Biased agonists of the chemokine receptor CXCR3 differentially control chemotaxis and inflammation

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The chemokine receptor CXCR3 plays a central role in inflammation by mediating effector/memory T cell migration in various diseases; however, drugs targeting CXCR3 and other chemokine receptors are largely ineffective in treating inflammation. Chemokines, the endogenous peptide ligands of chemokine receptors, can exhibit so-called biased agonism by selectively activating either G protein– or β-arrestin–mediated signaling after receptor binding. Biased agonists might be used as more targeted therapeutics to differentially regulate physiological responses, such as immune cell migration. To test whether CXCR3-mediated physiological responses could be segregated by G protein– and β-arrestin–mediated signaling, we identified and characterized small-molecule biased agonists of the receptor. In a mouse model of T cell–mediated allergic contact hypersensitivity (CHS), topical application of a β-arrestin–biased, but not a G protein–biased, agonist potentiated inflammation. T cell recruitment was increased by the β-arrestin–biased agonist, and biopsies of patients with allergic CHS demonstrated coexpression of CXCR3 and β-arrestin in T cells. In mouse and human T cells, the β-arrestin–biased agonist was the most efficient at stimulating chemotaxis. Analysis of phosphorylated proteins in human lymphocytes showed that β-arrestin–biased signaling activated the kinase Akt, which promoted T cell migration. This study demonstrates that biased agonists of CXCR3 produce distinct physiological effects, suggesting discrete roles for different endogenous CXCR3 ligands and providing evidence that biased signaling can affect the clinical utility of drugs targeting CXCR3 and other chemokine receptors.

INTRODUCTION

The chemokine receptor CXCR3 is a heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptor (GPCR) that is expressed primarily on activated effector/memory T cells and plays an important role in atherosclerosis, cancer, and inflammatory disease. Activation of CXCR3 by chemokines causes the migration of activated T cells in a concentration-dependent manner. Increased tissue concentrations of activated T cells initiate inflammatory responses, and the ability to modulate T cell chemotaxis would likely be therapeutically useful in many disease processes. Despite the importance of the more than 20 chemokine receptors in various disease states, there are currently only three U.S. Food and Drug Administration (FDA)–approved drugs that target chemokine receptor family members (1–3). This is somewhat surprising, because GPCRs constitute the plurality of FDA-approved medications, with >30% of therapeutics targeting this class of receptors (4). The difficulty in successfully targeting chemokine receptors was originally thought to be due to redundancy across the multiple chemokine ligands and chemokine receptors that bind to one another (5). However, this presumed redundancy appears to be more granular than was initially appreciated. Similar to most other chemokine receptors, CXCR3 signals through both Gαi family G proteins and β-arrestins. GPCR signaling deviates at critical junctions, including G protein and β-arrestins, which signal through distinct intracellular pathways. For example, β-arrestins promote interactions with kinases independently from their interactions with G proteins to induce downstream signaling (6).

It is now appreciated that many chemokines that bind to the same chemokine receptor can selectively activate such distinct signaling pathways downstream of the receptor (7–9). This phenomenon is referred to as biased agonism (10, 11). Biased ligands at other GPCRs, such as the μ-opioid receptor (MOR) (12, 13), the κ-opioid receptor (14), and the type 1 angiotensin II receptor (AT1R) (15), have shown promise in improving efficacy while reducing side effects through differential activation of G protein– and β-arrestin–mediated signaling pathways (16). Animal and human studies suggest that G protein–mediated signaling by the MOR primarily mediates analgesic efficacy, whereas β-arrestin–mediated signaling causes many adverse effects, such as respiratory depression, constipation, tolerance, and dependence (12, 13). Furthermore, relative degrees of G protein and β-arrestin bias can predict safer μ-opioid analogs (17). At the AT1R, biased and balanced AT1R agonists have distinct physiologic responses: Gqα–dependent signaling mediates vasoconstriction and cardiac hypertrophy, whereas β-arrestin–mediated signaling activates antiapoptotic signals and promotes calcium sensitization (15). At chemokine receptors, both pertussis toxin (PTX)–sensitive G protein signaling and β-arrestin–mediated signaling contribute to chemotaxis (18–23). However, chemokines with distinct G protein– and β-arrestin–biased signaling properties often induce chemotaxis to similar degrees (9). The relative contribution of β-arrestin– or G protein–mediated signaling to chemotaxis and inflammation is unclear, and it is experimentally challenging to discern the physiological relevance of biased signaling with peptide agonists in many assays because of the high molecular weight and short half-life of chemokines relative to those of small molecules. It is unknown whether endogenous or synthetic chemokine receptor ligands that preferentially target G protein or β-arrestin pathways would result in different physiological outcomes in models of disease and inflammation. If such differences in selective pathway activation result in distinct physiological outcomes, then biased agonism could be used to develop new insights into chemokine biology that could be harnessed to increase the therapeutic utility of drugs targeting chemokine receptors while reducing on-target side effects.

Given its prominent role in effector T cell function, we focused on biased signaling at CXCR3-A, the dominantly expressed CXCR3 isoform...
on T cells in humans and mice. CXCR3 signaling is implicated in various disease processes, including cancer (24), atherosclerosis (25), vitiligo (26, 27), and allergic contact dermatitis (ACD) (28). The chemokines CXCL9, CXCL10, and CXCL11, the endogenous ligands of CXCR3, stimulate the chemotaxis of CXCR3-expressing T cells (29). These chemokines are secreted in response to interferon-γ (IFN-γ) by various cell types, including monocytes, endothelial cells, keratinocytes, and fibroblasts. We previously demonstrated that the three ligands of CXCR3 act as biased agonists, displaying different efficacies at signaling through G proteins and β-arrestins and stimulating receptor internalization (8, 30), suggesting that biased agonists targeting CXCR3 may have distinct physiological effects. Given the importance of CXCR3 signaling in disease, we tested whether small-molecule biased agonists of CXCR3 could elicit biochemically and physiologically distinct effects in mice and humans.

**RESULTS**

**Screening enabled identification of biased agonists of CXCR3 with distinct signaling profiles**

To explore the physiological relevance of biased signaling at CXCR3, we screened a panel of publicly available small-molecule CXCR3 ligands and identified two agonists with biased signaling properties: VUF10661 (31) and VUF11418 (Fig. 1, A and B). These two small molecules were previously shown to have similar affinities for CXCR3 (table S1) (32). We first confirmed previous reports that both compounds signal through Gq G proteins (Fig. 1A) (33, 34), displaying similar potencies. Because the G protein–dependent signal did not saturate at high concentrations of agonist, differences in the efficacies of both compounds to activate Gq signaling could not be completely assessed. However, VUF10661 increased β-arrestin2 recruitment to CXCR3 compared to VUF11418 (Fig. 1B). These small-molecule CXCR3 agonists also displayed differential abilities to recruit β-arrestin1, recruit GPCR kinases (GRKs), and stimulate CXCR3 internalization (fig. S1, A to G), consistent with their biased signaling properties.

Because bias is a relative measurement, an appropriate reference must be used. Here, we used the endogenous agonist CXCL11 as a reference, which is a full agonist of CXCR3 for both G protein–and β-arrestin–mediated pathways (30, 35). As expected, CXCL11 displayed increased potency for CXCR3 relative to the small-molecule agonists (fig. S1, H and I). Because it exhibited increased efficacy in β-arrestin recruitment but a similar degree of efficacy in G protein activation, VUF10661 was considered to be a β-arrestin–biased agonist relative to VUF11418 and CXCL11. Similarly, the preserved G protein activation property of VUF11418 together with its reduced ability to induce β-arrestin recruitment demonstrated that it is relatively G protein biased compared to VUF10661 and CXCL11 (Fig. 1, A and B, and fig. S1, H and I). Consistent with its ability to stimulate increased β-arrestin recruitment, VUF10661 also induced a distinct β-arrestin2 conformation, as assessed with an intramolecular biosensor (Fig. 1C), increased the β-arrestin–dependent internalization of CXCR3 (Fig. 1D), and promoted serum response element (SRE) response factor–mediated transcription [which was previously correlated with increased β-arrestin signaling (30)] relative to VUF11418 (Fig. 1E).

**A β-arrestin–biased agonist of CXCR3 potentiates T cell–mediated inflammation and chemotaxis**

After confirming that ligand bias was conserved at the murine CXCR3 receptor, which has 86% sequence homology to human CXCR3 (fig. S2, on October 15, 2019 http://stke.sciencemag.org/ Downloaded from Smith et al., Sci. Signal. 11, eaq1075 (2018) 6 November 2018
A to D), we next studied how these small molecules affected a mouse model of allergic contact hypersensitivity (CHS), an inflammatory condition that is dependent on effector memory T cells (Fig. 2A). The CHS-delayed type IV hypersensitivity reaction enables an assessment of T cell–mediated inflammation and the recruitment of effector T cells to the sensitizer dinitrofluorobenzene (DNFB). Topical application of the β-arrestin–biased agonist, but not the G protein–biased agonist, potentiated the inflammatory response (Fig. 2B). The potentiation induced by VUF10661 was not observed in either β-arrestin2 knockout (KO) mice (Fig. 2C) or CXCR3 KO mice (Fig. S3), consistent with the effects of VUF10661 requiring both CXCR3 and β-arrestin2, the predominant arrestin isoform in T cells (23). Furthermore, the small molecules did not cause inflammation in the absence of DNFB-induced T cell allergy and therefore did not act as irritants or haptens (Fig. 2D).

Given the established role of CXCR3 in T cell chemotaxis (2) and the increased abundance of CXCR3 on CD8+ T cells (36), we hypothesized that the increased inflammation induced by the β-arrestin–biased agonist was due to increased effector memory CD8+ T cell chemotaxis compared to that induced by the G protein–biased agonist. Supporting this, we found that the β-arrestin–biased agonist induced more chemotaxis of effector memory T cells from wild-type (WT) mice in a Transwell migration assay than did the G protein–biased agonist (Fig. 2E). This difference in migration was not observed for activated CD8+ T cells from β-arrestin2 KO mice (Fig. 2E) and was reduced in activated CD4+ T cells (fig. S4A). Consistent with a role for β-arrestin–mediating chemotaxis stimulated by other chemokine receptors (7, 37, 38), migration of effector memory T cells to the endogenous murine chemokine CXCL10 was also markedly attenuated in β-arrestin2 KO mice (fig. S4, B to E). T cells from CXCR3 KO mice failed to migrate to either the β-arrestin–biased agonist or the G protein–biased agonist (Fig. 2E).

With the relative differences in biased agonist–induced chemotaxis, we then investigated whether topical application of the biased agonists caused an increase in the number of effector memory T cells in

![Fig. 2. A β-arrestin–biased, but not G protein–biased, CXCR3 agonist increases inflammation and the chemotaxis of effector/memory T cells. (A) Experimental design of the DNFB CHS model of inflammation. DNFB sensitization was induced with 0.5% DNFB, and contact allergy was elicited 5 days later with 0.3% DNFB. (B) Ear thickness after topical application of vehicle, the β-arrestin–biased agonist VUF10661 (50 μM), or the G protein–biased agonist VUF11418 (50 μM) on the ear of WT mice after DNFB elicitation. Data are means ± SEM of 7 to 11 mice per treatment group. (C) Ear thickness after topical application of the β-arrestin–biased agonist VUF10661 or vehicle on the ear of the β-arrestin2 KO mice. Data are means ± SEM of eight or nine mice per treatment group. (D) As a negative control, in the absence of DNFB treatment, VUF10661 or VUF11418 was applied to the ears. Data are means ± SEM of 7 to 10 mice per treatment group. (E) Measurement of the chemotaxis of CD8+CD44+ T cells isolated from the indicated mice toward the indicated concentrations of the β-arrestin–biased agonist VUF10661 or the G protein–biased agonist VUF11418. VUF11418 (1 μM) did not cause statistically significant chemotaxis compared to the 0 nM treatment (P < 0.05 by two-tailed t test). Data are means ± SEM of seven or three mice per treatment group. (F) Skin infiltration by effector T cells in either vehicle or DNFB allergen–elicited WT mouse ears induced by topical application of vehicle, the β-arrestin–biased agonist VUF10661 (50 μM), or the G protein–biased agonist VUF11418 (50 μM). Data are means ± SEM of six to nine mice per treatment group. (G) Skin infiltration by effector T cells in DNFB allergen–elicited β-arrestin2 KO mouse ears induced by topical application of VUF10661 (50 μM) or VUF11418 (50 μM). Data are means ± SEM of six to eight mice per treatment group. For (B), *P < 0.05 by two-way ANOVA analysis. For (E), *P < 0.05 by two-way ANOVA analysis, showing statistically significant effects of drug for WT VUF10661 versus WT VUF11418 (Tukey post hoc analysis for 1 μM; also, P < 0.05 corrected for multiple comparisons), of genotype for WT VUF10661 versus β-arrestin2 KO VUF10661, and of genotype for WT VUF10661 versus CXCR3 KO VUF10661.
the skin. The β-arrestin–biased agonist increased the number of effector memory cytotoxic T cells in the ear relative to the numbers induced by either vehicle or G protein–biased agonist after treatment of WT mice with DNFB (Fig. 2F). Treatment with the β-arrestin–biased agonist VUF10661 also increased the number of effector memory T helper cells in the ear relative to the number of cells induced by treatment with the G protein–biased agonist (Fig. S5, A and B). The ears of β-arrestin2 KO mice accumulated fewer effector memory T cells than did those of WT mice in response to VUF10661 (Fig. 2G and fig. S5C). No difference in the number of CD4+ regulatory T cells was observed (Fig. S5, D and E). Together, these findings are consistent with the β-arrestin–biased agonist increasing skin inflammation by promoting the chemotaxis and recruitment of effector memory T cells.

Patients with CHS coexpress CXCR3 and β-arrestin in T cells in the skin

A major confounder in studies of chemokine biology is differences in expression patterns and function between species (39). To correlate CXCR3 signaling with CHS in human patients, we sampled skin from patch-tested patients (Fig. 3A). Skin patch testing involves the placement of contact allergens directly on the skin. Positive patch test reactions include erythematic induration, with severe reactions causing ulceration. Patch testing is the gold standard for the diagnosis of ACD, and the role of T cells in the pathophysiology of allergic CHS that underlies this disease is well established (40). Ninety-six to 120 hours after allergen application, the patch test was read, and biopsies of positive and negative sites were collected and stained for the T cell marker CD3, CXCR3, and β-arrestin (Fig. 3B). More T cells and more T cells coexpressing CXCR3 and β-arrestin were observed in ACD biopsies than in nonlesional (negative) biopsies (8 of 23, or 35%). The ears of β-arrestin2 KO mice accumulated fewer effector memory T cells than did those of WT mice in response to VUF10661 (Fig. 2G and fig. S5C). No difference in the number of CD4+ regulatory T cells was observed (Fig. S5, D and E). Together, these findings are consistent with the β-arrestin–biased agonist increasing skin inflammation by promoting the chemotaxis and recruitment of effector memory T cells.

**Fig. 3.** Human CXCR3+ T cells display differential responses to biased agonists of CXCR3, and CXCR3 and β-arrestin are coexpressed in T cell clusters within lesions from human allergen patch-tested skin. (A) Diagram of ACD patch testing. Colored regions on day 5 signify a positive response. (B) Representative immunohistochemistry of CXCR3 (green), CD3 (red), β-arrestin (purple), and Hoechst (blue) in skin from human allergen patch-tested skin (+) and matched nonlesional (NL; –) controls from the same patient. Data are representative of three patient samples with similar results. Original magnifications were ×200 (left) and ×400 (right), with scale bars of 100 μm and 50 μm, respectively. White boxes in ×200 images indicate the area of magnified images in subsequent pictures and are located at the epidermal–dermal junction. In allergen patch-tested skin, this area was where most T cell clusters were found, as previously described (66), whereas the same area in nonlesional skin was devoid of such immune cell conglomerates and served as a control. (C and D) Quantitative analysis of the number of dermal CD3+ T cells (C) and coexpression of CD3 with CXCR3 and β-arrestin (D) in skin from allergen patch-tested skin (+) and matched nonlesional (NL; –) controls from the same patient. Data are representative of three patient samples with similar results. Original magnifications were ×200 (left) and ×400 (right), with scale bars of 100 μm and 50 μm, respectively. White boxes in ×200 images indicate the area of magnified images in subsequent pictures and are located at the epidermal–dermal junction. In allergen patch-tested skin, this area was where most T cell clusters were found, as previously described (66), whereas the same area in nonlesional skin was devoid of such immune cell conglomerates and served as a control. (C and D) Quantitative analysis of the number of dermal CD3+ T cells (C) and coexpression of CD3 with CXCR3 and β-arrestin (D) in skin from allergen patch-tested skin (+) and matched nonlesional (NL; –) controls from the same patient. Data are representative of three patient samples with similar results. Original magnifications were ×200 (left) and ×400 (right), with scale bars of 100 μm and 50 μm, respectively. White boxes in ×200 images indicate the area of magnified images in subsequent pictures and are located at the epidermal–dermal junction. In allergen patch-tested skin, this area was where most T cell clusters were found, as previously described (66), whereas the same area in nonlesional skin was devoid of such immune cell conglomerates and served as a control. (E and F) T cells isolated from patch-tested patients were tested for chemotaxis toward the indicated concentrations of the β-arrestin–biased agonist VUF10661 or the G protein–biased agonist VUF11418. (E) CXCR3+CD8+ T cells (n = 3) and (F) CD44+CD8+ T cells (n = 5). Patch test quantitative analyses are expressed as positive cells ± SEM from a microscopic field with six views at ×400, from a total of three patients. For (C) and (D), *P < 0.05 by unpaired two-tailed t test. For (E) and (F), *P < 0.05 by two-way ANOVA.
Akt is activated by β-arrestin and promotes T cell chemotaxis

Given the overlap of signaling pathways mediated by both β-arrestin and G proteins, it was unclear which specific signaling effectors downstream of β-arrestin were responsible for differences in chemotaxis and inflammation induced by β-arrestin– and G protein–biased agonists. To identify potential pathways, we performed a targeted flow cytometry–based analysis of intracellular phosphorylation in human peripheral blood mononuclear cells (PBMCs) after incubation with vehicle, VUF10661, or VUF11418. The Akt pathway, which can be activated by β-arrestin, displayed qualitative differences in these targeted analyses. The β-arrestin–biased agonist increased the phosphorylation of Akt residues Thr308 and Ser473, which are necessary for full-efficacy activation of peripheral blood mononuclear cells (PBMCs) after incubation with vehicle, VUF10661, or VUF11418. The Akt pathway, which can be activated by β-arrestin, displayed qualitative differences in these targeted analyses. These findings are consistent with a similar role for CXCR3-mediated signaling through β-arrestin in T cells in human CHS responses.

Although PTX eliminated T cell chemotaxis, Akt inhibition did not fully inhibit migration, suggesting that multiple CXCR3-mediated pathways mediate T cell chemotaxis. Note that Akt has a well-established role in mediating cell polarity and migration. Akt translocates to the leading edge of migrating cells (47, 48), and this pathway is known to be red stimulated by endogenous CXCR3 chemokines (49, 50). Moreover, β-arrestin alters Akt activation through the formation of signaling complexes, such as with protein phosphatase 2A, leading to Akt dephosphorylation downstream of the D2 dopamine receptor (51, 52), or with Src, in which both are phosphorylated and activated downstream of the insulin receptor (53). We found that the G protein–biased agonist failed to induce substantial chemotaxis above baseline, whereas at high concentrations it reduced migration below basal levels, which could be due to reduced Akt phosphorylation or off-target effects at high drug concentrations.

Despite previous studies that implicated both β-arrestin– and G protein–mediated signaling as being critical to cell function and migration downstream of other chemokine receptors, such as CXCR4 (7, 23, 54, 55), it was unclear how biased agonists could differentially affect physiological models of T cell movement and disease, if at all. Despite the fact that the β-arrestin–biased agonist caused increased chemotaxis internalization than did the G protein–biased agonist, the β-arrestin–biased agonist caused increased chemotaxis and potentiated a T cell–mediated inflammatory response. Consistent with studies of other chemokine receptors (18, 56), the fact that PTX eliminated CXCR3-mediated chemotaxis toward the β-arrestin–biased agonist implicates both G proteins and β-arrestins in chemotaxis. Studies classify CXCL11 as a β-arrestin–biased ligand relative to the other two CXCR3 endogenous ligands CXCL9 and CXCL10 (8, 30). CXCL10 and CXCL11 bind to nonoverlapping region(s) of CXCR3. In addition, CXCL11 binds to both G protein–coupled and PTX-uncoupled forms of CXCR3. In contrast, the relative G protein–biased endogenous ligand CXCL10 has no affinity for the PTX-uncoupled form of CXCR3 (57), providing an illustrative example of multisite chemokine binding observed at CXCR3 (58). These data suggest that a different transducer, such as β-arrestin, may stabilize a distinct active receptor conformation when bound to CXCL11. Functionally, CXCL11 induces a greater chemotactic response than those induced by CXCL9 and CXCL10 (29, 59), which
suggests that CXCL11 may be the most proinflammatory of the endogenous CXCR3 ligands. Consistent with β-arrestin playing a central role in T cell migration, we found that a β-arrestin–biased agonist was more effective than a G protein–biased agonist at increasing CXCR3-mediated T cell chemotaxis and enhancing a T cell–mediated inflammatory response. In addition, T cells isolated from β-arrestin2 KO mice displayed defective chemotaxis to an endogenous CXCR3 chemokine as evidenced by a ~10-fold decrease in potency, consistent with previous observations (23). Our data are consistent with a model in which the activation of β-arrestin–mediated signaling can increase T cell migration and function, whereas inhibiting either β-arrestin–mediated signaling or the association between β-arrestin and Akt would oppose migration and inflammation.

On the basis of our findings, it is perhaps unsurprising that small-molecule screens of CXCR3 ligands that focus primarily on receptor affinity or G protein–mediated signaling could potentially underperform in identifying promising preclinical agonists or antagonists. The role of β-arrestin in other chemokine receptor signaling pathways suggests that an expanded screen to include the β-arrestin pathway (and potentially Akt) would improve candidate selection. In summary, targeting different CXCR3 pathways with biased agonists that have similar receptor affinities but different efficacies for G protein– and β-arrestin–mediated signaling pathways produces distinct physiological differences in chemotaxis and CHS reactions, which has implications for drug development within the chemokine receptor family.
MATERIALS AND METHODS
Study design
The main research objectives of these studies were to investigate the physiological effects of biased agonists of CXCR3 and determine the mechanisms by which engagement of the β-arrestin–mediated signaling pathway potentiates chemotaxis and inflammation. These studies were performed with a combination of patient leukocytes, patient skin, genetically modified mice, and pharmacologic treatments supported by in vitro mechanistic data. The number of patients and animals used in each group for each experiment is reported in the figure legends. Animals were randomly assigned to treatment groups once their genotypes were confirmed, and investigators were blinded to mouse pharmacological treatments. Statistical details are provided at the end of this section and within the figure legends.

Small molecules and peptides
VUF10661 (Sigma-Aldrich), VUF11418 (Aobious), LY294002 (Sigma-Aldrich), and AZD5363 (Axon Medchem) were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and were stored at 20°C in a desiccator cabinet. Recombinant human CXCL11 and murine CXCL11 proteins (PeproTech) were diluted according to the manufacturer’s specifications, and aliquots were stored at −80°C until needed for use.

BRET assays
Intermolecular and intramolecular β-arrestin–based biosensor BRET experiments were performed as previously described (30) and were similar to those outlined originally by the Bouvier laboratory (60, 61). Briefly, HEK 293T cells were transiently transfected with CXCR3-Rluc together with plasmids encoding β-arrestin–YFP, GRK2-YFP, or GRK6-YFP and plated on a 96-well plate at 50,000 cells per well (Corning) or with untagged CXCR3 and 50 ng of the Nanoluc–β-arrestin2–YFP biosensor, which was previously determined to be the optimal amount of biosensor expression vector (30). Forty-eight hours after transfection, the cells were incubated with the compounds indicated in the figure legends in assay buffer consisting of Hanks’ balanced salt solution (HBSS) supplemented with 20 mM Hepes and 3 μM coelenterazine-h (Promega). The plate was read by a Mithras LB940 instrument (Berthold, Germany), and the net BRET ratio was calculated by subtracting the YFP:Rluc ratio in vehicle-treated wells from the YFP:Rluc ratio in the ligand-stimulated wells. Cells tested negative for mycoplasma contamination.

The DiscoveRx β-arrestin–dependent internalization assay
This assay was conducted as previously described (30) and in accordance with the manufacturer’s protocols. Briefly, an Enzyme Acceptor–tagged β-arrestin and a ProLink tag localized to endosomes were stably expressed in U2OS cells. The cells were transiently transfected with plasmid encoding untagged CXCR3. β-Arrestin–mediated internalization resulted in the complementation of the two β-galactosidase enzyme fragments that hydrolyzed a substrate (DiscoveRx) to produce a chemiluminescent signal.

SRE/serum response factor pathway assay
SRE/serum response factor (SRF) experiments were performed as previously described (30). Briefly, HEK 293T cells were transiently transfected with plasmid encoding CXCR3 and either the SRE or SRF luciferase reporters. Four hours later, the cells were plated on a 96-well plate at a concentration of 25,000 cells per well. The next day, the cells were serum starved overnight, incubated with the compounds indicated in the figure legends for 5 hours, and subsequently lysed with passive lysis buffer (Promega). Luciferin was added to the lysate, and the resulting luminescence was quantified using a Mithras LB940 instrument.

TGF-α–shedding assay
The ability of CXCR3 to stimulate Gq activity was assessed by the TGF-α–shedding assay as previously described (62). Briefly, HEK 293 cells lacking Gqα, Gqβ, Gqγ, and Gq12/13 were transiently transfected with plasmids encoding CXCR3, a modified TGF-α–containing alkaline phosphatase (AP-TGF-α), and either the Gq12/13 assay subunit [because Gq12 is observed to be dispensable in CXCR3 signaling in T cells (63)] or, as a negative control, the Ac subunit (which lacks the distal amino acid residues of G protein α subunits required for receptor interaction) and were reseeded 24 hours later in HBSS (Gibco, Gaithersburg, MD) supplemented with 5 mM Hapes in a Costar 96-well plate (Corning Inc., Corning, NY). Cells were then stimulated with the indicated concentration of ligand for 1 hour. Conditioned medium (CM) containing the shed AP-TGF-α was transferred to a new 96-well plate. Both the cell and CM plates were treated with para-nitrophenylphosphate (p-NPP; 100 mM) (Sigma-Aldrich, St. Louis, MO) substrate for 1 hour, which is converted to para-nitrophenol (p-NP) by AP-TGF-α. This activity was measured at OD405 (optical density at 405 nm) in a Synergy Neo2 Hybrid Multi-Mode (BioTek) plate reader immediately after the addition of p-NPP and a 1-hour incubation. Gq activity was calculated by first determining the amount of p-NP by absorbance through the following equation:

$$\frac{100}{\text{OD}_{405}\text{cell}} \left( \frac{\Delta\text{OD}_{405}\text{CM}}{\Delta\text{OD}_{405}\text{CM} + \Delta\text{OD}_{405}\text{CM}} \right)$$

where $\Delta\text{OD}_{405}$ = OD405 1 hour − OD405 0 hour, and $\Delta\text{OD}_{405}$ cell and $\Delta\text{OD}_{405}$ CM represent the changes in absorbance after 1 hour in the cell and CM plates, respectively. Data were normalized to those from the vehicle-treated sample, and the nonspecific ΔC signal was subtracted from Gq signal percentage AP-TGF-α release as follows:

$$\frac{\text{AP activity} \left( \frac{\text{ligand} - \text{vehicle}}{\text{vehicle}} \right)}{\text{AP activity} \left( \frac{\text{ligand} - \text{vehicle}}{\text{vehicle}} \right)} - \Delta\text{C}$$

Only the two highest concentrations of VUF11418 ligand (8 and 16 μM) resulted in consistent appreciable background ΔC signals, which resulted in larger errors relative to those from all other conditions (fig. S1G).

Generation of CXCR3* Jurkat cells
Jurkat cells (an immortalized human CD4 T cell line) stably expressing CXCR3 were generated by transfecting a linearized pcDNA3.1 expression vector encoding geneticin (G-418) resistance, selecting for transfected cells with geneticin (1000 μg/ml), and collecting cells that highly expressed CXCR3 by fluorescence-activated cell sorting (FACS). Cells were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 1% penicillin/streptomycin, 0.23% glucose, 10 mM Hapes, 1 mM sodium pyruvate, and geneticin (250 μg/ml).
Cells were serum starved for at least 4 hours, incubated with the ligands indicated in the figure legends, subsequently washed once with ice-cold phosphate-buffered saline (PBS), lysed in ice-cold radioimmunoprecipitation assay buffer containing phosphatase and protease inhibitors [PhosSTOP (Roche) and cComplete EDTA-free (Sigma-Aldrich)] for 15 min, sonicated, and cleared of insoluble debris by centrifugation at >12,000g at 4°C for 15 min, after which the supernatant was collected. Proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and analyzed by Western blotting at 4°C overnight with the indicated primary antibody. Antibodies against phosphorylated ERK (pERK) (#9106, Cell Signaling Technology) and total ERK (#06-182, Millipore) were used to assess ERK activation. Antibodies against pAkt-Thr308 (#13038, Cell Signaling Technology), pAkt-Ser473 (#9271, Cell Signaling Technology), and total Akt (#4691, Cell Signaling Technology) were used to assess Akt phosphorylation. The A1-CT antibody, which recognizes both isoforms of β-arrestin, was supplied by the laboratory of R. J. Lefkowitz. Horseradish peroxidase–conjugated polygonal mouse anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG was used as secondary antibodies. Immune complexes on nitrocellulose membranes were imaged by SuperSignal enhanced chemiluminescent substrate (Thermo Fisher Scientific). After the detection of phosphorylated kinases, the nitrocellulose membranes were stripped and rebotted for total kinases. For quantification, band intensities corresponding to phosphorylated proteins were normalized to signals corresponding to the appropriate total protein on the same membrane with Image Lab software (Bio-Rad).

**Immunoprecipitation**

HEK 293N cells were cultured in 100-mm tissue culture plates and transiently transfected with 5 μg of CXCR3-encoding plasmid and 2.5 μg of plasmid encoding FLAG-tagged β-arrestin2 or were cultured in six-well tissue culture plates and transfected with 1 μg of CXCR3-encoding plasmid and 0.5 μg of plasmid encoding FLAG-tagged β-arrestin2. The cells were lysed as described earlier, and the lysates were incubated for 4 hours at 4°C with anti-FLAG magnetic beads (Thermo Fisher Scientific) and washed according to the manufacturer’s protocol. Samples were then immediately eluted with 2× SDS, resolved by 10% SDS-PAGE, and analyzed by Western blotting as described earlier.

**siRNA-mediated knockdown**

HEK 293N cells in six-well tissue culture plates were transiently transfected with 1 μg of plasmid encoding CXCR3A and 3.5 μg of either control siRNA or with previously validated β-arrestin2–specific siRNA ("wem2") (64) with Lipofectamine 3000 (Thermo Fisher Scientific) as per the manufacturer’s specifications. Seventy-two hours later, the cells were stimulated with 1 μM VUF10661 for 60 min and the cells were then lysed and analyzed by Western blotting as described earlier.

**Study subjects and skin samples**

All studies involving human subjects were approved by the Institutional Review Board of Duke University Health System. Study participation inclusion was offered to patients undergoing patch testing in a specialty contact dermatitis clinic. Inclusion criteria were ≥18 years of age and completion of patch testing. Exclusion criteria were pregnancy, topical corticosteroids at patch site, oral corticosteroids, systemic immunosuppressants, phototherapy, known bleeding disorders, and allergy to lidocaine or epinephrine. Skin biopsies and venipunctures were obtained from male and female volunteers under a protocol approved by the Institutional Review Board of Duke University. Patches containing test allergens were applied to study participants on day 1, removed on day 3, and analyzed after 96 to 120 hours. If a study participant had a positive patch test, then a 4-mm punch biopsy was obtained at the positive test site and a 4-mm punch biopsy was obtained at a negative site (normal skin) from normal regions of the skin nearby. Immunofluorescence analysis was performed as previously described (65). Preceding cell counting, skin images from allergen patch-tested skin and matched nonlesional controls from the same patient were separated by color channel. All collected images were processed with ImageJ software. The ImageJ cell counter tool recorded mouse clicks on cells that were labeled with colored dots. Cell numbers were expressed as counts per view in a microscopic field with six views.

**Mice**

WT C57BL/6; C57BL/6 CXCR3−/-; and C57BL/6 ARRB2+/− mice were bred and maintained under specific pathogen–free conditions in accredited animal facilities at the Duke University. C57BL/6 CXCR3−/-; CXCR3+/− mice were acquired from the Jackson Laboratory (Bar Harbor, ME, USA; stock #005796), and C57BL/6 ARRB2+/− were provided by R. J. Lefkowitz (Duke University, USA). Mice were between 6 and 15 weeks of age when first used.

**Mouse allergic CHS**

Mice were sensitized by topical application of 50 μl of 0.5% DNFB (Sigma-Aldrich) in 4:1 acetone/olive oil on their shaved back and were challenged on their ears 4 days later with 10 μl of 0.3% DNFB or vehicle control. Then, 4, 24, and 48 hours later, 10 μl of either vehicle, VUF10661 (50 μM), or VUF11418 (50 μM) dissolved in 72:18:10 acetone:olive oil:DMSO was topically applied to the indicated ear by an investigator blinded to treatment. Ear thickness was measured at different time points with an engineer’s micrometer (Standard Gage).

**Chemotaxis assays**

Chemotaxis assays were conducted similarly to those previously described (56). Briefly, mouse leukocytes, obtained by passing cells isolated from the spleen and subjected to erythrocyte lysis through a 70-μm filter, were suspended in RPMI 1640 medium containing 5% FBS. For the assay, 1 × 10⁶ cells in 100 μl of medium were added to the top chamber of a 6.5-mm-diameter, 5-µm-pore polycarbonate Transwell insert (Costar) and incubated in duplicate with the indicated concentrations of ligand suspended in 600 μl of the medium in the bottom chamber for 2 hours at 37°C. Cells that migrated to the bottom chamber were resuspended, washed, stained with a Live/Dead marker (Aqua Dead, Thermo Fisher Scientific) and antibodies to cell surface markers (CD3, CD4, CD8, CD44, and CD45), fixed with paraformaldehyde, and subjected to cell counting flow cytometric analysis with a BD LSR II flow cytometer. Flow cytometry was performed in the Duke Human Vaccine Institute Research Flow Cytometry Facility (Durham, NC). We were unable to identify a reliable anti-murine CXCR3 antibody for flow cytometry suitable for surface staining. CountBright beads (Thermo Fisher Scientific) were added immediately after bottom chamber resuspension to correct for differences in final volume and any sample loss during wash steps. A 1:10 dilution of input cells was similarly analyzed. Migration was calculated by dividing the number of migrated cells by the number of
input cells. In some studies, cells were preincubated for 1 hour at 37°C with PTX (100 ng/ml; List Biological Laboratories), 100 μM LY294002 (Sigma-Aldrich) to inhibit PI3K, or 100 nM AZD5363 (Axon Medchem) to inhibit Akt. Human peripheral blood leukocytes were obtained by venipuncture in accordance with the Duke Institutional Review Board, subjected to erythrocyte lysis, and assayed as described earlier, with the addition of an anti-human CXCR3 antibody. For some samples, reliable anti-human CXCR3 staining was not obtained, which resulted in two fewer replicates in the CD8+CXCR3+ group compared to the CD8+CD44+ group. Analysis was conducted with FlowJo (Ashland, OR) version 10 software. A representative gating tree is shown in Fig. S11.

Assessment of T cells in the skin
Mice were subjected to the mouse allergic CHS assay as described earlier, with the exception that both ears received 0.3% DNFβF to induce inflammation. Two ears were pooled from a single mouse to produce one biological replicate. About 4 hours after the last topical drug treatment, mouse ears were dissected and transferred dorsal side up to a dish containing ice-cold PBS. Dorsal and ventral layers were separated and added to 5 ml of digestion media consisting of HBSS supplemented with 5% FBS (Corning), 10 mM Hepes, liberase (0.04 mg/ml; Sigma-Aldrich), and deoxyribonuclease (0.3 mg/ml; Sigma-Aldrich). Ears were incubated at 37°C for 10 min, minced, and incubated for an additional 30 min at 37°C, vortexing gently every 10 min. After 40 min, 25 ml of PBS was added and the sample was vortexed and strained through a 70-μm mesh into a fresh 50-ml conical tube. Cells were spun down at 200g at 4°C and resuspended in PBS supplemented with 10 mM Hepes, 5 mM EDTA, and 1% bovine serum albumin. To obtain leukocyte cell counts, cells were manually counted with Turks solution by an investigator blinded to treatment group. Cells were transferred to round-bottom FACS tubes, washed twice with 2 ml of ice-cold PBS, and stained with a Live/Dead marker (Aqua Dead, #L34957, Thermo Fisher Scientific). Cells were blocked in PBS supplemented with 3% FBS and 10 mM EDTA (FACS buffer) with anti-CD16/32 (Fcγ block), 5% normal mouse serum (Thermo Fisher Scientific), and 5% normal rat serum (Thermo Fisher Scientific). Cells were then stained with antibodies to cell surface markers (CD3, CD4, CD8, CD44, and CD45) for 30 min at 4°C, washed with FACS buffer, and fixed with 0.4% paraformaldehyde. Foxp3 intracellular staining was performed with an anti-Foxp3 antibody using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Flow cytometry was performed in the Duke Human Vaccine Institute Research Flow Cytometry Facility on a BD LSRII flow cytometer (Durham, NC). Analysis was conducted with FlowJo version 10 software. Total cells were calculated by multiplying the relative abundance of total live cells by the manual leukocyte count. A list of flow cytometry antibodies used is listed in table S2.

Immunofluorescence analysis of human skin
Sections of frozen human specimens were incubated overnight at 4°C with anti-human CD3 (OKT3, BioLegend) and anti-human β-arrestin (provided by R. J. Lefkowitz), which was followed by reaction with Cy3- and Alexa Fluor 647–conjugated secondary antibodies (Thermo Fisher Scientific). The sections were then incubated with Alexa Fluor 488–conjugated mouse IgG1 antibodies against human CXCR3 (R&D Systems Inc.) or mouse IgG1 isotype control (Tonbo Biosciences). Nuclei were counterstained with Hoechst 33342, washed in PBS, and mounted with Anti-fade Mounting Media (Thermo Fisher Scientific).

Targeted phosphoprotein analysis
Human PBMCs were stimulated with saturating concentrations of the ligands indicated in the figure legends for 1, 2, 5, or 15 min; fixed with 1% paraformaldehyde for 10 min at room temperature; and prepared for antibody staining as previously described (66). Briefly, the fixed cells were permeabilized in 90% methanol for 15 min. The cells were then stained with a panel of antibodies specific to the markers indicated in the figure (Primity Bio Pathway Phenotyping service) and analyzed on an LSRII flow cytometer (BD Biosciences). The log₂ ratio of the mean fluorescence intensity (MFI) of the stimulated samples divided by that of the unstimulated control samples was calculated as a measure of the response.

Statistical analyses
Dose-response curves were fitted to a log agonist versus stimulus with four parameters [Span, Baseline, Hill coefficient, and EC₅₀ (median effective concentration)] with the minimum baseline constrained to zero using Prism 7.0 software (GraphPad). To compare ligands in concentration-response assays or time-response assays, a two-way ANOVA of ligand and concentration or ligand and time, respectively, was conducted. If a statistically significant interaction or main effect of treatment, depending on the experiment, was observed (P < 0.05), then comparative two-way ANOVAs between individual ligands were performed. Further details of statistical analysis and replicates are included in the figure legends. For mouse ear T cell counts, two mice corresponding to the highest and lowest values in the WT VUF10661 treatment group were excluded from analysis (CD8⁺CD44⁺ counts 79,515 and 2709, respectively). Lines indicate the mean, and error bars signify the SEM throughout the manuscript, unless otherwise noted. Asterisk indicates P < 0.05 throughout the paper to indicate statistical significance from pertinent comparisons detailed in the figure legends, unless otherwise noted.

SUPPLEMENTARY MATERIALS
www.sciencesignaling.org/cgi/content/full/11/555/eaaq1075/DC1
Fig. S1. Additional signaling analyses of CXCR3 ligands.
Fig. S2. Biased signaling is conserved at murine CXCR3.
Fig. S3. The inflammatory effects of the β-arrestin-biased agonist VUF10661 are absent in CXCR3 KO mice.
Fig. S4. Loss of β-arrestin2 attenuates chemotaxis to mCXCL10, and both VUF10661 and mCXCL10 induce chemotaxis of only CD44⁺ T cell populations.
Fig. S5. Biased ligands of CXCR3 differentially increased the numbers of CD4⁺CD44⁺ T cells and total T cells in DNFβF-treated ears.
Fig. S6. Human T cell chemotaxis.
Fig. S7. Targeted phosphoprotein data in T cells, monocytes, and natural killer cells.
Fig. S8. Co-immunoprecipitation of pAkt-Thr308 with β-arrestin2.
Fig. S9. Differential phosphorylation of Akt, but not ERK1/2, in a T cell line stably expressing CXCR3 after stimulation with VUF10661 or VUF11418.
Fig. S10. Both PTX and a PI3K inhibitor eliminate effector T cell migration to VUF10661.
Fig. S11. Flow cytometry gating strategy.
Table S1. Pharmacological properties of the biased agonists of CXCR3.
Table S2. Flow cytometry antibodies.

REFERENCES AND NOTES


Acknowledgments: We thank R. J. Leffkowitz (Duke University, USA) for guidance, mentorship, and thoughtful feedback throughout this work and for supplying CS7BL/6 ABR22–/– mice; R. Preamont (Harrington Discovery Institute, USA) for providing the GRK-VPY constructs; A. Inoue (Tohoku University, Japan) for G protein KO cells; M. Caron, S. Shenoy, and N. Freedman for the use of laboratory equipment; T. Pack, A. Wisdom, and M.-N. Huang for many helpful discussions; N. Nako for laboratory assistance; and K. Hines and K. Scoggins for assistance in patient sample acquisition. Funding: This work was supported by T32GM71717 (J.S.S.), the Duke Medical Scientist Training Program (J.S.S.), IR01GM122798-01A1 (S.R.), K08HL114643-01A1 (S.R.), Burroughs Wellcome Career Award for Medical Scientists (S.R.), R21AI28727 (A.S.M.), R01AI39207 (A.S.M.), Duke Physician-Scientist Strong Start Award (A.S.M.), Dermatology Foundation Research Grant (A.S.M.), and the Duke Pinnell Center for Investigative Dermatology (J.S.S., A.S.M., and S.R.). Author contributions: J.S.S. and S.R. conceived and planned the study. J.S.S., L.T.N., T.S.W., A.R.A., M.D.G., A.S.M., and S.R. helped plan and review experiments. J.S.S., L.T.N., J.S., R.A.G, N.M.K., P.A., J.N.G., T.S.W., and A.S.M. performed and analyzed flow cytometry data. J.S.S., L.T.N., J.S., R.A.G, N.M.K., P.A., J.N.G., T.S.W., and A.S.M. performed and analyzed immunohistochemistry data. J.S.S. and S.R. analyzed all other data. J.S.S. and S.R. wrote the paper. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 2 October 2017
Resubmitted 23 February 2018
Accepted 19 October 2018
Published 6 November 2018
10.1126/scisignal.aaaq1075

Biased agonists of the chemokine receptor CXCR3 differentially control chemotaxis and inflammation

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Sci. Signal. 11 (555), eaaq1075.  
DOI: 10.1126/scisignal.aaq1075

Biased chemokine responses
Like many other G protein–coupled receptors (GPCRs), chemokine receptors exhibit so-called biased agonism, whereby different ligands can stimulate either G protein– or β-arrestin–dependent signaling. Smith et al. investigated biased signaling by the receptor CXCR3, which directs T cell migration to sites of inflammation. The authors found that topical application of a small-molecule agonist that was β-arrestin biased, but not one that was G protein biased, exacerbated inflammation in a mouse model of contact hypersensitivity. The β-arrestin–biased agonist was more potent at stimulating mouse and human T cell chemotaxis in vitro and activated the kinase Akt, which promoted migration. Together, these data suggest that biased agonists of CXCR3, and perhaps other chemokine receptors, result in different physiological outcomes.

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