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Classification: Biological sciences, Immunology

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

Graeme O'Boyle[1]*, Christopher Fox[1], Hannah R Walden[2], Joseph DP Willet[1], Emily R Mavin[1], Dominic W Hine[1], Jeremy M Palmer[1], Catriona E Barker[1], Christopher A Lamb[1], Simi Ali[1], John A Kirby[1]

1 Institute of Cellular Medicine, Newcastle University, NE2 4HH

2 School of Life Sciences, Northumbria University, NE1 8ST

*Correspondence to:

Dr G O'Boyle
Applied Immunobiology Group
Institute of Cellular Medicine
Newcastle University
Medical School
NE2 4HH
graeme.oboyle@ncl.ac.uk

The authors have no conflicting interests to declare

Abstract

The recruitment of T lymphocytes to tissues during inflammatory diseases such as rheumatoid arthritis is regulated by stimulation of the chemokine receptor repertoire expressed by these cells. This complex system has precluded therapeutic modification by blockade of any single chemokine receptor. 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 then 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 the 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 also showed that intravenous 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 that form spontaneously 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 chemokine mixture present in synovial fluid from patients with active rheumatoid arthritis.

Introduction

Chemokine-driven lymphocyte recruitment plays a central role in the development of inflammatory diseases such as rheumatoid arthritis (1). The pathogenic importance of the chemokine system has led to the development of a range of neutralising antibodies targeted at receptors or ligands, and small molecule receptor antagonists (2). However, existing pre-clinical animal models have failed to estimate drug efficacy and many clinical trials have failed (2-4); it remains unclear whether these approaches to therapy will ever prove effective (4, 5).

The human chemokine system is complex with some 44 ligands and 21 G protein-coupled receptors identified to date (3). This is further complicated by the formation of active 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 are capable of activating more than one receptor. The chemokines are generally 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 the reactive joints of patients with active rheumatoid arthritis (8).

Activation of the chemokine receptor CXCR3 by the interferon-inducible chemokines CXCL9, CXCL10 and CXCL11 provides an example of a pro-inflammatory interaction that will recruit activated T cells which 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 the pathogenesis of 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, 15). However, only one molecule, T487, has entered clinical trial for psoriasis. This agent lacked clinical efficacy and the trial was terminated at phase II (16).

Our group (5, 17, 18) and others (19, 20) 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 in mice by a mechanism believed to involve the induction of chemokine receptor desensitisation (18).

A recent report identified the 589Da small molecule N^{α}-[2-(4-Phenyl-4-oxobutyryl)-1,2,3,4-tetrahydroisoquinoline-3α-ylcarbonyl]-N-(cyclohexylmethyl)argininamide (PS372424) as an agonist specific for the human form of CXCR3 (21). The current study was designed to investigate 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 then defined.

Results

T cell activation by PS372424

Initial experiments were performed to compare the effects of chemokine receptor engagement on activated T cells by PS372424 or the endogenous CXCR3 ligand CXCL11. Treatment with both the small molecule agonist and 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, a diffusion gradient of 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 (22) or the agonist PS372424. The CXCR3 antagonist inhibited migration towards the natural CXCR3 ligand CXCL11 (Figure 2A). However treatment with 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 was 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 joints of 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 treatment with 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. In total, 6 groups of mice were humanized using peripheral blood mononuclear cells (PBMC) from different donors. Splenocytes from these mice contained 71.3 ± 10.7% (mean ± 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 also almost exclusively $CD14^-$ $CD19^-$ $CD56^-$ $CD45RO^+$ $CD69^{lo}$ $CCR5^+$.

Humanized mice animals were used to examine human T cell migration *in vivo* using a standard air pouch model of inflammation. Injection of human CXCL11 into air pouches induced a significant recruitment of human $CD45^+$ cells compared with air pouches filled with PBS (Figure 3D); the recruitment produced by CXCL11 was significantly reduced when mice received intravenous PS372424 ($p<0.05$). Humanized mice did not show any apparent acute adverse effect for up to 24 hours after intravenous 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 compared to untreated animals. Furthermore, whole human blood showed no significant production of CCL2, CCL4, CCL5, CCL11, CCL13, CCL17, CXCL8, CXCL10 , TNF-α, IFN-γ, IL-1β and IL-6 when incubated for up to 24 hours with 1μM PS372424 and no acute leukocyte respiratory burst was observed (Figure 3E).

Anti-inflammatory activity of PS372424

Intravenous administration of 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 migration 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 a significant recruitment of human $CD45^+$ cells (Figure 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, this chemotactic recruitment by RASF was reduced to a background level by intravenous administration of the CXCR3 agonist PS372424 ($p<0.01$; Figure 4C).

Mechanism of chemokine receptor cross-desensitisation

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

Examination of ligand-dependent 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 the CCR5-specific ligand CCL5 (Figure 5B). The PS372424-induced phosphorylation of CCR5 on $CXCR3^+$ T cells was abolished in the presence of the PKC inhibitor staurosporine (Figure 5B).

Flow cytometric analysis of immunochemically labelled mitogen-activated T cells showed that CXCR3 and CCR5 form a chemokine receptor heterodimer, with a FRET efficiency of 9% (Figure 5C). The theoretical maximum energy transfer between the APC and PE fluorochromes used in this experiment is approximately 10% (23). The FRET signal was reduced by removal of cell-surface antibodies with a brief acid wash ($p<0.05$) and no signal was observed when CD45 rather than CCR5 was labelled with an APC-conjugated antibody (Figure 5C). Results from a control matrix titration of both anti-CXCR3-PE and anti-CCR5-APC indicated this was a true FRET signal (Figure 5D) rather than the

consequence of spectral overlap as the signal was sensitive to increasing acceptor antibody concentration and not affected by donor antibody concentration (23).

Discussion

PS372424 is a small molecule agonist for the receptor CXCR3 that was identified during a combinatorial library screen for antagonist compounds (21). The agonist has a peptidic structure containing a tetrahydroisoquinoline-arginine motif that closely aligns with a Pro-Arg motif at residues 35-39 of the endogenous ligand CXCL10; this motif is known to be important for CXCR3 activation (24). 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 (25, 26). Other sequelae of chemokine receptor ligation including CXCR3 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 of the slow cycling of this receptor (27).

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 (28). Importantly, unlike natural chemokine ligands, 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 (18, 19, 29).

The potential of PS372424 to differentially 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 (22) only prevented migration towards CXCL11. This result 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 normally 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 polyclonal activation by antibody stimulation of CD3 and CD28. Natural Treg were isolated from peripheral blood by selecting the $CD4^{+}CD25^{hi}$ phenotype and validated for nuclear expression of FOXP3 (30); their chemokine

receptor phenotype was largely $CCR5^{+}CXCR3^{-}CXCR4^{+}$. Although the expression of CXCR3 by some human Treg has been described (7, 31), the majority of natural Treg are $CXCR3^{-}$ (32, 33).

Chemokine receptors are known to possess antigenic variability which can limit their detection by flow cytometry (34). For this reason the result of flow cytometric analysis of the 3 T cell subpopulations was confirmed by functional chemotaxis assays. These showed a clear concordance between the observed expression of CCR5, CXCR3 and CXCR4 and migration towards CCL5, CXCL11 and CXCL12 respectively. Rheumatoid arthritis synovial fluid (RASF) from inflamed joints of patients with active disease was used as a broad-spectrum, chemokine-rich stimulus and induced equal migration of each functionally-defined T cell subpopulation (17).

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 symptomatic inflammation is produced by a mixture of chemokines (18). Importantly, the PS372424 agonist 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 (28). 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 (35) to produce a reproducible peripheral T cell population which, after 28 days, was almost exclusively human and $CXCR3^{+}$. The induction of the activation marker CXCR3 on these cells is consistent with the observed co-expression of CD45RO and CCR5 and may be indicative of the partial activation produced by homeostatic T cell proliferation (36).

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 (18), unlike granulocyte activating chemokines such as CXCL8

(37). Stimulation of whole human blood with a high (1μM) concentration of the CXCR3 agonist for up to 24 hours induced no increase in the concentration of TNF-α, IFN-γ, IL-1β, IL-6, CCL2, CCL4, CCL5, CCL11, CCL13, CCL17, CXCL8 or CXCL10 above baseline levels. Stimulation with the agonist for 10 min also produced no acute oxidative burst. Whilst these observations are consistent with the absence of any apparent adverse effect associated with administration of the CXCR3 agonist to humanized mice for 24 hours, they do not exclude subtle pharmacological toxicity.

Stable air pouches were generated on humanized mice by subcutaneous injection of sterile air over six days (17). Inflammatory factors were then injected into these pouches and the migration of human cells was measured by lavage and cell counting. Addition of human CXCL11 to the pouch resulted in a significant recruitment of human $CD45^+$ cells. This migration was reduced to background by intravenous injection of sufficient PS372424 to produce an estimated initial 1μM concentration in the blood. Injection of PS372424 also significantly inhibited the migration of human cells into air pouches filled with either CXCL12 or CCL5.

The small molecule CXCR3 antagonist NBI-74330 (38) did not modify human cell migration towards CCL5. NBI-74330 is a true receptor antagonist with no partial agonist activity (22). 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 treatment with a CXCR3 neutralising antibody or the CXCR4 antagonist AMD3100 (39).

As the air pouch develops a pseudosynovial membrane (18, 40, 41), 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 recruitment was not inhibited by intravenous administration of AMD3100 at 2μM, the maximum achievable clinical concentration (39), anti-CXCR3 or NBI-74330 at 100mg/kg, a dose that has been used in other *in vivo* models (42). However, the inflammatory response produced by RASF within the air pouches was reduced to background levels by intravenous administration of PS372424. This provides the first *in vivo* demonstration, to our knowledge, that systemic administration of a small

molecule chemokine receptor agonist can limit the migration of human cells towards the broad range of chemokines present in RASF in the absence of apparent toxicity.

Analysis of cell surface chemokine receptor expression by human T cells recovered from the spleen of humanized mice which had been treated for 24 h with PS372424 demonstrated a significant reduction in both CXCR3 and CCR5. Chemokine receptor heterodimerisation is increasingly recognised as a source of diversity within the chemokine system and suggests a mechanism by which specific activation of one receptor can induce desensitisation of another (6, 43). Importantly, FRET analysis of activated human T cells demonstrated that CXCR3 and CCR5 can form such a heterodimer on the cell surface. This is the first description of this heterodimer, to our knowledge, although a physical interaction between the chemokine receptors CXCR4 and CCR5 has been reported previously (44).

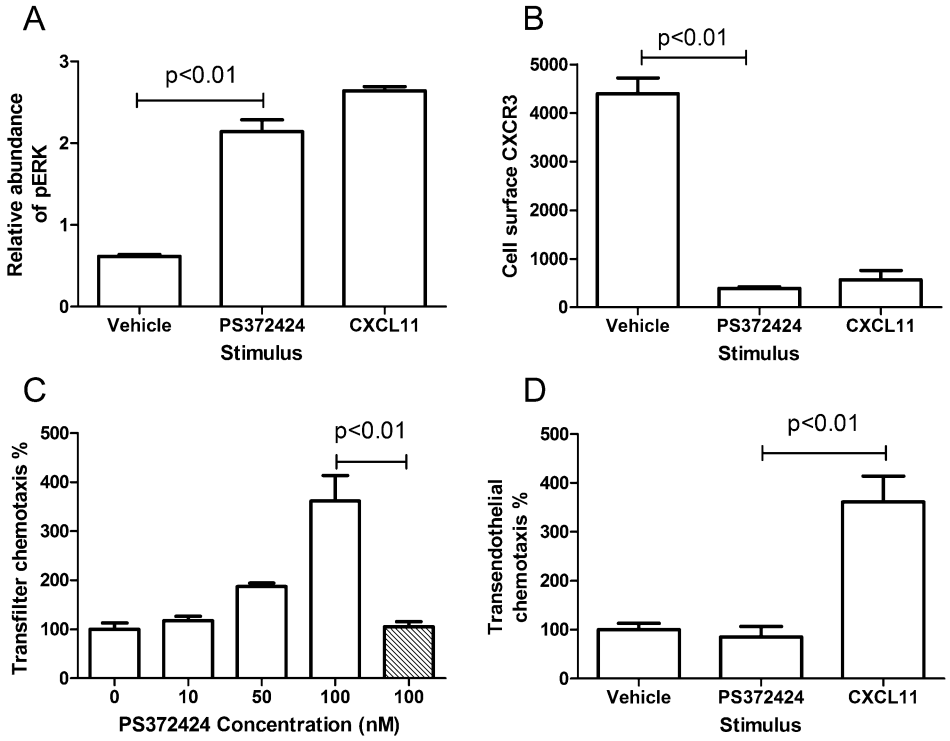
Phosphorylation of chemokine receptors disrupts their ability to recruit heterotrimeric G-proteins and therefore inhibits signalling (6). This phosphorylation can also cause the loss of cell surface receptors by activation of β-arrestin-mediated internalisation (45), leading to receptor recycling or degradation (6). Restoration of cell surface CXCR3 is known to require *de novo* receptor synthesis (27). 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 novel observation is consistent with previous reports suggesting that CCR5 cross-phosphorylation is variably mediated by protein kinase C or G protein-coupled receptor kinases depending on the nature of the agonist-stimulated partner within the heterodimeric receptor complex (45, 46). 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 (47). Previous studies have suggested that this induction of heterologous chemokine receptor phosphorylation, internalization and desensitization might provide a novel route to anti-inflammatory therapy (18).

The current study demonstrates a 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 further exploration of agonist-induced chemokine receptor desensitisation strategies for the treatment of important inflammatory diseases.

Figures

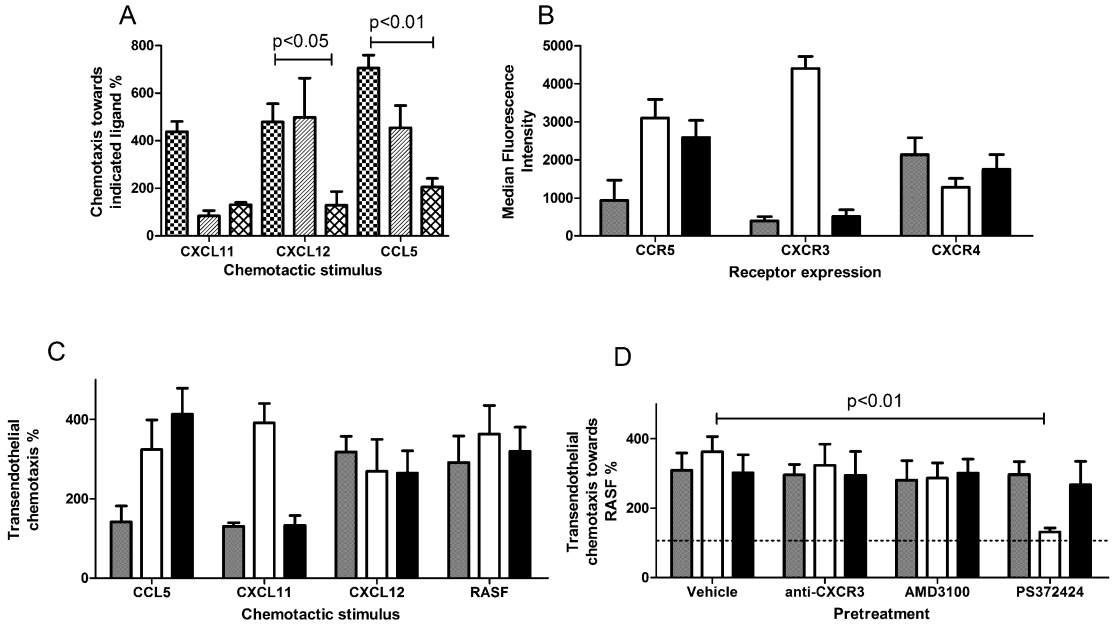
Figure 1 the consequences for human T cells of activation of CXCR3 by PS372424



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. Transenodothelial chemotaxis of activated T cells toward ligand

Data represent three independent experiments performed in triplicate ± SEM

Figure 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)

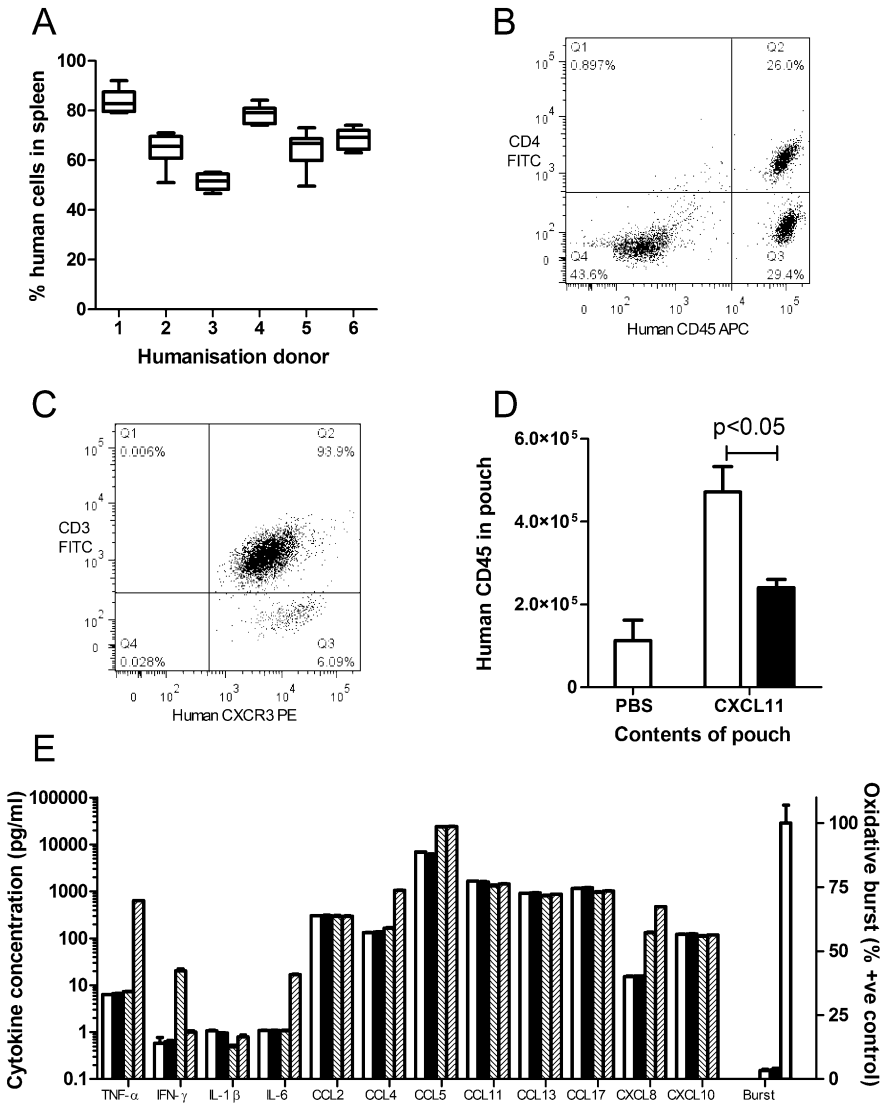
B. Cell surface chemokine receptor expression determined by flow cytometry on freshly isolated (grey bars), activated (white bars) and $CD4^{+}CD25^{hi}$ (black bars) T cells

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 rheumatoid arthritis synovial fluid (RASF)

D. Migration of indicated freshly isolated (grey bars), activated (white bars) and $CD4^{+}CD25^{hi}$ (black bars) T cells towards rheumatoid arthritis synovial fluid (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

Figure 3 A humanised air pouch model of inflammation

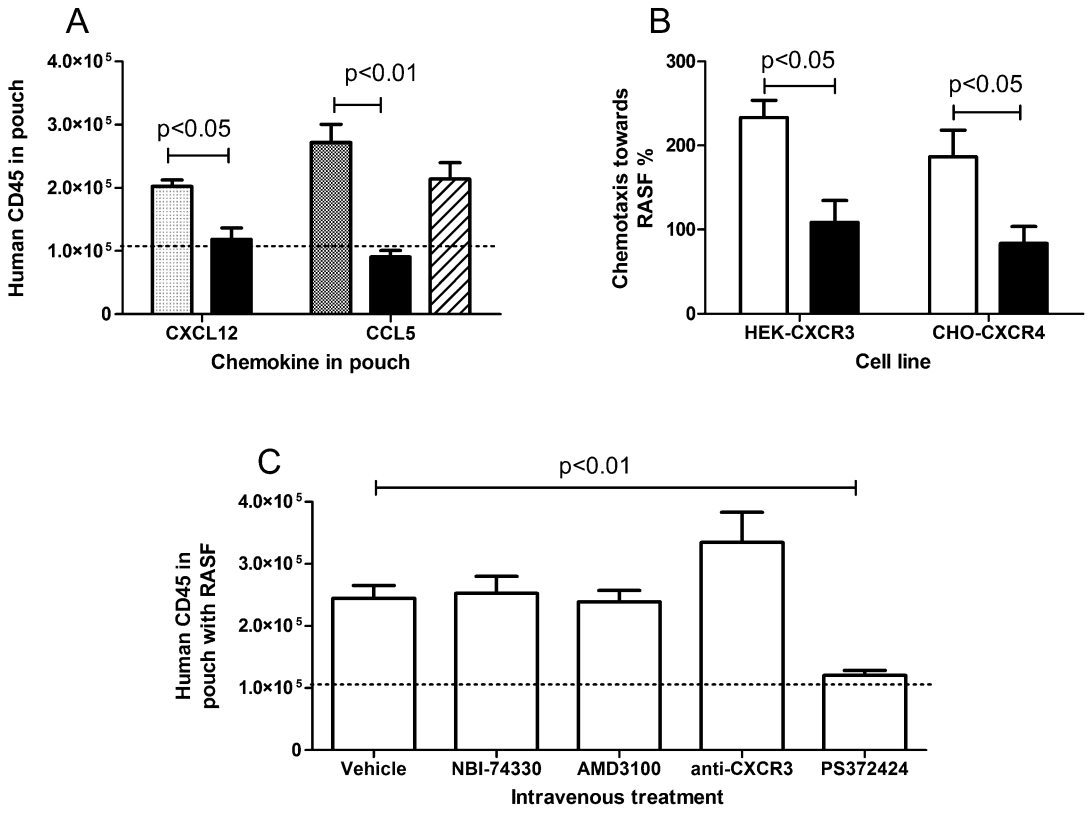


A. Flow cytometric analysis of human CD45 in spleen of humanised mice using 6 different human donors
B. Representative flow cytometric analysis of all spleen cells taken from mice at 28 days post humanisation
C. Expression of CXCR3 subsequent to human CD45 gating
D. Human cell migration into air pouches 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

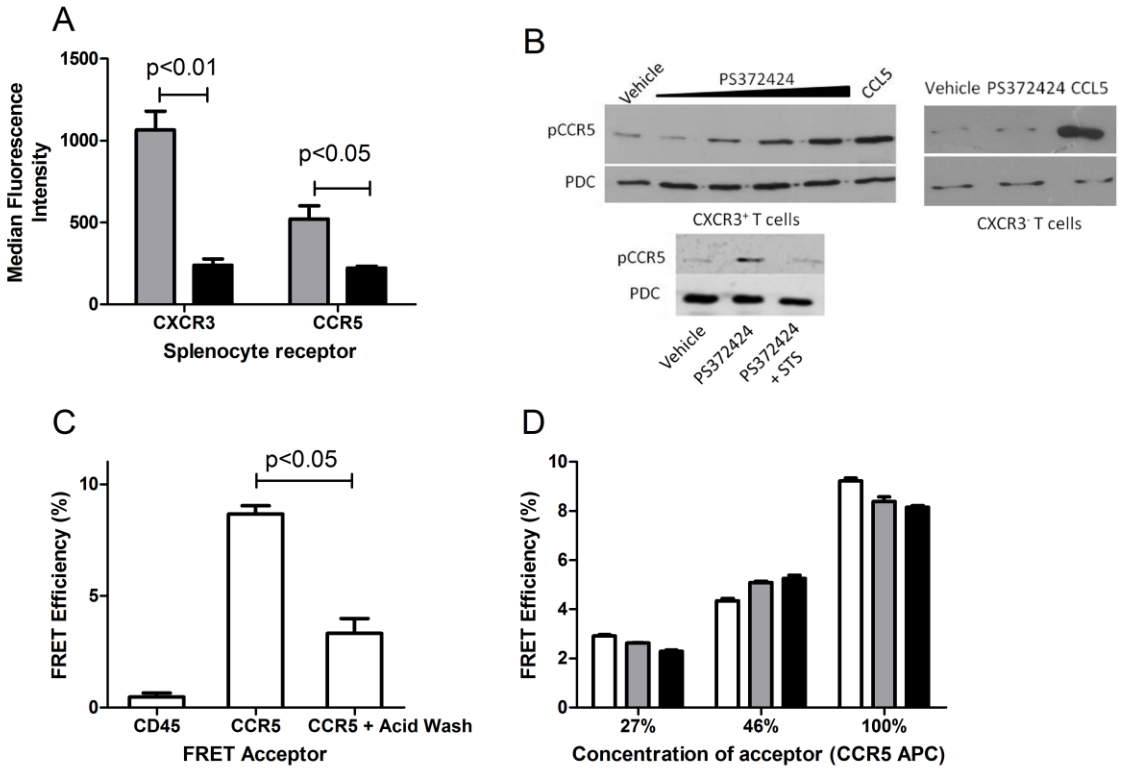
Figure 4 Heterologous chemokine receptor regulation in vivo



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 rheumatoid arthritis synovial fluid (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

Figure 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% saturating of saturating CXCR3-PE, grey bars 43%, and black bars 100%

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

Materials and Methods

The animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and local Ethics Committee approval under the authority of Home Office Licence PPL60/3772.

Cytokines and Reagents

All human chemokines were obtained from R&D Systems (Abingdon, UK) and reconstituted in PBS with 0.1% bovine serum albumin (BSA). Chemokines were typically used *in vitro* at a concentration of 50nM.

The small molecule CXCR3 agonist PS372424 was supplied by Ligand Pharmaceuticals (La Jolla, California) (21). This compound was dissolved in DMSO (Sigma-Aldrich) at 100mM and typically used *in vitro* at a concentration of 100nM; a final DMSO dilution of 1 in 10^6 was used as the vehicle control.

An anti-CXCR3 neutralising antibody (Clone 49801; R&D Systems) was resuspended in PBS and used at a concentration of 2µg/ml for *in vitro* studies. The CXCR3 antagonist NBI-74330 was kindly supplied by M Smit and M Wijtmans (Amsterdam Centre for Drug Research)(48). A stock of 10mM was prepared in DMSO and the inhibitor was used at a final concentration of 1µM (38). The CXCR4 antagonist AMD3100 (Sigma-Aldrich) was prepared to 25mM in DMSO and used at a final concentration of 2µM, the maximum achievable plasma concentration (39).

Following informed consent, rheumatoid arthritis synovial fluid (RASF) was collected into sterile tubes from the knee joint of patients with active rheumatoid arthritis undergoing therapeutic aspiration at the Freeman Hospital, Newcastle upon Tyne NHS Foundation Trust. The samples were centrifuged and the cell-free supernatant was stored at –20°C; samples from four patients were pooled in order to standardise the RSAF used across all experiments.

Chemokine levels in RASF were quantified by multiplex electrochemoluminescent immunosorbent assay using the MesoScale Discovery System (MesoScale, Gaithersburg, MD). Undiluted 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. The fluid was diluted 1 in 10 into PBS for use in the lower chamber of transendothelial

chemotaxis assays or 1in 5 into PBS for use in air pouch experiments, as described below (17, 18). All experiments involving human blood cells were performed independently using at least three different donors.

Human T cell Isolation

All primary T cells were isolated from anonymised National Blood Service aphaeresis cones after informed consent and approval from the local Research Ethics Committee. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as described previously (18). Purified, non-activated T lymphocytes were extracted using RosetteSep human T cell enrichment cocktail antibody (StemCell Technologies, UK) according to manufacturer's instructions as described previously (9).

Activated T cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI; Sigma-Aldrich), containing 10% foetal calf serum (FCS; Sigma-Aldrich), 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 automated RoboSep immunomagnetic $CD25^{hi}$ positive selection (human $CD25^{bright}$ positive selection kit; StemCell Technologies) as described previously (30).

ERK Activation

Phosphorylation of ERK1 at T202/Y204 and ERK2 at T185/Y187 were measured by ELISA (KCB1018; R&D Systems) according to manufacturer's instructions. Activated T cells were stimulated with 50nM CXCL11 or 100nM PS372424 for 10 mins at 37ºC. Ice-cold PBS was added and cells were pelleted by centrifugation at 800g. Cell lysis was performed by sonication in Phosphosafe Extraction Reagent (Novagen). Lysates were clarified by centrifugation at 20000g for 20 mins at 4ºC prior to analysis by ELISA.

Chemotaxis Assays

The chemotactic migration of T cells was assessed using a 3µm pore Transwell filter system developed using polyethylene terepthalate chemotaxis filters (BD Biosciences) and corresponding 24-well companion plates (18). In some cases, transendothelial chemotaxis was assessed by first culturing $5x10^4$ EAhy926 cells on the filter for 72 hours before the migration assay.

For T cell migration, $2x10^5$ cells were placed in upper chamber whereas the lower chamber contained serum-free RPMI 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 migrated cells counted by FACS using Flow-Count beads (BD Biosciences).

Chinese Hamster Ovary cells stably transfected with CXCR4 (CHO-CXCR4) and Human Embryonic Kidney cells stably transfected with CXCR3 (HEK-CXCR3) were generated previously (49). These were used in an 8µm pore chemotaxis assay towards RASF. These cells adhere to the filter and were enumerated by light microscopy following haemotoxylin staining as described previously (18).

All chemotaxis data is expressed relative to the background cell migration seen in the absence of migratory stimulus.

Flow Cytometry

Immunofluoresence flow cytometric analysis of surface receptor expression was performed as described previously using a BD FACS Canto II (18). Receptor internalisation was measured at 30 mins following stimulated with ligand. Antibodies used in this study were anti-human CD45 (clone 2D1; BD Biosciences), anti-human CXCR3 (clone 49801; R&D Systems), anti-CD3 (clone HCHT-1; Sigma-Aldrich), anti-CD4 (clone B-A1; Abcam), anti-CD8 (clone B-Z31; Abcam), and anti-CCR5 (clone CTC5; R&D Systems). Data were acquired using BD FACSDiva software and analysed using FlowJo software v.7.6.4 (Treestar).

Examination of fluorescent resonance transfer (FRET) was performed as described previously (9). The potential for 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 the FRET was a non-random event both antibodies were titrated to a saturating concentration as recommended by Batard

et al. (23). Control experiments were performed in which anti-CCR5 was substituted with APC conjugated anti-CD45 antibodies as described previously (50). To determine whether any FRET detected was occurring intracellularly following endocytosis of receptor complexes, a 5 min acid wash (20 mM HCl/HBSS; pH 2) at 4 °C was performed to remove surface antibodies. In all cases FRET was examined and energy transfer efficiencies calculated as described by Batard et al. (23).

CCR5 Phosphorylation

Cells were briefly serum starved for 2 hours and stimulated with vehicle control, 10, 50, 100, or 200 nM PS372424 or 50nM CCL5 for 30 mins at 37ºC. In control experiments, cells were incubated with PS372424 in the presence of 10ng/ml staurosporine (Calbiochem). Lysates were prepared as above, fractionated by SDS-PAGE and Western blotting performed as described previously (25). Membranes were blocked using 5% BSA and probed using 250ng/ml anti-pCCR5 (Ser349; Clone E11/19; Biolegend, Cambridge, UK) in 5% non-fat milk. 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-Aldrich) was used at 1:2000.

Humanized Air Pouch Assay

Female NOD.Cg-*Prkdc*^{scid} *Il2rg*tm1$^{Wjl/SzJ}$ (35) mice (Charles River; 8 week old) were given 10^7 human PBMC in 0.5ml PBS by i.p. injection. 21 days later air pouches were generated as described previously (17, 18). Briefly, air pouches were induced by injecting 3 ml of sterile air subcutaneously into the back of each animal, followed by 1 ml of air on 3 further occasions (days 2, 4, and 5, respectively); this produced stable fluid-filled pouches. On day 6, each pouch was injected with 1 ml of PBS containing either 5µg of chemokine or RASF. Groups of animals received Intravenous injections of 0.1ml volumes of vehicle control, PS372424 (20µM, to produce an initial concentration of approximately 1µM in 2ml murine blood), CXCR3 neutralising antibody (25µg) or AMD3100 (to produce an approximate concentration of 2µM in blood). NBI-74330 was administered by subcutaneous injection (100mg/kg) (42).

After 24 hours, blood was harvested from humanized mice by cardiac puncture under terminal anesthesia. Recruited cells were recovered by gently lavaging the pouch twice with 0.75 ml of PBS containing 3 mM EDTA. The exudates were centrifuged at 500g for 5 min, and the supernatants were

removed. The cell pellets were resuspended in and human cell migration enumerated by FACS with counting beads. Splenocytes were isolated by trituration of splenic tissue before filtration through a 70µm filter. Red cell lysis buffer (Sigma-Aldrich) was added and cells were stained for analysis by flow cytometry.

Treatment of Whole Blood with PS372424

Humanized mice receiving PS372424 were closely monitored following i.v. administration and no apparent adverse affect was observed. Heparanised 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 37ºC. Cells were pelleted and the supernatants analysed by multiplex electrochemoluminescent immunosorbent assay using the MesoScale Discovery System (MesoScale, Gaithersburg, MD). ProInflammatory-4 I Ultra-Sensitive, RANTES Ultra-Sensitive and Chemokine 7-Plex Ultra-Sensitive kits allowed measurement of CCL2, CCL4, CCL5, CCL11, CCL13, CCL17, CXCL8, CXCL10 , TNF-α, IFN-γ, IL-1β and IL-6.

To examine the oxidative burst activity of whole blood, a Burst Test kit (Orpegen Pharma, Heidelberg, Germany) was used (18). A volume of 100µl of heparinized human blood was stimulated with either vehicle, 1µM PS372424, or $2x10^7$ opsonized *Escherichia coli* for 10 min at 37°C. On stimulation, leukocyte production of reactive oxygen metabolites, such as superoxide anion, hydrogen peroxide, and hypochlorous acid, was monitored by the addition and oxidation of dihydrorhodamine 123 to rhodamine 123 and 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 post test. All data were analyzed using Prism 5 software (GraphPad, San Diego, CA, USA).

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