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Citation: O'Boyle, Graeme, Fox, Christopher, Walden, Hannah, Willet, Joseph, Mavin, Emily, Hine, Dominic, Palmer, Jeremy, Barker, Catriona, Lamb, Christopher, Ali, Simi and Kirby, John (2012) Chemokine receptor CXCR3 agonist prevents human T-cell migration in a humanized model of arthritic inflammation. Proceedings of the National Academy of Sciences of the United States of America, 109 (12). pp. 4598-4603. ISSN 0027-8424

Published by: National Academy of Sciences

URL: <http://dx.doi.org/10.1073/pnas.1118104109>
<<http://dx.doi.org/10.1073/pnas.1118104109>>

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Classification: Biological sciences, Immunology

A CXCR3 agonist prevents human T cell migration in a humanized model of arthritic inflammation

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The authors have no conflicting interests to declare

Abstract

The recruitment of T lymphocytes during diseases such as rheumatoid arthritis is regulated by stimulation of the chemokine receptors expressed by these cells. The current study was designed to assess the potential of a CXCR3-specific small molecule agonist to inhibit the migration of activated human T cells towards multiple chemokines. Further experiments defined the molecular mechanism for this anti-inflammatory activity.

Analysis *in vitro* demonstrated that the agonist induced internalisation of both CXCR3 and other chemokine receptors co-expressed by CXCR3 positive T cells. Unlike chemokine receptor-specific antagonists, the CXCR3 agonist inhibited migration of activated T cells towards the chemokine mixture in synovial fluid from patients with active rheumatoid arthritis. A humanized mouse air pouch model showed that IV treatment with the CXCR3 agonist prevented inflammatory migration of activated human T cells towards 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 generalised chemokine receptor desensitisation 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 desensitisation inhibits the inflammatory response that is normally produced by the chemokines present in synovial fluid from patients with active rheumatoid arthritis.

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Introduction

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 neutralising antibodies targeted at receptors or ligands, and small molecule receptor antagonists (2). However, pre-clinical 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 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, whilst inflammatory chemokines are induced by localised stress, pro-inflammatory 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 CXCL9, CXCL10 and CXCL11 provides an example of a pro-inflammatory 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 (TCR) complex (9). In this situation both CXCR3 and the TCR share downstream signalling 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 (GAG) binding chemokines. These molecules retain full agonist activity for their respective receptors but, unlike normal chemokines, persist in the blood as they do not bind to the apical surface of vascular

endothelium. Significantly, they also mediate a powerful anti-inflammatory effect by a mechanism believed to involve chemokine receptor desensitisation (17).

A recent report identified the 589Da 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 desensitise 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 3-times higher than unstimulated cells ($p < 0.01$; Figure 1A). The PS372424 agonist caused internalisation of 87% of cell-surface CXCR3 within 30 minutes ($p < 0.01$; Figure 1B); the receptor did not return to the cell surface within 5 hours of stimulation. In a transfilter chemotaxis assay, PS372424 stimulated significant T cell migration at starting concentrations above 50nM ($p < 0.05$); greater migration occurred using the agonist at 100nM (Figure 1C). Pre-treatment of the T cells with a CXCR3 neutralising antibody prevented this agonist-induced migration ($p < 0.01$). Unlike CXCL11, PS372424 did not stimulate transendothelial T cell migration (Figure 1D).

Chemokine receptor expression and modulation of chemotaxis

The potential to modify the migration of activated T cells was examined by measuring chemotaxis towards CXCL11, CXCL12 or CCL5 following treatment with either the CXCR3 antagonist NBI-74330 (20) or the agonist PS372424. The CXCR3 antagonist inhibited migration towards CXCL11 (Figure 2A). However the CXCR3 agonist PS372424 significantly inhibited migration towards each of the 3 chemokine receptor ligands (Figure 2A). To investigate this 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 Treg was examined by immunofluorescence flow cytometry after labelling CCR5, CXCR3 and CXCR4 (Figure 2B); CCR5 was largely expressed by activated T cells and Treg, 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 (Figure 2C). Rheumatoid arthritis synovial fluid (RASf) from patients with active inflammation induced significant migration of each T cell subpopulation (Figure 2C). Chemotactic migration of the 3 T cell subpopulations towards RASf was not reduced by treatment with the CXCR3 neutralising antibody or the CXCR4 antagonist AMD3100 (Figure 2D). However, the migration of activated T cells towards RASf was inhibited specifically by PS37242 ($p < 0.01$), suggesting a potential for anti-inflammatory activity.

Novel *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. 6 groups of mice were humanized using peripheral blood mononuclear cells (PBMC) from different donors. Splenocytes from these mice contained $71.3 \pm 10.7\%$ (mean \pm std dev, $n=82$) cells expressing human CD45 by 28 days after the injection of human PBMC (Figure 3A). Typically, 40% of the human CD45⁺ cells co-expressed CD4⁺ (Figure 3B), the remainder expressed CD8⁺. More than 90% of the human cells were CD3⁺ T cells, of which >90% co-expressed CXCR3 (Figure 3C). These human cells were almost exclusively CD14⁻ CD19⁻ CD56⁻ CD45RO⁺ CD69^{lo} 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 (Figure 3D); the recruitment produced by CXCL11 was reduced when mice received IV PS372424 ($p < 0.05$). Humanized mice did not show any apparent adverse effect for up to 24 hours after IV administration of PS372424. Daily administration of PS372424 to humanised mice for 5 days did not result in any apparent adverse effect, with no changes in weight or behaviour. Whole human blood showed no significant production of a range of cytokines when incubated for up to 24 hours with 1 μ M PS372424; no acute leukocyte respiratory burst was observed (Figure 3E).

Anti-inflammatory activity of PS372424

IV PS372424 inhibited human cell recruitment to air pouches filled with either CXCL12 or CCL5 to a background level ($p < 0.05$, Figure 4A). However, treatment with the CXCR3 antagonist NBI-74330 did not inhibit T cell migration towards CCL5 (Figure 4A). Cell lines transfected to express only CXCR3 or CXCR4 migrated towards RASF *in vitro* (Figure 4B); this was blocked respectively by treatment with a CXCR3 neutralising antibody or the CXCR4 antagonist AMD3100 (in both cases $p < 0.05$). Administration of RASF into air pouches on humanized mice induced significant recruitment of human CD45⁺ cells (Figure 4C). IV 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 IV administration of PS372424 ($p < 0.01$; Figure 4C).

Mechanism of chemokine receptor cross-desensitisation

Splenocytes from humanized mice were examined by flow cytometry 24 hours after IV 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 ($p < 0.05$; Figure 5A).

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

FRET analysis showed that CXCR3 and CCR5 form a chemokine receptor heterodimer, with a FRET efficiency of 9% (Figure 5C), the maximum energy transfer between APC and PE is $\leq 10\%$ (21). An acid wash reduced the FRET ($p < 0.05$) and no FRET occurred using CD45 as a control (Figure 5C). A titration of both antibodies indicated this was a true FRET rather than spectral overlap (Figure 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 signalling and is an absolute requirement for directional cell migration (23). Other effects of CXCR3 ligation including receptor internalization and Ca^{2+} flux were also similar for T cells activated with PS372424 and CXCL11. CXCR3 did not return to the cell surface within 5 hours 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 100nM PS372424; this concentration is consistent with reports of the receptor affinity of PS372424 (25). Importantly, PS372424 did not elicit T cell migration across an endothelial monolayer. This is a typical feature of non-GAG binding chemokine receptor agonists as 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 towards CXCL11, CXCL12 and CCL5 whereas the CXCR3 antagonist NBI-74330 (20) only prevented migration towards CXCL11. This is consistent with stimulation of CXCR3 by PS372424 causing additional desensitisation of both CXCR4 and CCL5, whilst 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 $\text{CCR5}^- \text{CXCR3}^- \text{CXCR4}^+$ but these cells were mostly $\text{CCR5}^+ \text{CXCR3}^+ \text{CXCR4}^+$ following activation by stimulation of CD3 and CD28. Natural Treg were isolated from peripheral blood by selecting the $\text{CD4}^+ \text{CD25}^{\text{hi}}$ phenotype and validated for nuclear expression of FOXP3 (26); their chemokine receptor phenotype was largely $\text{CCR5}^+ \text{CXCR3}^- \text{CXCR4}^+$. Although the expression of CXCR3 by some human Treg has been described (7), the majority of natural Treg 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 3 T cell subpopulations was confirmed by functional chemotaxis assays. A clear concordance was observed between expression of CCR5, CXCR3 and CXCR4 and migration towards 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 subpopulation (16).

The effect of chemokine receptor blockade on the migration of each T cell subpopulation towards RASF was measured. As expected, neither the small molecule CXCR4 antagonist AMD3100 nor a CXCR3 neutralising antibody disrupted cell migration towards the chemokines present in RASF. This 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 towards RASF whilst allowing 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). This 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 days, was almost exclusively human and CXCR3⁺. The induction of the activation marker CXCR3 on these cells is consistent with the co-expression of CD45RO and CCR5 and may be indicative of homeostatic T cell proliferation (30).

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 μ M) concentration of the CXCR3 agonist for up to 24 hours induced no increase in the concentration of a range of cytokines. Stimulation with the agonist for 10 min also produced no acute oxidative burst. Whilst this is consistent with the absence of any apparent adverse effect following administration of the CXCR3 agonist, they do not exclude subtle pharmacological toxicity.

Air pouches were generated on humanized mice by subcutaneous injection of sterile air over six days (16). Addition of human CXCL11 to the pouch resulted in significant recruitment of human CD45⁺

cells. This migration was reduced to background by IV injection of sufficient PS372424 to produce an initial 1 μ M 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 towards CCL5. NBI-74330 has no agonist activity (20). These data indicate that PS372424 mediates its heterologous anti-inflammatory effect by signalling 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 towards RASF was respectively inhibited by a CXCR3 neutralising antibody or the CXCR4 antagonist AMD3100 (32).

As the air pouch develops a pseudosynovial membrane (33), instillation of RASF provides a pre-clinical 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 was not inhibited by IV administration of AMD3100 at 2 μ M, the maximum achievable clinical concentration (32), anti-CXCR3 or NBI-74330 at 100mg/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 IV administration of PS372424. This provides the first *in vivo* demonstration that systemic administration of a small molecule chemokine receptor agonist can limit the migration of human cells towards 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 heterodimerisation suggests a mechanism by which specific activation of one receptor can induce desensitisation 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 signalling (6). This phosphorylation can also cause the loss of cell surface receptors by activation of β -arrestin-mediated internalisation (35), leading to receptor recycling or degradation

(6). Treatment of CCR5⁺CXCR3⁺ T cells with PS372424 caused a dose dependent increase in the phosphorylation of CCR5; this was not seen following stimulation of CCR5⁺CXCR3⁻ T cells with this agonist. This 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 10ng/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. Whilst CCR1 may be considered a non-redundant 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 current study has the potential to desensitise multiple chemokine receptors expressed by activated T cells but did not affect the migration of potentially beneficial Treg. These data make a clear argument for exploration of agonist-induced chemokine receptor desensitisation strategies for the treatment of important inflammatory diseases.

Materials and Methods

Studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and local Ethics Committee approval (Home Office Licence PPL60/3772).

Cytokines and Reagents

Chemokines (R&D Systems) were used *in vitro* at a concentration of 50nM. The small molecule CXCR3 agonist PS372424 was supplied by Ligand Pharmaceuticals (La Jolla, California) (19). PS372424 was typically used *in vitro* at a concentration of 100nM; a DMSO (Sigma) dilution of 1 in 10⁶ was used as the vehicle control. CXCR3 neutralising antibody (Clone 49801; R&D Systems) was used at 2µg/ml *in vitro*. CXCR3 antagonist NBI-74330 supplied by M Smit and M Wijtmans (Amsterdam Centre for Drug Research) (37) was used at 1µM (31). CXCR4 antagonist AMD3100 (Sigma) was used at 2µM, 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. Following centrifugation, the cell-free supernatant was stored at -20°C; samples from 4 patients were pooled in order to standardise the RASF used. Chemokine levels in RASF were quantified by multiplex electrochemoluminescent immunosorbent assay (MesoScale). Undiluted pooled RASF contained 3652±62 pg/ml CCL2, 12327±207 pg/ml CCL4, 2592±28 pg/ml CCL5, 10962±440 pg/ml CCL11, 2540±18 pg/ml CCL13, 9528±269 pg/ml CCL17, 618±11 pg/ml CXCL8, 3078±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 ≥3 donors.

Human T cell Isolation

Primary T cells were isolated from National Blood Service aphaeresis cones after approval from the local Research Ethics Committee. Peripheral blood mononuclear cells (PBMC) were isolated as described previously (17). Purified T lymphocytes were extracted using RosetteSep human T cell enrichment cocktail antibody (StemCell Technologies, UK) (9). Activated T cells were cultured in RPMI 1640 medium (Sigma), containing 10% foetal calf serum, 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 Treg, defined as CD4⁺CD25^{hi}FOXP3⁺, were isolated from aphaeresis cones using RosetteSep negative enrichment of CD4⁺ T cells followed by RoboSep immunomagnetic CD25^{hi} positive selection (human CD25^{bright} 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 50nM CXCL11 or 100nM PS372424 for 10 mins 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 20000g for 20 mins at 4°C prior to analysis.

Chemotaxis Assays

T cell migration was assessed using a 3µm pore Transwell filter system (BD Biosciences). In some cases, transendothelial chemotaxis was assessed by culturing 5x10⁴ EAhy926 cells on the filter for 72 hours before assay (17, 38). 2x10⁵ 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). Chinese Hamster Ovary cells transfected with CXCR4 (CHO-CXCR4) and Human Embryonic Kidney cells transfected with CXCR3 (HEK-CXCR3) were generated previously (39). These were used in an 8µm pore chemotaxis assay towards RASF. These cells were enumerated on the filter by light microscopy (17). All chemotaxis data is expressed relative to migration in the absence of stimulus.

Flow Cytometry

Cell surface receptor expression was measured using a BD FACS Canto II. Receptor internalisation was measured 30 mins 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-A1; Abcam), CD8 (clone B-Z31; Abcam), and CCR5 (clone CTC5; R&D Systems). Data were analysed using FlowJo software v.7.6.4 (Treestar). FRET was assessed by flow cytometry with excitation of CXCR3-PE at 488nm and measurement of emission from CCR5-APC at 675nm. To

demonstrate that FRET was non-random both antibodies were titrated to a saturating concentration (21). A 5 min acid wash (20mM 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 hours and stimulated with vehicle control, 10, 50, 100, or 200nM PS372424 or 50nM CCL5 for 30 mins at 37°C. In control experiments, cells were incubated with PS372424 in the presence of 10ng/ml staurosporine (Calbiochem). Lysates were prepared as above and fractionated by SDS-PAGE for Western blotting (23). Membranes were blocked using 5% BSA and probed using 250ng/ml anti-pCCR5 (Ser349; Clone E11/19; Biolegend). For loading control, blots were stripped and probed with anti-pyruvate dehydrogenase complex (PDC) at a concentration of 1:5000. Anti-mouse HRP-conjugated secondary antibody (Sigma) was used at 1:2000.

Humanized Air Pouch Assay

Female NOD.Cg-*Prkdc*^{scid} *Il2rgtm1*^{Wjl/SzJ} (29) mice (Charles River; 8 week old) were given 10⁷ human PBMC in 0.5ml PBS by i.p. injection. 21 days later air pouches were generated as described previously (17). On day 6, each pouch was injected with 1ml PBS containing 5µg chemokine or RASF, animals received IV 0.1ml of vehicle control, PS372424 (1µM in blood), CXCR3 neutralising antibody (25µg) or AMD3100 (2µM in blood). NBI-74330 was administered by s.c. (100mg/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 500g for 5 min. The cell pellets resuspended for enumeration of human cells by FACS. Splenocytes were isolated and analysed by flow cytometry.

Treatment of Blood with PS372424

Heparinised whole human blood was treated with vehicle control, 1µM PS372424 or 100ng/ml LPS or 20ng/ml PMA and incubated for up to 24 hours at 37°C. Cells were pelleted and the supernatants analysed for cytokines. To examine the oxidative burst of whole blood, a Burst Test kit (Orpegen Pharma) was used (17). 100µl of heparinized human blood was stimulated with either vehicle, 1µM

PS372424, or 2×10^7 opsonized *E.coli* for 10 min at 37°C. Reactive oxygen metabolites were measured by flow cytometry.

Statistical analysis

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

Acknowledgements

The authors thank JD Isaacs (University of Newcastle) for sourcing RASF and M Kirkley for technical support. JAK receives support from the Newcastle upon Tyne NIHR Biomedical Research Centre. This work was funded by ARUK grant #19369.

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Figure Legends

1 Consequences for human T cells of activation of CXCR3 by PS372424

- A. Relative pERK in activated T cells measured by ELISA following 10 minute treatment with ligand
 - B. Flow cytometric analysis of surface CXCR3 expression in activated T cells 30 minutes following treatment with ligand
 - C. Dose-response curve using PS372424 in activated T cell chemotaxis assay. Hatched bar denotes cells pretreated with CXCR3 neutralising antibody
 - D. Transendothelial chemotaxis of activated T cells toward ligand
- Data represent three independent experiments performed in triplicate \pm SEM

2 Effect of a CXCR3 agonist on T cell migration

- A. Migration of activated T cells towards indicated ligand following pretreatment with vehicle (chequered bars), NBI-74330 (hatched bars), or PS372424 (cross-hatched bars)
- B. Cell surface chemokine receptor expression determined by flow cytometry on freshly isolated (grey bars), activated (white bars) and CD4⁺CD25^{hi} (black bars) T cells

- C. Transendothelial chemotaxis of freshly isolated (grey bars), activated (white bars) and CD4⁺CD25^{hi} (black bars) T cells towards 10nM of indicated ligand or RASF
 - D. Migration of indicated freshly isolated (grey bars), activated (white bars) and CD4⁺CD25^{hi} (black bars) T cells towards RASF following 30 min pretreatment with indicated agents. Dashed line is background migration in the absence of RASF
- Data represent three independent experiments performed in triplicate ± SEM

3 Humanised air pouch model of inflammation

- A. Flow cytometric analysis of human CD45 in spleen of humanised mice using 6 different human donors
 - B. Representative flow cytometric analysis of spleen cells taken from mice 28 days post humanisation
 - C. Expression of CXCR3 subsequent to human CD45 gating
 - D. Human cell migration into air pouches 24 hours following intra pouch administration of PBS or CXCL11. Mice also received intravenous injection of vehicle (white bars) or PS372424 (black bars)
 - E. Multiplex electrochemoluminescent immunosorbent analysis of cytokine production (24 hours) and flow cytometric analysis of respiratory burst (10 minutes) following treatment of human blood with vehicle (white bars), PS372424 (black bars), LPS (left hatched bars), PMA (right hatched bars) or opsonised bacteria (grey bar)
- Data represent three independent experiments performed in triplicate ± SEM performed with 6 mice per group

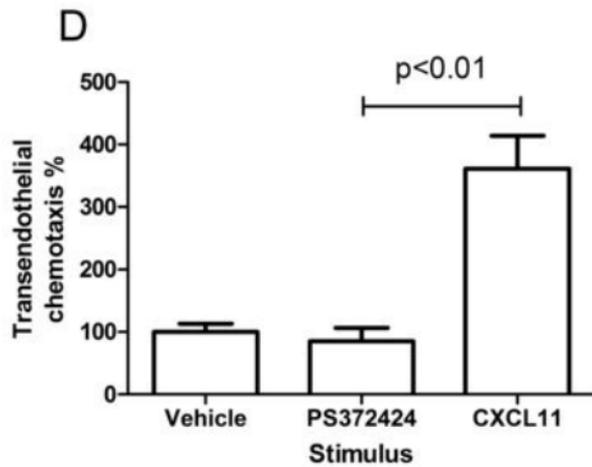
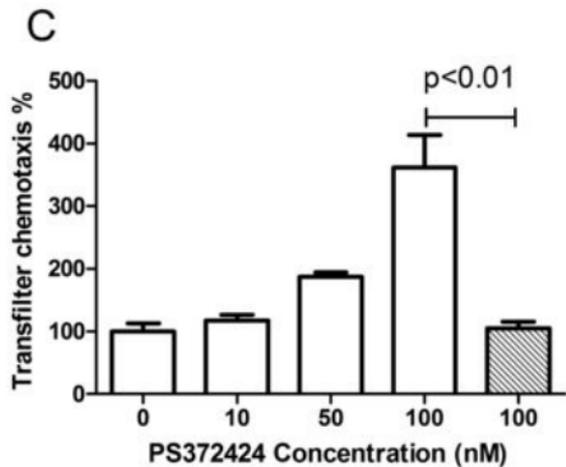
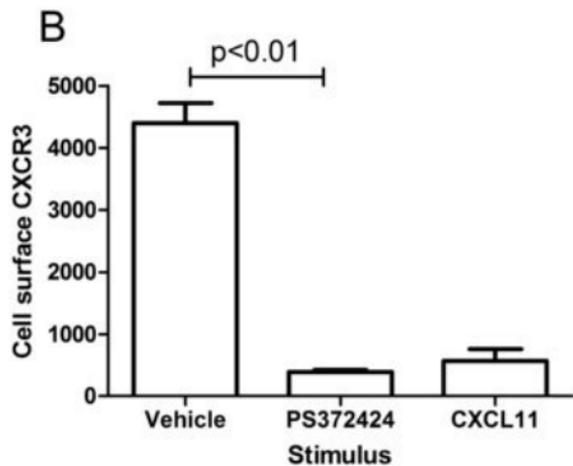
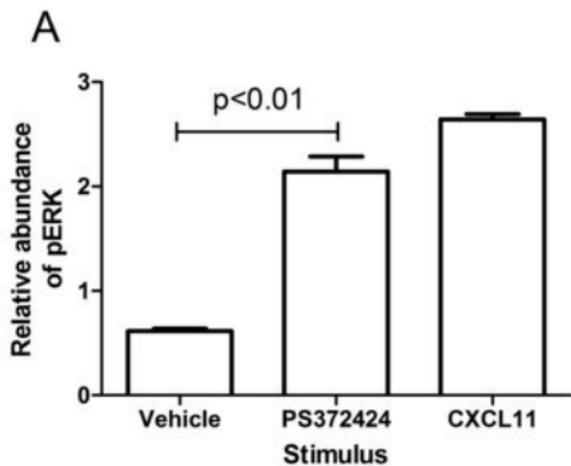
4 Heterologous chemokine receptor regulation in vivo

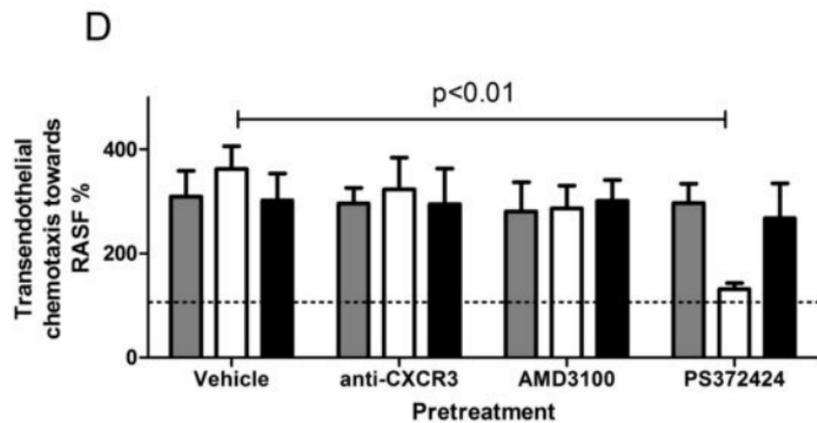
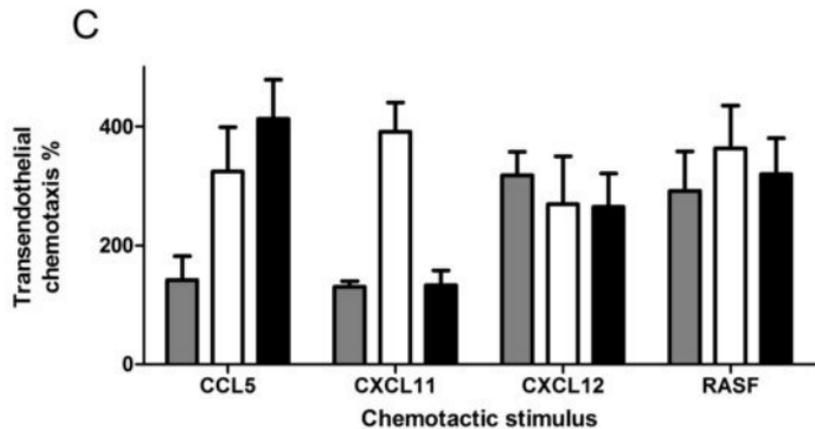
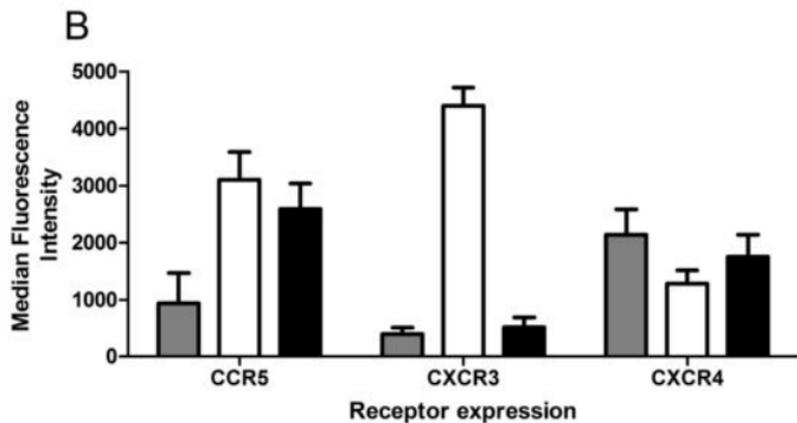
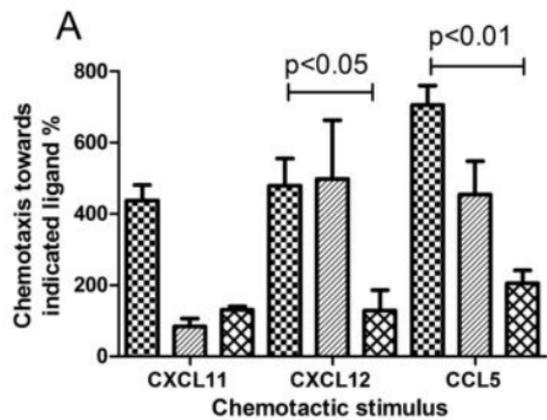
- A. Human cell migration into air pouches 24 hours following intra pouch administration of indicated ligand and intravenous administration of vehicle (grey bars), PS372424 (black bars), or NBI-74330 (hatched bar). Dashed line indicates level of migration without chemokine
 - B. Migration of indicated cell line towards RASF following pretreatment with vehicle (white bars) or specific receptor blockade (NBI-74330 and AMD3100 for CXCR3 and CXCR4 respectively; black bars).
 - C. Human cell migration into air pouches 24 hours following intra pouch administration of rheumatoid arthritis synovial fluid (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 8 mice per group

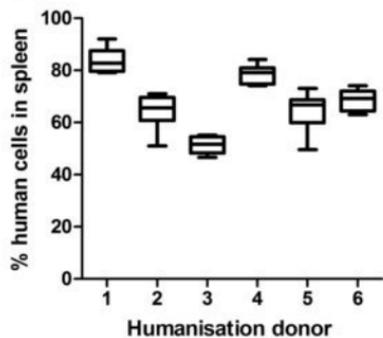
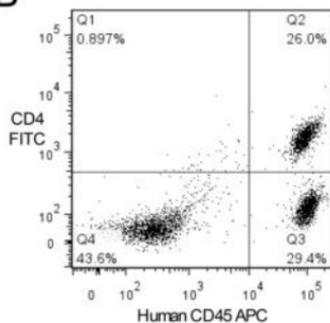
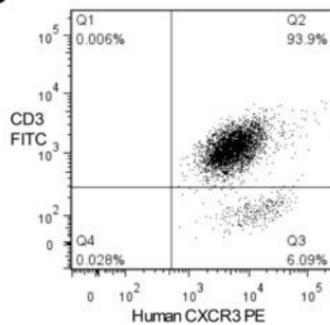
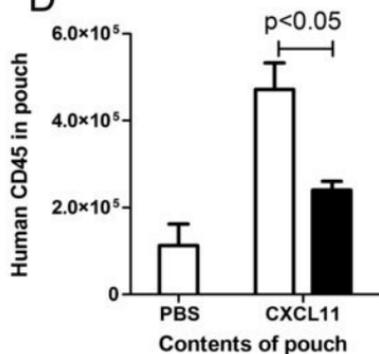
5 Mechanism for heterologous chemokine receptor desensitisation

- A. Flow cytometric analysis of surface CXCR3 and CCR5 on splenic human T cells 24 hours following intravenous administration of vehicle (grey bars) or PS372424 (black bars)
- B. Western blot analysis of pCCR5 in activated T cells or in CD4⁺CD25^{hi}CXCR3⁻ T cells following 30 min treatment with ligand; in indicated lane, cells were treated in the presence of 10ng/ml staurosporine (STS). Pyruvate dehydrogenase complex (PDC) blotting demonstrates equal loading.
- C. Fluorescence resonance energy transfer (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 denotes 15% saturation CXCR3-PE, grey bars 43%, and black bars 100%

Data represent three independent experiments \pm SEM performed in triplicate with 8 mice per group





A**B****C****D****E**