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Review

Biased signaling pathways via CXCR3 control the development and function of CD4$^+$ T cell subsets

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ABSTRACT

Structurally related chemotactic cytokines (chemokines) regulate cell trafficking through interactions with 7-transmembrane domain, G protein-coupled receptors. Biased signaling or functional selectivity is a concept that describes a situation where a 7-transmembrane domain receptor preferentially activates one of several available cellular signaling pathways. It can be divided into 3 distinct cases: ligand bias, receptor bias, and tissue or cell bias. Many studies, including those coming from our lab, have shown that only a limited number of chemokines are key drivers of inflammation. We have referred to them as "driver chemokines." They include the CXCR3 ligands CXCL9 and CXCL10, the CCR2 ligand CCL2, all 3 CCR5 ligands, and the CCR9 ligand CCL25. As for CXCR3, despite the proinflammatory nature of CXCL10 and CXCL9, transgenic mice lacking CXCR3 display an aggravated manifestation of different autoimmune disease, including Type I diabetes and experimental autoimmune encephalomyelitis. Recently, we showed that whereas CXCL9 and CXCL10 induce effector Th1/Th17 cells to promote inflammation, CXCL11, with a relatively higher binding affinity to CXCR3, drives the development of the forkhead box P3-negative IL-10^{high} T regulatory 1 cell subset and hence, dampens inflammation. We also showed that CXCL9/CXCL10 activates a different signaling cascade than CXCL11, despite binding to the same receptor, CXCR3, which results in these diverse biologic activities. This provides new evidence for the role of biased signaling in regulating biologic activities, in which CXCL11 induces ligand bias at CXCR3 and receptor-biased signaling via atypical chemokine receptor 3. *J. Leukoc. Biol.* 99: 857–862; 2016.

Introduction

Chemokines are small (8–14 kDa), secreted proteins that regulate cell trafficking through interactions with a subset of 7TMD GPCRs [1–3]. They are the principal attractants of leukocytes to sites of inflammation and through the activation of adhesion molecules. promote leukocyte extravasation [4–8]. This makes chemokines key drivers of inflammation.

The question of why as many as ~50 chemokines and 20 receptors are required for inducing and regulating immune responses is complex and could be explained partially by the complex interplay between them, enabling a rapid, yet regulated, response. This includes not only competition of binding receptors but also partial agonistic and antagonistic effects, for example, CCL11 that functions as a partial agonist of CCR2b [9].

Several reports, including ours, reveal that only a limited number of chemokines (8–10 of almost 50 known chemokines) and their cognate receptors are mainly involved in promoting an inflammatory response [10–15]. Our working hypothesis is that as drivers of inflammation, these chemokines not only attract leukocytes but also direct their proinflammatory biologic properties [12, 13].

Many investigations analyzed the role of chemokines as proinflammatory mediators [16–18], with the aim to make them and their respective receptors as targets for therapeutic interventions in inflammatory autoimmune diseases [2, 19, 20]. We focused on chemokines that bind 2 receptors on CD4$^+$ T cells—CXCR4 and CXCR3—and found that the CXCR3 ligand CXCL11 and the CXCR4 ligand CXCL12, aside from attracting leukocytes to sites of inflammation, also direct their polarization of CD4$^+$ T cells into Tr1 cells, which leads to a restraining inflammation. CXCL11 is not the only ligand of CXCR3, but the receptor also binds CXCL9 and CXCL10. However, the 3 chemokines differ in their ability to activate signal transduction downstream of the receptors with opposing outcomes of the cell phenotype. Whereas CXCL11, which binds CXCR3 with higher affinity, induced T cell polarization into Tr1 cells, CXCL9 and CXCL10, with lower affinity for CXCR3, promote CD4$^+$ T cell polarization toward Th1/Th17 effector cells [21]. The recent finding is an example of how ligand-based

Abbreviations: 7TMD = 7-transmembrane domain, ACKR3 = atypical chemokine receptor 3, DC = dendritic cell, EAE = experimental autoimmune encephalomyelitis, FOXP3 = forkhead box P3, GPCR = G protein-coupled receptor, GVHD = graft-versus-host disease, mTOR = mammalian target of rapamycin, nT$_{reg}$ = naturally occurring regulatory T cell, Tr1 = T regulatory 1, T$_{reg}$ = regulatory T cell

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biased signaling leads to diverse biologic functions of $CD4^+$ T cells that regulate immunity.

LIGAND-BIASED SIGNALING AND ITS ROLE IN CHEMOKINE BIOLOGY

Biased signaling or functional selectivity is a concept that describes a situation where a 7TMDR preferentially activates 1 of several available cellular signaling pathways. It can be divided into 3 distinct cases: ligand bias, receptor bias, and tissue or cell bias (**Fig. 1**) [22–24]. Ligand bias describes a situation where different ligands bind the same receptor but induce diverse responses. It is not exclusive for chemokines [25, 26]. As for chemokines, 1 of the possible relevant examples is the CCR7 and its ligands CCL19 and CCL21. Both ligands are able to activate a variety of G proteins, but with different efficacies and efficiencies [22], and stimulate common signaling pathways; however, it has been suggested that only CCL19 induces internalization of the receptor [27]. Moreover, CCR7 can trigger different responses upon stimulation with CCL19 and CCL21 in T cells and DCs, thus dependent on its cellular context [28, 29]. The biologic consequences of these findings are yet to be addressed, as both chemokines hold similar properties with respect to cell migration.

Likewise, it was found that of the 2 CXCR2 ligands CXCL8 and CXCL7, CXCL8 is much more efficient in receptor internalization [30]. On the other hand, the binding of CXCL8 to the highly related receptors CXCR1 and CXCR2 induces different responses. Despite that both receptors couple to pertussis toxin-sensitive G proteins, only CXCR1 activates phospholipase D and the NADPH oxidase in response to CXCL8 in human neutrophils [31]. The chemokines CCL2 and CCL11 bind CCR2, although with different affinities [31], but activate opposing signaling mechanisms. CCL11, initially reported as a natural antagonist that competes for binding of CCL2 to CCR2, was shown, in addition via the MAPK cascade, to attenuate CCR2 signaling [32]. The observation that upon binding of CCL2 or CCL11, the receptor activates different isoforms of PI3K suggested ligand-induced different receptor active states [32]. More recently, it was reported that ligands of CCR2 and CCR5, which trigger typical cellular responses, such as cell migration, differ in their ability to activate available downstream signaling pathways [22, 24]. Likewise, for CXCR3 and its ligands, differences in signaling were reported [33–35]. The first observation that may implicate bias signaling of CXCR3 ligands came from Cox et al. [36], showing differential binding to receptor states of these ligands to their receptor. More recently, Thompson et al. [37] used $G\alpha_i$ knockout mice to show that $G\alpha_{i2}$ is the key G protein needed for CXCR3 signaling in the mouse, whereas $G\alpha_{i3}$ actually inhibits this process. In addition Colvin et al. [34] showed that CXCL9- and CXCL10-induced internalization requires serine/threonine residues (putative phosphorylation sites) on the receptor C terminus, whereas CXCL11-induced internalization depends on the third intracellular receptor loop. Notably, CXCL11 is much more efficient in inducing receptor internalization, making it less accessible to CXCL9 and CXCL10 [34, 35]. Very recently, we uncovered the biologic significance of these findings. Chemokines possess very short half-life time (in vivo). To facilitate a stable form of CXCL11 to be used as a drug, we have generated a stabilized form by generating a fusion protein in which CXCL11 was linked to IgG_1 (Fc; CXCL11-Ig) [21]. When administered during ongoing EAE, this fusion protein could rapidly suppress the disease by increasing the relative number of IL-10-producing Tr1-like cells (direct effect) and at the same time, leading to reduced polarization of Th1 and Th17 cells, which could be a result of CXCR3 desensitization, making it less accessible to CXCL9/CXCL10 [21]. A classic example for receptor-biased signaling is the interplay of CXCL12 with its receptors. This chemokine triggers full chemokine receptor signaling on CXCR4, including G protein-dependent cell migration and IL-10 production [38], whereas binding to the ACKR3 (CXCR7) induces G protein-independent arrestin recruitment and activation of the MAPK cascade [39].

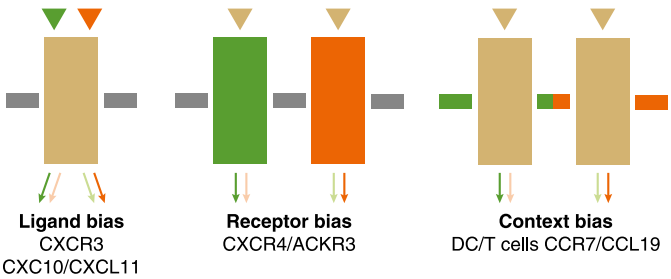

Figure 1. Biased signaling by chemokine receptors. Biased signaling or functional selectivity preferentially activates distinct cellular signaling pathways. Three cases are illustrated: ligand bias (left), receptor bias (center), and context/tissue or cell bias (right). Two different pathways (green and orange arrows in all panels) are preferentially activated by 2 distinct ligands (triangles) on the same receptor (left); by the same ligand acting on 2 distinct receptors (center); or by the same ligand/receptor pair but embedded in a distinct cellular context (right).

Nevertheless, the interplay of the 2 receptors, CXCR4 and ACKR3, is critical for CXCL12-mediated signaling. Initially, tentatively called "eccentric" [32], the trio displays tight interdependent regulation of biologic systems. ACKR3 functions mainly as sink and is often found in apposition to sites of CXCL12 production to ensure the formation of efficient chemotactic gradients [40–43]. In addition, the scavenging activity of the receptor is essential to prevent CXCR4 down-regulation, e.g., during migration of interneurons [44, 45]. Recently, it was shown that ACKR3 expression on $CXCR4^+$ plasma blasts licenses them to leave CXCL12-rich germinal centers [46]. Under inflammatory conditions when endothelial cells up-regulate ACKR3, the receptor promotes infiltration of leukocytes into the brain parenchyma through scavenging of perivascular CXCL12 at the basal site of brain blood microvessels [47].

$CD4^+$ T CELL SUBSETS AS REGULATORS OF THE INFLAMMATORY PROCESS

The FOXP3-negative $CD4^+$ T cells include several subsets, among them, Th1, Th2, Th3, Th17, Th9, Th22, follicular Th, and Tr1 cells. These subsets differ in their cytokine production and thereby, their biologic functions. For example, Th1 cells produce IFN-γ, as well as IL-2 and TNF-α [48, 49], to support cell-mediated immunity, whereas Th2 cells that produce IL-4 [50] promote humoral immunity and to some extent, restrain the

inflammation by 2 complementary mechanisms: direct effect on macrophages [51] and shifting the Th1/Th2 balance into Th2, resulting in reduced polarization and activity of Th1 cells [52]. Several reports, including ours, previously showed that indeed, the skewing of the Th1/Th2 balance into Th2 could effectively suppress inflammatory autoimmunity within the CNS [12, 53–55] and other organs [13, 56–60].

The Th17 subset [61] primarily produces 2 of the 6 IL-17 members—IL-17A and IL-17F—that share the same IL-17R [62]. IL-17A and IL-17F, in particular, IL-17A, direct tissue inflammation and play a major part in antimicrobial and anti-fungal immunity [63]. Its relevance for anti-fungal immunity in human has been shown in subjects with an immunodeficiency in IL-17 production that suffers from recurrent fungal infections, which could be restrained by the administration of GM-CSF or G-CSF to increase Th17 activities [64–66]. Other key cytokines produced by these cells are IL-22, which mediates mucosal host defense [67], and IL-21, which further promotes the polarization of Th17 [68, 69]. Currently, clinical trials aiming at targeting IL-17, directly or via its receptor, are being carried out in psoriasis [70–72].

The effector function of Th1/Th17 cells is tightly regulated by 2 major types of T_{regs}: those that express the FOXP3, also known as the nT_{regs}, and those that do not but rather, produce a large amount of IL-10, also known as Tr1 cells [73]. These cells suppress the activities of effector T cells and of inflammatory macrophages by various mechanisms, thus maintaining self-tolerance [74–77]. Aside from nT_{regs}, FOXP3-positive T cells could be polarized from FOXP3-negative T cells (in vitro) in the presence of TGF-β [78].

The polarization and potentiation of both types of T_{regs} are also dependent on the cytokine milieu. As for Tr1 cells, their initial discovery was when being selected (in vitro) in cultures supplemented with IL-10 (and IL-2), implicating that IL-10, produced by these cells, has an autocrine effect on their selection [73]. Later, it has been shown that TGF-β, together with IL-27, polarizes Thnp cells into Tr1 cells [79, 80], as opposed to TGF-β alone, which polarizes Th3 [81] or FOXP3-positive induced T_{regs} [78].

CHEMOKINES AS POTENTIAL REGULATORS OF INFLAMMATION

A little more than 20 y ago, 1 of us (N.K.), together with Ted Yednock, Lawrence Steinman, and colleagues [82], identified the α4β1 integrin (VLA-4) as the key adhesion molecule that drives the accumulation of inflammatory macrophages and T cells at the autoimmune site within the CNS, during EAE and that the mAb-based blockade of VLA-4 (the α4 chain) effectively suppresses the disease. This particular antibody became the leading biologic drug for multiple sclerosis [83]. CXCL12 is a key driver in the activation of VLA-4 [84]. Therefore, we (N.K. lab) aimed to treat EAE by blocking CXCL12. While targeting CXCL12 at the early stage, before the clinical onset of disease indeed postponed it onset, its later targeting aggravated the manifestation of disease [38]. Subsequently, we showed that at this time, CXCL12 shifts $CD4^+$ T cell polarization into Tr1, thus regulating the dynamics of disease [38]. This has been the first evidence that chemokines may also hold anti-inflammatory properties. The translational implication of this study has been the generation of stabilized CXCL12-Ig for therapy of inflammatory autoimmunity [38]. Nevertheless, the multibiological properties of this chemokine may question its future use as a stabilized chemokine for treating autoimmunity [85, 86].

BIASED SIGNALING VIA CXCR3 DIRECTS T CELL POLARIZATION AND BIOLOGIC FUNCTION

We have investigated the interplay between CXCR3 and its 3 ligands—CXCL9, CXCL10, and CXCL11—on directing the polarization of $CD4^+$ T cells and observed that whereas CXCL9 and CXCL10 skew T cell polarization into Th1/Th17 effector cells, CXCL11 drives $CD4^+$ T cell polarization into IL-10-producing Tr1 [21]. We also uncovered the signaling basis of this biased response and learned that it is $G\alpha_i$ independent [21]. Whereas CXCL10/CXCR3 interactions drive effector Th1 polarization via STAT1, STAT4, and STAT5 phosphorylation, CXCL11/CXCR3 binding induces an immunotolerizing state that is characterized by $IL\text{-}10^{high}$ (Tr1) and $IL\text{-}4^{high}$ (Th2) cells and mediated via p70 kinase/mTOR in STAT3- and STAT6-dependent pathways (**Fig. 2**) [21]. CXCL11 binds CXCR3 with a higher affinity than CXCL10, suggesting that CXCL11 has potential to mediate and restrain inflammatory autoimmunity. This may explain, in part, why CXCR3-deficient mice develop an extremely severe form of EAE and Type I diabetes mellitus [87, 88].

The ability of GPCRs to transmit diverse signaling cascades upon binding different ligands [25, 26, 39, 89, 90] has been already raised by others. First, by the Nobel prizewinner Robert J. Lefkowitz and his team [90–92], showing that different ligands binding the same GPCR may induce diverse signaling cascades, called biased signaling, resulting in distinct biologic activities. Even though the mechanistic basis of this feature is not fully understood, its biologic and clinical implications are highly significant [90]. Our studies were the first to uncover the relevance of these findings in $CD4^+$ T cell polarization and

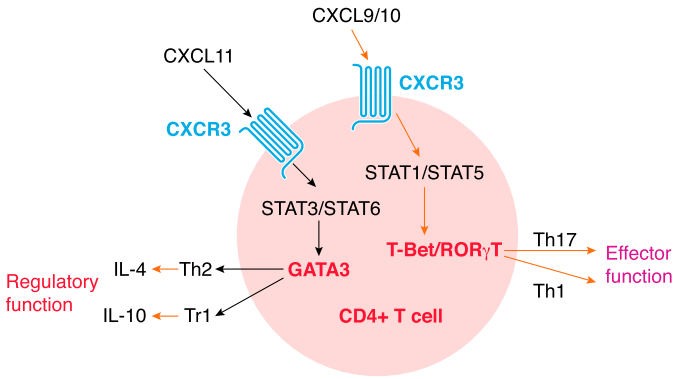

Figure 2. Biased signaling of CXCR3 ligands. Whereas CXCL10/CXCR3 interactions drive effector Th1 polarization via STAT1, STAT4, and STAT5 phosphorylation, CXCL11/CXCR3 binding induces an immunotolerizing state that is characterized by $IL\text{-}10^{high}$ (Tr1) and $IL\text{-}4^{high}$ (Th2) cells and mediated via p70 kinase/mTOR in STAT3- and STAT6-dependent pathways. RORγT, Retinoic acid-related orphan receptor γT.

explore its translational consequences in inflammatory autoimmunity.

Sierro et al. [93] previously showed that wild-type C57BL/6 mice display a shift in the open-reading frame of the CXCL11-encoding gene (insertion of 2 bases after nucleotide 39), resulting in the translation of a chimeric protein lacking the critical CXC motif. We have confirmed these data by PCR analysis. The lack of optimal production of this chemokine by this strain may explain, in part, why CXCL11-based therapy is more effective in C57BL/6 mice than in SJL [21]

COULD STABILIZED REGULATORY CHEMOKINES BE USED AS BIOLOGIC DRUGS FOR AUTOIMMUNITY AND GVHD?

The fundamental approach of applying T_{reg}-based therapies is based on their isolation, in vitro activation, and use for autogeneic therapy after enrichment. This approach has been applied recently for nT_{regs} and Tr1 therapy. As for nT_{regs} in humans, they could be isolated and purified by cell surface molecules ($CD4^+CD25^+CD127^{low}$). So far, this approach has been applied with limited success in human [94, 95]. Maria Grazia Roncarolo, from Stanford University, and colleagues [96–99] developed a reciprocal approach for Tr1 cells that includes their activation in the presence of IFN-α and IL-10 [100]. The efficiency of Tr1 cell-based cell therapy in human has yet to be explored.

An alternative approach for T_{reg}-based therapy is based on the amplification of their function. The first successful clinical trials included administration of low-dose IL-2 for treating GVHD [101] and hepatitis C virus-induced vasculitis [102]. The major potential pitfalls of this approach are that IL-2 also induces effector $CD4^+$ and $CD8^+$ T cells, as well as NK cells, and may potentially aggravate these diseases [103]. We believe that biologic drugs that would selectively induce $FOXP3^+$ T_{regs} or Tr1 cells, without a proinflammatory effect on other cells, could become a leading drug for inflammatory autoimmunity and GVHD. Hence, a potential risk in these drugs could still be that increased activity of T_{regs} would affect the ability of the immune system to combat cancer or to generate effective antimicrobial immunity. An additional pitfall refers to the stability of chemokines that begin to possess short half-life time as a result of enzymatic degradation. Ig-based stabilization may only partially challenge this obstacle.

CONCLUSIONS

Biased signaling or functional selectivity is a concept that describes a situation where a 7TMDR preferentially activates 1 of several available cellular signaling pathways. The current review focuses on exploring the outcome of this feature on the way the interaction between CXCR3 and its ligands shapes the development and function of $CD4^+$ T cell subsets. Thus, far most of the attention has been devoted to exploring the role of cytokines in this property. From a clinically oriented perspective, the findings that chemokines may also polarize T_{regs} (so far, our data show relevance only for FOXP3-negative T_{regs}) open the window of opportunities for use of stabilized chemokines for therapy of inflammatory autoimmunity and GVHD. Among the chemokines that polarize Tr1 cells (i.e., CXCL12 and CXCL11), we find some major differences: CXCL12 also renders anti-inflammatory properties in macrophages [38], whereas CXCL11 also polarizes $IL\text{-}4^{high}$ Th2 cells [21]. We assume that CXCL11 could be a better candidate for being a potential drug, as CXCL12 is involved in many biologic activities, aside from being an immunoregulator, such as neutrophil homeostasis or stem cell homing [85].

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DISCLOSURES

N.K. and G.W. hold a pending patent on the use of CXCL11 and CXCL11-Ig for therapy of autoimmunity and GVHD.

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