Correction

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The authors note that Fig. 5 appeared incorrectly. The corrected figure and its corresponding legend appear below.

Fig. 5. Mechanism for heterologous chemokine receptor desensitization. (A) Flow cytometric analysis of surface CXCR3 and CCR5 on splenic human T cells 24 h following intravenous administration of vehicle (gray bars) or PS372424 (black bars). (B) Western blot analysis of pCCR5 in activated T cells or in CD4+CD25hiCXCR3− T cells following 30-min treatment with ligand; in indicated lane, cells were treated in the presence of 10 ng/mL staurosporine (STS). Pyruvate dehydrogenase complex (PDC) blotting demonstrates equal loading. (C) FRET efficiency between indicated acceptor and CXCR3-PE determined by flow cytometry using activated T cells. (D) FRET efficiency between CCR5-APC and CXCR3-PE determined by flow cytometry using activated T cells. White bars denote 15% saturation CXCR3-PE, gray bars 43%, and black bars 100%. Data represent three independent experiments ± SEM performed in triplicate with eight mice per group.

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Chemokine receptor CXCR3 agonist prevents human T-cell migration in a humanized model of arthritic inflammation

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The recruitment of T lymphocytes during diseases such as rheumatoid arthritis is regulated by stimulation of the chemokine receptors expressed by these cells. This study was designed to assess the potential of a CXCR3-specific small-molecule agonist to inhibit the migration of activated human T cells toward multiple chemokines. Further experiments defined the molecular mechanism for this anti-inflammatory activity. Analysis in vitro demonstrated agonist induced internalization of both CXCR3 and other chemokine receptors coexpressed by CXCR3+ T cells. Unlike chemokine receptor-specific antagonists, the CXCR3 agonist inhibited migration of activated T cells toward the chemokine mixture in synovial fluid from patients with active rheumatoid arthritis. A humanized mouse air-pouch model showed that intravenous treatment with the CXCR3 agonist prevented inflammatory migration of activated human T cells toward this synovial fluid. A potential mechanism for this action was defined by demonstration that the CXCR3 agonist induces receptor cross-phosphorylation within CXCR3-CCR5 heterodimers on the surface of activated T cells. This study shows that generalized chemokine receptor desensitization can be induced by specific stimulation of a single chemokine receptor on the surface of activated human T cells. A humanized mouse model was used to demonstrate that this receptor desensitization inhibits the inflammatory response that is normally produced by the chemokines present in synovial fluid from patients with active rheumatoid arthritis.

autoimmune | humanization | chemotaxis | PS372424

Chemokine-driven lymphocyte recruitment plays a central role in inflammatory diseases, such as rheumatoid arthritis (1). The importance of the chemokine system has led to the development of neutralizing antibodies targeted at receptors or ligands, and small-molecule receptor antagonists (2). However, preclinical animal models have failed to estimate drug efficacy and many clinical trials have failed (2–4); it is unclear whether these approaches will ever prove effective (4, 5). The human chemokine system is complex, with 44 ligands and 21 G protein-coupled receptors identified to date (3). This system is further complicated by the formation of heterodimers of both chemokines and their receptors (6). Although some chemokine receptors have a single ligand, most are activated by more than one chemokine and many chemokines can activate more than one receptor. The chemokines are divided into two functional groups. Homeostatic chemokines are constitutively active in specific tissues, and inflammatory chemokines are induced by localized stress, proinflammatory cytokines, or activation of Toll-like receptors (7). More than 12 chemokines have been identified within synovial fluid samples from patients with rheumatoid arthritis (8). Activation of the chemokine receptor CXCR3 by CXCL19, CXCL10, and CXCL11 provides an example of a proinflammatory interaction that will recruit activated T cells that specifically express CXCR3. As well as promoting T-cell migration, CXCR3 forms a ligand-dependent association with the T-cell receptor complex (9). In this situation both CXCR3 and the T-cell receptor share downstream signaling molecules, potentially allowing cross-regulation (9). Cells expressing CXCR3 have been implicated in a range of diseases, including psoriasis (10), multiple sclerosis (11), inflammatory bowel disease (12), and rheumatoid arthritis (13). Small-molecule CXCR3 antagonists have shown anti-inflammatory activity in rodent models (14). However, only one molecule, T487, has entered clinical trial. This agent lacked clinical efficacy in psoriasis and the trial was terminated at phase II (15).

Our group (5, 16, 17) and others (18) have generated non-glycosaminoglycan-binding chemokines. These molecules retain full agonist activity for their respective receptors but, unlike normal chemokines, persist in the blood because they do not bind to the apical surface of vascular endothelium. Significantly, these molecules also mediate a powerful anti-inflammatory effect by a mechanism believed to involve chemokine receptor desensitization (17). A recent report identified the 589 Da small molecule PS372424 as an agonist specific for the human form of CXCR3 (19). The present study investigated the potential of this agonist to functionally desensitize CXCR3 and other chemokine receptors expressed by activated human T cells. The anti-inflammatory activity of this compound was then validated by measurement of the recruitment of human T cells to murine air pouches filled with chemokines or synovial fluid from patients with active rheumatoid arthritis. A candidate mechanism for this activity was defined.

Results

T-Cell Activation by PS372424. Treatment of activated T cells with the small-molecule agonist or CXCL11 induced ERK phosphorylation to a level three-times higher than unstimulated cells (P < 0.01) (Fig. 1A). The PS372424 agonist caused internalization of 87% of cell-surface CXCR3 within 30 min (P < 0.01) (Fig. 1B); the receptor did not return to the cell surface within 5 h of stimulation. In a transfilter chemotaxis assay, PS372424 stimulated significant T-cell migration at starting concentrations above 50 nM (P < 0.05); greater migration occurred using the agonist at 100 nM (Fig. 1C). Pretreatment of the T cells with a CXCR3 neutralizing antibody prevented this agonist-induced migration (P < 0.01). Unlike CXCL11, PS372424 did not stimulate transendothelial T-cell migration (Fig. 1D).

Chemokine Receptor Expression and Modulation of Chemotaxis. The potential to modify the migration of activated T cells was

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examined by measuring chemotaxis toward CXCL11, CXCL12, or CCL5 following treatment with either the CXCR3 antagonist NBI-74330 (20) or the agonist PS372424. The CXCR3 antagonist inhibited migration toward CXCL11 (Fig. 2A). However, the CXCR3 agonist PS372424 significantly inhibited migration toward each of the three chemokine receptor ligands (Fig. 2A). To investigate this finding further, a range of T-cell phenotypes were studied. The repertoire of chemokine receptors expressed by resting and activated human T cells and natural human regulatory T cells (Tregs) was examined by immunofluorescence flow cytometry after labeling CCR5, CXCR3, and CXCR4 (Fig. 2B). CCR5 was largely expressed by activated T cells and Tregs. CXCR3 was almost exclusively expressed by activated T cells, and CXCR4 was expressed almost equally by each T-cell subpopulation. A clear concordance was observed between this receptor expression profile and the migratory response of each cell type to the corresponding chemokine ligands CCL5, CXCL11, and CXCL12 (Fig. 2C). Rheumatoid arthritis synovial fluid (RASF) from patients with active inflammation induced significant migration of each T-cell subpopulation (Fig. 2C). Chemotactic migration of the three T-cell subpopulations toward RASF was not reduced by treatment with the CXCR3 neutralizing antibody or the CXCR4 antagonist AMD3100 (Fig. 2D). However, the migration of activated T cells toward RASF was inhibited specifically by PS372424 (P < 0.01), suggesting a potential for anti-inflammatory activity.

Unique in Vivo Human T-Cell Migration Assay. PS372424 is specific for human CXCR3 but does not activate murine CXCR3. To examine the effect of this small molecule agonist in vivo, a murine model of human T-cell migration was established. Six groups of mice were humanized using peripheral blood mononuclear cells (PBMC) from different donors. Splenocytes from these mice contained 71.3 ± 10.7% (mean ± SD, n = 82) cells expressing human CD45 by 28 d after the injection of human PBMC (Fig. 3A). Typically, 40% of the human CD45+ cells coexpressed CD4+ (Fig. 2B), the remainder expressed CD8+. More than 90% of the human cells were CD3+ T cells, of which >90% coexpressed CXCR3 (Fig. 3C). These human cells were almost exclusively CD14+CD19−CD56−CD45RO−CD69−CCR5+.

Humanized mice were used to examine human T-cell migration in vivo using an air-pouch model of inflammation. Injection of human CXCL11 into air pouches induced significant recruitment of human CD45+ cells compared with air pouches filled with PBS (Fig. 3D); the recruitment produced by CXCL11 was reduced when mice received intravenous PS372424 (P < 0.05). Humanized mice did not show any apparent adverse effect for up to 24 h after intravenous administration of PS372424. Daily administration of PS372424 to humanized mice for 5 d did not result in any apparent adverse effect, with no changes in weight or behavior. Whole human blood showed no significant production of a range of cytokines when incubated for up to 24 h with 1 μM PS372424; no acute leukocyte respiratory burst was observed (Fig. 3E).

Fig. 1. Consequences for human T cells of activation of CXCR3 by PS372424. (A) Relative pERK in activated T cells measured by ELISA following 10-min treatment with ligand. (B) Flow cytometric analysis of surface CXCR3 expression in activated T cells 30 min following treatment with ligand. (C) Dose-response curve using PS372424 in activated T-cell chemotaxis assay. Hatched bar denotes cells pretreated with CXCR3 neutralizing antibody. (D) Transendothelial chemotaxis of activated T cells toward ligand. Data represent three independent experiments performed in triplicate ± SEM.
Intravenous PS372424 following activation T cells with PS372424 did not result in phosphorylation hatched bars), PMA (0.05). Administration 0.05) and no <

Chemokine receptors possess antigenic variability, fl Splenocytes from humanized mice were examined by flow cytometry 24 h after intravenous administration of PBS or PS372424. A

Anti-Inflammatory Activity of PS372424. Intravenous PS372424 inhibited human cell recruitment to air pouches filled with either CXCL12 or CCL5 to a background level (P < 0.05) (Fig. 4A). However, treatment with the CXCR3 antagonist NBI-74330 did not inhibit T-cell migration toward CCL5 (Fig. 4A). Cell lines transfected to express only CXCR3 or CXCR4 migrated toward RASF in vitro (Fig. 4B); this was blocked by treatment with a CXCR3 neutralizing antibody or the CXCR4 antagonist AMD3100, respectively (in both cases P < 0.05). Administration of RASF into air pouches on humanized mice induced significant recruitment of human CD45+ cells (Fig. 4C). Intravenous administration of the CXCR4 antagonist AMD3100 did not inhibit the recruitment of human cells to the air pouch in response to RASF. A similar failure to inhibit human cell recruitment in response to RASF was observed after administration of the CXCR3-blocking antibody or the small-molecule CXCR3 antagonist NBI-74330. However, cell recruitment by RASF was reduced to a background level by intravenous administration of PS372424 (P < 0.01) (Fig. 4C).

Mechanism of Chemokine Receptor Cross-Desensitization. Splenocytes from humanized mice were examined by flow cytometry 24 h after intravenous administration of PBS or PS372424. A reduction in cell-surface CXCR3 expression was observed in animals that had received the PS372424 agonist (P < 0.01); a decrease in CCR5 expression was also observed (P < 0.05) (Fig. 5A).

Examination of chemokine receptor phosphorylation showed that PS372424 caused a concentration-dependent phosphorylation of CCR5 on CXCR3+ T cells (Fig. 5B). Treatment of CXCR3+ T cells with PS372424 did not result in phosphorylation of CCR5, but this receptor was phosphorylated by treatment with CCL5 (Fig. 5B). The PS372424-induced phosphorylation of CCR5 on CXCR3+ T cells was prevented by the PKC inhibitor staurosporine (Fig. 5B).

FRET analysis showed that CXCR3 and CCR5 form a chemokine receptor heterodimer, with a FRET efficiency of 9% (Fig. 5C), the maximum energy transfer between APC and PE is ≤10% (21). An acid wash reduced the FRET (P < 0.05) and no FRET occurred using CD45 as a control (Fig. 5C). A titration of both antibodies indicated this was a true FRET rather than spectral overlap (Fig. 5D) (21).

Discussion

PS372424 is a small-molecule agonist for CXCR3 that was identified during a screen for antagonist compounds (19). The agonist has a peptidic structure containing a tetrahydroisoquinoline-arginine motif that closely aligns with a Pro-Arg motif at residues 35–39 of CXCL10; this motif is important for CXCR3 activation (22). PS372424 activated ERK in T cells with a similar potency and kinetic to the natural ligand CXCL11. The activation of ERK is commonly used to assess chemokine receptor signaling and is an absolute requirement for directional cell migration (23). Other effects of CXCR3 ligation, including receptor internalization and Ca2+ flux, were also similar for T cells activated with PS372424 and CXCL11. CXCR3 did not return to the cell surface within 5 h of stimulation, which is consistent with previous reports that this receptor requires de novo synthesis (24).

T-cell chemotaxis occurred efficiently in a concentration gradient formed by the diffusion of 100 nM PS372424; this concentration is consistent with reports of the receptor affinity of PS372424 (25). Importantly, PS372424 did not elic iT-cell migration across an endothelial monolayer. This is a typical feature of nonglycosaminoglycan-binding chemokine receptor agonists because apical presentation of chemokines by endothelial cells is crucial in this assay (17).

The potential of PS372424 to inhibit the migration of activated T cells was examined using single chemokine ligands. PS372424 inhibited the migration of these T cells toward CXCL11, CXCL12, and CCL5, whereas the CXCR3 antagonist NBI-74330 (20) only prevented migration toward CXCL11. This finding is consistent with stimulation of CXCR3 by PS372424 causing additional desensitization of both CXCR4 and CCR5, but the antagonist can only modulate the response produced by the activation of CXCR3.

Flow cytometric analysis demonstrated that freshly isolated human T cells were largely CCR5+ CXCR3−CXCR4+, but these cells were mostly CCR5+ CXCR3−CXCR4+ following activation by stimulation of CD3 and CD28. Natural Tregs were isolated from peripheral blood by selecting the CD4+CD25+ phenotype and validated for nuclear expression of FOXP3 (26); their chemokine receptor phenotype was largely CCR5+ CXCR3− CXCR4+. Although the expression of CXCR3 by some human Tregs has been described (7), the majority of natural Tregs are CXCR3− (27). Chemokine receptors possess antigenic variability, which can limit their detection by flow cytometry (28). For this reason flow cytometric analysis of the three T-cell subpopulations was confirmed by functional chemotaxis assays. A clear concordance was observed between expression of CCR5, CXCR3, and CXCR4 and migration toward CCL5, CXCL11, and CXCL12, respectively. RASF from patients with active disease was used as a broad-spectrum, chemokine-rich stimulus and induced equal migration of each functionally defined T-cell subgroup (16).
The effect of chemokine receptor blockade on the migration of each T-cell subpopulation toward RASF was measured. As expected, neither the small-molecule CXCR4 antagonist AMD3100 nor a CXCR3 neutralizing antibody disrupted cell migration toward the chemokines present in RASF. This result reflects the situation during active human disease, where inflammation is produced by a myriad of chemokines (17). Importantly, PS372424 inhibited the migration of activated T cells toward RASF but allowed normal chemotactic recruitment of resting and regulatory T cells, which both express little CXCR3, suggesting a potential to modify the composition of the inflammatory infiltrate in vivo.

As a consequence of a Q196E amino acid change between human and murine CXCR3, PS372424 is only agonistic for the human chemokine receptor (25), which prevents in vivo examination of the potential anti-inflammatory properties of this agonist using conventional murine models. For this reason, immunodeficient mice were injected with human PBMC (29) to produce a reproducible peripheral T-cell population that, after 28 d, was almost exclusively human and CXCR3⁺. The induction of the activation marker CXCR3 on these cells is consistent with the coexpression of CD45RO and CCR5 and may be indicative of homeostatic T-cell proliferation (30).

**Fig. 4.** Heterologous chemokine receptor regulation in vivo. (A) Human cell migration into air pouches 24 h following intrapouch administration of indicated ligand and intravenous administration of vehicle (gray bars), PS372424 (black bars), or NB7-74330 (hatched bar). Dashed line indicates level of migration without chemokine. (B) Migration of indicated cell line toward RASF following pretreatment with vehicle (white bars) or specific receptor blockade (NB7-74330 and AMD3100 for CXCR3 and CXCR4, respectively; black bars). (C) Human cell migration into air pouches 24 h following intrapouch administration of RASF and intravenous administration of indicated agent. Dashed line indicates level of migration without RASF. Data represent three independent experiments ± SEM performed in triplicate or eight mice per group.

**Fig. 5.** Mechanism for heterologous chemokine receptor desensitization. (A) Flow cytometric analysis of surface CXCR3 and CCR5 on splenic human T cells 24 h following intravenous administration of vehicle (gray bars) or PS372424 (black bars). (B) Western blot analysis of pCCR5 in activated T cells or in CD4⁺CD25⁺CXCR3⁻ T cells following 30-min treatment with ligand; in indicated lane, cells were treated in the presence of 10 ng/mL staurosporine (STS). Pyruvate dehydrogenase complex (PDC) blotting demonstrates equal loading. (C) FRET efficiency between indicated acceptor and CXCR3-PE determined by flow cytometry using activated T cells. (D) FRET efficiency between CCR5-APC and CXCR3-PE determined by flow cytometry using activated T cells. White bars denote 15% saturation CXCR3-PE, gray bars 43%, and black bars 100%. Data represent three independent experiments ± SEM performed in triplicate with eight mice per group.
Experiments were performed to examine potential side effects produced by systemic administration of PS372424. In previous studies, lymphocyte-activating chemokines did not elicit activation of effector mechanisms, such as degranulation (17). Stimulation of whole human blood with a high (1 μM) concentration of the CXCR3 agonist for up to 24 h induced no increase in the concentration of a range of cytokines. Stimulation with the agonist for 10 min also produced no acute oxidative burst. Although this finding is consistent with the absence of any apparent adverse effect following administration of the CXCR3 agonist, it does not exclude subtle pharmacological toxicity.

Air pouches were generated on humanized mice by subcutaneous injection of sterile air over 6 d (16). Addition of human CXLCL11 to the pouch resulted in significant recruitment of human CD45+ cells. This migration was reduced to background by intravenous injection of sufficient PS372424 to produce an initial 1-μM concentration in the blood. Injection of PS372424 also inhibited recruitment of human cells into air pouches filled with CXCL12 or CCL5.

The small-molecule CXCR3 antagonist NBI-74330 (31) did not modify human cell migration toward CCL5. NBI-74330 has no agonist activity (20). These data indicate that PS372424 mediates its heterologous anti-inflammatory effect by signaling through CXCR3, rather than by simply blocking the CXCR3/CCR5 heterodimer. Chemotaxis experiments using cell lines transfected with single chemokine receptors indicated that RASF contained ligands for the receptors CXCR3 and CXCR4. The migration of CXCR3- and CXCR4-expressing cells toward RASF was inhibited by a CXCR3-neutralizing antibody or the CXCR4 antagonist AMD3100, respectively (32).

Because the air pouch develops a pseudosynovial membrane (33), instillation of RASF provides a preclinical assay system to test agents of relevance for the treatment of rheumatoid arthritis. Injection of RASF into these pouches produced a significant recruitment of human CD45+ cells. This production was not inhibited by intravenous administration of AMD3100 at 2 μM, the maximum achievable clinical concentration (32), or anti-CXCR3 or NBI-74330 at 100 mg/kg, a dose that has been used in other in vivo models (34). However, the inflammatory response produced by RASF was reduced to background levels by intravenous administration of PS372424. This finding provides in vivo demonstration that systemic administration of a small-molecule CXCR3 receptor agonist can limit the migration of human cells toward the range of chemokines present in RASF.

Analysis of cell-surface chemokine receptor expression by human T cells recovered from the spleen of humanized mice that had been treated with PS372424 demonstrated a significant reduction in both CXCR3 and CCR5. Chemokine receptor heterodimerization suggests a mechanism by which specific activation of one receptor can induce desensitization of another (6). Importantly, FRET analysis of activated human T cells demonstrated that CXCR3 and CCR5 could form such a heterodimer on the cell surface.

Phosphorylation of chemokine receptors disrupts their ability to recruit heterotrimeric G proteins and therefore inhibits signaling (6). This phosphorylation can also cause the loss of cell-surface receptors by activation of β-arrestin-mediated internalization (35), leading to receptor recycling or degradation (6). Treatment of CCR5+ CXCRI+ T cells with PS372424 caused a dose-dependent increase in the phosphorylation of CCR5; this was not seen following stimulation of CCR5+ CXCRI− T cells with this agonist. This finding is consistent with previous reports, suggesting that CCR5 cross-phosphorylation is variably mediated by protein kinase C or G protein-coupled receptor kinases (35).

The observation that 10 ng/mL staurosporine inhibits CCR5 phosphorylation following treatment with the CXCR3 agonist suggests that the cross-phosphorylation produced in this system is dependent on the activity of protein kinase C (36).

The current study demonstrates potent anti-inflammatory activity associated with the CXCR3 agonist PS372424 both in vitro and in a novel humanized in vivo model of rheumatoid arthritis. Significant effort has been expended in targeting receptors such as CCR1 for clinical benefit in rheumatoid arthritis. Although CCR1 may be considered a nonredundant receptor in the genesis of this disease (3), the joints of patients with active disease contain ligands for many chemokine receptors. The compound described in the present study has the potential to desensitize multiple chemokine receptors expressed by activated T cells but does not affect the migration of potentially beneficial Tregs. These data make a clear argument for exploration of agonist-induced chemokine receptor desensitization strategies for the treatment of important inflammatory diseases.

Materials and Methods

Studies were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and local Ethics Committee approval (Home Office License PPL603772).

Cytokines and Reagents. Chemokines (R&D Systems) were used in vitro at a concentration of 50 nM. The small-molecule CXCR3 agonist PS372424 was supplied by Ligand Pharmaceuticals (19). PS372424 was typically used in vitro at a concentration of 100 nM; a DMSO (Sigma) dilution of 1 in 106 was used as the vehicle control. CXCR3-neutralizing antibody (Clone 49801; R&D Systems) was used at 2 μg/mL in vitro. CXCR3 antagonist NBI-74330 supplied by M. Smit and M. Wijtmans (Leiden/Amsterdam Centre for Drug Research, Amsterdam, The Netherlands) (37) was used at 1 μM (31). CXCR4 antagonist AMD3100 (Sigma) was used at 2 μM, the maximum plasma concentration (32). Following informed consent, RASF was collected from the knee joint of patients with active rheumatoid arthritis undergoing therapeutic aspiration at the Freeman Hospital, Newcastle upon Tyne, United Kingdom. Following centrifugation, the cell-free supernatant was stored at −20 °C; samples from four patients were pooled to standardize the RASF used. Chemokine levels in RASF were quantified by multiplex electrochemoluminescent immunoassay (Mesoscale). Undiluted pooled RASF contained 3,652 ± 62 pg/mL CCL2, 12,327 ± 207 pg/mL CCL4, 2,592 ± 28 pg/mL CCL5, 10,962 ± 640 pg/mL CCL11, 2,540 ± 384 pg/mL CCL13, 9,528 ± 269 pg/mL CCL17, 618 ± 11 pg/mL CXCL8, and 3,078 ± 0.3 pg/mL CXCL10. RASF was diluted 1 in 10 into PBS for use in vitro or 1 in 5 into PBS for use in vivo, as described below. Experiments involving human blood cells were performed independently with greater than or equal to three donors.

Human T-Cell Isolation. Primary T cells were isolated from National Blood Service apheresis cones after approval from the Newcastle and North Tyneside Research Ethics Committee. PBMC were isolated from whole blood by density centrifugation (Ficoll, 100 U/mL penicillin, and 100 μg/mL streptomycin). T cells were activated with CD3/CD28 T-cell expander Dynabeads (Invitrogen) at a 1:1 bead:T cell ratio. Natural Tregs, defined as CD4+CD25hiFOXP3+CD45RO−, were isolated from apheresis cones using RosetteSep-negative enrichment of CD4+ T cells followed by RoboSep immunomagnetic CD25+ positive selection (human CD25+CD4+CD45RO−, positive selection kit; StemCell Technologies) (26).

ERK Activation. Phosphorylation of ERK1 at T202/Y204 and ERK2 at T185/Y187 were measured by ELISA (KCB1018; R&D Systems). Activated T cells were stimulated with 50 nM CCL5 or 100 nM PS372424 for 10 min at 37 °C. Ice-cold PBS was added and cells were pelleted. Cell lysis was performed in PhosphoSafe Extraction Reagent (Novagen). Lysates were centrifuged at 20,000 × g for 20 min at 4 °C before analysis.

Chemotaxis Assays. T-cell migration was assessed using a 3-μm pore Transwell filter system (BD Biosciences). In some cases, transendothelial chemotaxis was assessed by culturing 5 × 105 EAhY926 cells on the filter for 72 h before assay (17, 38). Next, 2 × 105 T cells were placed in upper chamber; the lower chamber was supplemented with chemokine or RASF. The assay was incubated for 90 min at 37 °C before removal of the insert. The medium in the lower chamber was aspirated and migrant cells counted by FACs using flow-count beads (BD Biosciences). CHO cells transfected with CXCR4 (CHO-CXCR4) and HEK cells transfected with CXCR3 (HEK-CXCR3) were generated as previously described (39). These cells were used in an 8-μm pore chemotaxis assay toward RASF. These cells were enumerated on the filter by light
microscopy (17). All chemokataxis data are relative to migration in the absence of stimulus.

Flow Cytometry. Cell-surface receptor expression was measured using a BD FACs Canto II. Receptor internalization was measured 30 min following stimulation. Antibodies used in this study were human CD45 (clone 2D1; BD Biosciences), human CXCR3 (clone 49801; R&D Systems), CD3 (clone HCHT-1; Sigma), CD4 (clone B-2; Abbam), CD8 (clone B-231; Abbam), and CCR5 (clone CT5; R&D Systems). Data were analyzed using FlowJo software v7.6.4 (TreeStar). FRET was assessed by flow-cytometry with excitation of CXCR3-PE at 488 nm and measurement of emission from CCR5-APC at 675 nm. To demonstrate that FRET was nonrandom, both antibodies were titrated to a saturating concentration (21). A 5-min acid wash (20 mM HCl/HBSS; pH 2) at 4°C was also performed to remove surface antibodies. CD45-APC was used as a negative control (40). Energy transfer efficiencies calculated as described by Batard et al. (21).

CCR5 Phosphorylation. Cells were serum-starved for 2 h and stimulated with vehicle control, 10, 50, 100, or 200 nM PS372424 or 50 nM CCL5 for 30 min at 37°C. In control experiments, cells were incubated with PS372424 in the presence of 10 ng/mL staurosporine (Calbiochem). Lysates were prepared as above and fractionated by SDS/PAGE for Western blotting (23). Membranes were blocked in 5% (w/v) BSA and probed using 250 ng/mL anti-pCCRs (Ser309; Clone E11/19; Biolegend). For loading control, blots were stripped and probed with antippyruvate dehydrogenase complex at a concentration of 1:5,000. Anti-mouse HRP-conjugated secondary antibody (Sigma) was used at 1:2,000.

Humanized Air-Pouch Assay. Female NOD.Cg-PkdcreERT2 tm1Il2rgtm1KnjF1 (29) mice (Charles River; 8 wk old) were given 105 human PBMC in 0.5 mL PBS by intraperitoneal injection. Twenty-one days later, air pouches were generated as described previously (17). On day 6, each pouch was injected with 1 mL PBS containing 5 μg chemokine or RASF, animals received intravenously 0.1 mL of vehicle control, PS372424 (1 μM in blood), CXCR3 neutralizing antibody (25 μg) or AMD3100 (2 μM in blood). NBI-74330 was administered subcutaneously (100 ng/kg) (34).

Recruited cells were recovered by lavaging the pouch twice with 0.75 mL of PBS containing 3 mM EDTA. The exudates were centrifuged at 500 × g for 5 min. The cell pellets resuspended for enumeration of human cells by FACS. Splenocytes were isolated and analyzed by flow cytometry.

Treatment of Blood with PS372424. Heparinized whole human blood was treated with vehicle control, 1 μM PS372424, or 100 ng/mL LPS or 20 ng/mL phorbil myristate acetate (PMA) and incubated for up to 24 h at 37°C. Cells were pelleted and the supernatants analyzed for cytokines. To examine the oxidative burst of whole blood, a Burst Test kit (Orsgepen Pharma) was used (17). Next, 100 μL of heparinized human blood was stimulated with either vehicle, 1 μM PS372424, or 2 × 107 opsonized Escherichia coli for 10 min at 37°C. Reactive oxygen metabolites were measured by flow cytometry.

Statistical Analysis. All results are expressed as mean values ± SEM of replicate samples. The significance of changes was assessed by the application of an ANOVA with Bonferroni posttest. Data were analyzed using Prism 5 software (GraphPad).

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