absolute requirement for CS-decorated GAGs (76, 77).

There is ample evidence, both biochemical and structural, that chemokine receptors can form functional homodimers and heterodimers, most likely commencing during their biosynthesis and maturing as nascent proteins en route to the cell membrane. This notion raises the possibility that both positive and negative cooperativity come into play to further bias receptor signaling. The receptors CCR2 and -5 show considerable homology and have been shown to form heterodimers that exhibit both positive and negative cooperativity. Early studies suggested that heterodimer formation induced a degree of synergy in which there was a clear signaling bias, the switching of G protein coupling from $G\alpha_i$ to $G\alpha_q$, which resulted in leukocyte chemotaxis, giving way to cell adhesion (78). Subsequent studies from the group of Marc Parmentier (79) have reported that no synergy is apparent, with negative cooperativity observed between CCR2 and -5 heterodimers (i.e., the binding of a ligand for one receptor inhibited the binding of ligands at the receptor partner), with a receptor dimer able to bind just a single chemokine. The effects of heterodimerization on biased signaling clearly require further exploration, and the use of bivalent ligands probes to explore simultaneous interactions with both heterodimers may be a useful approach (80). Looking further afield, existing data also support the notion that chemokine receptors can form functional heterodimers, not only with other chemokine receptors, but also with other GPCRs. A recent example of is the discovery that CXCR4 heterodimerizes with $\alpha 1$ -adrenergic receptors in smooth muscle and plays a role in modulating adrenergic receptor function (81). In addition to demonstrations of physical association *in vitro*, administration of CXCR4 ligands to mice was shown to significantly affect their blood pressure, enhancing the response of the $\alpha 1$ -adrenergic receptor to the agonist phenylephrine. Whether chemokines can induce ligand bias at nonchemokine receptors within a dimer or higher order complex is currently unknown, but is clearly worthy of further investigation.

AUTHORSHIP

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DISCLOSURE

The authors declare no conflicts of interest.

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