The effect of a selective CXCR2 antagonist (AZD5069) on human blood neutrophil count and innate immune functions

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

• Although CXCR2 antagonists are now in clinical development, there have been no published studies to date assessing the impact of these potential anti-inflammatory agents on the innate host defence responses of neutrophils in humans.

WHAT THIS STUDY ADDS

• The study data show, for the first time, that neutrophils retain their ability to mobilize from the bone marrow and other compartments such as the marginating pool and to engage in their key antimicrobial immune functions (phagocytosis and the oxidative burst) during treatment with a CXCR2 antagonist (AZD5069) in human volunteers.

AIMS

The aim of the present study was to investigate whether selective antagonism of the cysteine-X-cysteine chemokine receptor-2 (CXCR2) receptor has any adverse effects on the key innate effector functions of human neutrophils for defence against microbial pathogens.

METHODS

In a double-blind, crossover study, 30 healthy volunteers were randomized to treatment with the CXCR2 antagonist AZD5069 (100 mg) or placebo, twice daily orally for 6 days. The peripheral blood neutrophil count was assessed at baseline, daily during treatment and in response to exercise challenge and subcutaneous injection of granulocyte-colony stimulating factor (G-CSF). Neutrophil function was evaluated by phagocytosis of *Escherichia coli* and by the oxidative burst response to *E. coli*.

RESULTS

AZD5069 treatment reversibly reduced circulating neutrophil count from baseline by a mean [standard deviation (SD)] of -1.67 (0.67) $\times 10^9$ l^{-1} vs. 0.19 (0.78) $\times 10^9$ l^{-1} for placebo on day 2, returning to baseline by day 7 after the last dose. Despite low counts on day 4, a 10-min exercise challenge increased absolute blood neutrophil count, but the effect with AZD5069 was smaller and not sustained, compared with placebo treatment. Subcutaneous G-CSF on day 5 caused a substantial increase in blood neutrophil count in both placebo- and AZD5069-treated subjects. Superoxide anion production in *E. coli*-stimulated neutrophils and phagocytosis of *E. coli* were unaffected by AZD5069 ($P = 0.375$, $P = 0.721$, respectively vs. baseline, Day 4). AZD5069 was well tolerated.

CONCLUSIONS

CXCR2 antagonism did not appear adversely to affect the mobilization of neutrophils from bone marrow into the peripheral circulation, phagocytosis or the oxidative burst response to bacterial pathogens. This supports the potential of CXCR2 antagonists as a treatment option for diseases in which neutrophils play a pathological role.

Introduction

Neutrophils regulate innate immune responses but can also contribute to tissue damage through the release of inflammatory mediators, and therefore represent a potential therapeutic target [1]. Many chronic inflammatory airway diseases, such as bronchiectasis, cystic fibrosis, chronic obstructive pulmonary disease (COPD) and more severe forms of asthma, are characterized by neutrophil-dominated mucosal inflammation [2–6]. Morphometric examination of the airways in COPD and asthma shows that sputum neutrophil count correlates with measures of disease severity, such as forced expiratory volume in 1 s (FEV_1) [7, 8]. There is also evidence that neutrophil numbers increase in the airways of patients with either COPD or asthma after acute exacerbations [9, 10].

Elevated concentrations of major neutrophil chemokines, including cysteine-x-cysteine ligand (CXCL)-1 [also known as growth-related oncogene α (GROα)], CXCL5 [also known as epithelial-derived neutrophil-activating protein 78 (ENA-78)] and CXCL8 [also known as interleukin-8 (IL-8)], have been reported in the airways of patients with COPD, cystic fibrosis and severe asthma, and may contribute towards persistent airway neutrophilia [4, 11–14]. These CXC chemokines act via the cysteine-X-cysteine chemokine receptor-2 (CXCR2) expressed on the surface of neutrophils and are thought to promote neutrophil homing to the inflamed airways [1, 15]. Considerable preclinical and clinical data support the development of selective CXCR2 antagonists for the treatment of inflammatory lung diseases characterized by neutrophilic inflammation [1, 16]. Preclinical evidence has shown that genetic and pharmacological inactivation of CXCR2 diminishes pulmonary neutrophilic inflammation induced by lipopolysaccharide (LPS) in several animal species [17, 18]. Oral administration of the small-molecule CXCR2 antagonists SCH527123 (navarixin) and SB-656933 has been shown to block airway neutrophilia induced by ozone challenge in healthy volunteers [19, 20]. Recent clinical studies demonstrated that a CXCR2 antagonist (AZD8309) given orally blocked the increase both in neutrophils (by 79%) and selected neutrophil chemoattractants, including IL-8 (by 52%), in induced sputum following inhaled LPS challenge [21], and significantly reduced LPS-induced neutrophil recruitment in the upper airways of healthy subjects [22]. Interestingly, however, treatment with the CXCR2 antagonists SB-656933 and AZD5069 caused a small but significant elevation in blood concentrations of IL-8 in studies of patients with cystic fibrosis and bronchiectasis, respectively [23, 24]. Clinical data from a recent Phase 2b clinical trial showed that CXCR2 antagonism using MK-7123 can improve measures of lung function and reduce COPD exacerbations in current smokers but was associated with a decrease in absolute peripheral neutrophil count [25].

In addition, recent clinical data from a Phase 2a trial in moderate-to-severe COPD patients indicated that treatment with AZD5069 50 mg or 80 mg bid was well tolerated and there were no safety issues, as judged by no further increments in infection rates in either dosage group compared with placebo [26].

However, understanding the neutrophil-mediated immune mechanisms underlying host–pathogen relationships is important and critical to the development of novel anti-inflammatory agents that target neutrophils, such as the CXCR2 antagonist AZD5069. Studies in healthy volunteers and patients with COPD or asthma have shown a rapid reduction in blood neutrophil count within the first 24 h of treatment with several CXCR2 antagonists in development, including AZD5069 [20, 26, 27]. Those changes were completely and rapidly reversible on cessation of treatment. However, there is a theoretical risk that reducing blood neutrophil counts through CXCR2 antagonism could compromise innate immune responses against invading pathogens [16, 28].

In order to fulfil their pathogen defence functions, neutrophils need to be able to respond rapidly to physiological signals that trigger their mobilization from the bone marrow and induce changes in migration patterns. Although CXCR2 antagonism affects neutrophil recruitment dynamics [16, 20, 28], it should still allow operational neutrophil responses to physiological stimuli, such as granulocyte-colony stimulating factor (G-CSF), to mobilize neutrophils from the bone marrow into the circulation. Neutrophils can also be recruited into the circulation from other compartments, such as the marginating pool, by different stimuli such as exercise and adrenaline [29, 30].

Although previous studies using isolated primary cells have demonstrated that selective antagonism of CXCR2 per se does not interfere with the key neutrophil antimicrobial activities in vitro [16, 31, 32], this has not been fully explored in a clinical setting. The primary aim of the present study, therefore, was to investigate whether neutrophils from healthy volunteers demonstrate any functional abnormalities in their key innate effector functions (e.g. neutrophil mobilization into the circulation, phagocytosis and respiratory burst activity) following treatment with AZD5069. To ascertain whether CXCR2 antagonism could also affect the neutrophil activation state, we measured the surface expression of two known neutrophil activation-associated markers, CD11b [also known as macrophage-1 antigen (MAC-1)] and CD62L (also known as L-selectin), using flow cytometry [33, 34]. CD16 (also known as FcγRIIIb), a potential marker of viable non-apoptotic neutrophils [35], was assessed at the same time points. Serum concentrations of CXCR2 ligands IL-8, GROα and ENA-78 were also assessed before, during and after treatment with AZD5069.

Methods

Clinical data

A single-centre, randomized, double-blind, placebo-controlled, crossover study (Clinicaltrials.gov Identifier NCT01480739) was conducted in 30 healthy subjects (Figure 1) at the Hammersmith Medicines Research (HMR) Medical Laboratory, London, UK. The first subject was enrolled on 4 May 2012; the last subject visit was on 15 July 2012. The primary objectives were to assess whether oral dosing with AZD5069 affects neutrophil number and function (measured in phagocytosis and oxidative burst assays) in peripheral blood and whether AZD5069 affects the release of neutrophils into the circulation in response to exercise or after an injection of G-CSF. A two-way crossover design was chosen to counter inter-individual variation in neutrophil counts. Owing to the diurnal variation in blood neutrophil count (lower neutrophil counts in the morning), the study drugs (AZD5069 or placebo) were administered consistently at the same time in the morning and evening during treatment with the active drug and placebo. Predose blood sampling was also done at the same time point each day.

Study drugs were administered orally twice daily (*bid*) for 6 days, followed by a washout period of at least 21 days and then a second 6-day treatment period. Subjects were randomized, using Quintiles standard methodology, to one of two treatment sequences, AB or BA, where A was 100 mg AZD5069 *bid* and B was matching placebo *bid*. The AZD5069 dose was relatively high compared with that used in previous studies with healthy volunteers and patients with COPD or bronchiectasis [24, 36, 37]. At three time points during each treatment period [baseline, steady state (day 4), and 7 days after the last dose], fresh neutrophils were collected for testing in functional assays using whole blood to assess phagocytosis and oxidative burst function; flow cytometry was used to assess the subpopulations of neutrophils affected. The subjects remained in the study centre for the duration of each treatment period.

The present study was performed in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with the International Conference on Harmonisation (ICH)/ Good Clinical Practice (GCP) and applicable regulatory requirements and the AstraZeneca policy on Bioethics (http://www.astrazeneca.com/Responsibility/Code-policies-standards/Our-global-policies).

Neutrophil effector function assays

Neutrophil function (phagocytosis and the oxidative burst) was considered to be normal in the clinical study if more than 80% of neutrophils were observed phagocytosing and oxidizing in whole-blood assay when stimulated with the mucosa-associated Gram-negative bacterium, *Escherichia coli*, at 37°C. To determine the overall metabolic integrity of phagocytosing neutrophils, the percentage of neutrophils phagocytosing opsonized fluorescein isothiocyanate (FITC)-labelled *E. coli* (4×10^7 bacteria) was measured *ex vivo*, as described previously [38–40]. Neutrophil phagocytic function was visualized using a multispectral imaging flow cytometry instrument (Imagestream-X, Amnis, Seattle, WA, USA).

Superoxide anion production by neutrophils was assessed by oxidation of dihydrorhodamine (DHR) 123 and quantified by flow cytometry, as described previously [40, 41]. Furthermore, in order to quantitate the numbers of ingested/phagocytosed *E. coli* per neutrophil and to measure the intensity of superoxide anion production, the mean fluorescence intensity (MFI) values were analysed.

In the prestudy assay qualification experiments, we determined the intra-assay coefficient of variation (CV) for neutrophil function assays. The CVs for the phagocytosis and superoxide anion production assays were 12.54% and 8.67%, respectively.

Activation state of the neutrophils

For the assessment of neutrophil activation and survival, the following antibodies were used: FITC-conjugated anti-CD11b, anti-CD62L anti-CD16 (all BD Biosciences, Oxford, UK), as described previously [34, 35]. The neutrophil activation status was defined on the basis of the
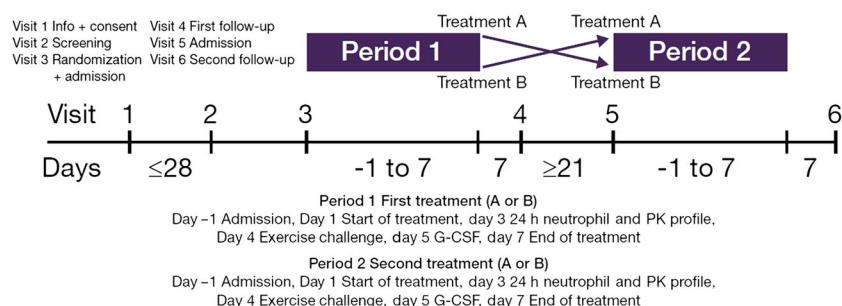

Figure 1

Study plan. Following screening and enrolment, AZD5069 or placebo were administered orally *bid* for 6 d, followed by a washout period of at least 21 d and then a second 6-d treatment period. Subjects were randomized to one of two treatment sequences, AB or BA, where A was 100 mg AZD5069 *bid* and B was matching placebo *bid*. A two-way crossover design was chosen to counter any inter-individual variation in neutrophil counts in human volunteers. PK, pharmacokinetic

magnitude and direction of changes in the expression of surface markers: >30% changes in the level of expression (MFI) in the expected direction (CD11b increase and CD62L decrease), based on published data [42]. In addition, the levels of expression of CD11b, CD16 and CD62L on the neutrophil surface were measured as MFI units and analysed independently for each of these activation markers. The CVs for these assays were determined in the prestudy investigation. The intra-assay CV for MFI measurements were 6.08% for CD11b, 13.62% for CD16 and 16.73% for CD62L.

Exercise and G-CSF-induced mobilization of neutrophils

To determine whether treatment with AZD5069 affects exercise-induced mobilization of human neutrophils *in vivo*, AZD5069-treated volunteers were challenged on day 4 with a standardized 10 min sub-maximal exercise test, up to 80% of age-related maximum heart rate, on an exercise bicycle. Exercise normally increases blood neutrophils by mobilizing them from other compartments, such as the marginating pool, into the circulation [43]. Blood samples were collected before and at 10 min (directly after the challenge), 2 h and 4 h after the end of the exercise challenge, to assess the effect of AZD5069 on the blood neutrophil and leukocyte differential counts. On day 5, after the morning dose, the subjects received 300 µg G-CSF subcutaneously, and blood samples for blood neutrophil counts and leukocyte differential counts were collected before and at 2, 4, 8, 12, 24 and 36 h after injection. This was to assess the effect of AZD5069 on the ability of G-CSF to stimulate the release of neutrophils into the circulation. The G-CSF-stimulated release of neutrophils from the bone marrow is an important component of the innate immune response to infectious organisms, and a normal release upon stimulus indicates that neutrophils can be mobilized from the bone marrow.

Effects of AZD5069 treatment on blood inflammatory mediators

As an exploratory objective, systemic concentrations of ligands for the CXCR2 receptor (IL-8, GROα and ENA-78) and G-CSF were measured using Luminex Fluorokine Multi-Analyte Profiling (MAP) reagents and an enzyme-linked immunosorbent assay (ELISA) kit for GROα (both R&D Systems, Abingdon, UK). The lower detection limits for Luminex assays were 2.47 pg ml^{-1} for IL-8, 4.00 pg ml^{-1} for ENA-78 and 2.02 pg ml^{-1} for G-CSF. The lower detection limit for the ELISA assay for GROα was 31.25 pg ml^{-1}. The CV for assays measuring inflammatory mediators determined in prestudy assay qualification tests were 12.01% for IL-8, 14.48% for GROα, 4.42% for ENA-78 and 14.93% for G-CSF. The within-assay CV for the high-sensitivity C-reactive protein (hsCRP) assay, a measure of the total variability at a low concentration, was shown to be 3.6% prior to its use in the study. [The drug/molecular target

nomenclature conforms to the *British Journal of Pharmacology* Guide to Receptors and Channels (GRAC) [44].]

Statistical methods

Statistical analyses were performed by Quintiles (Overland Park, KS, USA and Bangalore, India), using SAS® version 9.2 (SAS Institute Inc., Cary, NC, USA). Continuous variables were summarized using descriptive statistics. Missing data were not imputed. Any subject who withdrew before the last planned observation in a study period was included in the analyses up to the time of withdrawal.

The pharmacodynamic (PD) analysis of neutrophil counts was carried using Quintiles' standard operating procedures and work instructions. All PD computations were performed using SAS version 9.2. Graphics were prepared using SAS version 9.2 or SigmaPlot 9.0 (Systat Software Inc., San Jose, CA, USA).

A linear model (on the log scale) was used to analyse the data from the crossover designs on the functional assays and neutrophil markers. Fixed effects were included for baseline, treatment, sequence and period as well as a random effect for subjects.

This crossover analysis was performed using R version 3.1.2 using the nlme package version 3.1 (R Foundation for Statistical Computing, Vienna, Austria).

Safety and tolerability

The secondary objectives of the study were to evaluate the general safety and tolerability of AZD5069 and the steady-state 24-h profile of circulating blood neutrophils during *bid* administration of AZD5069. During both treatment periods, subjects were checked for signs and symptoms of infection on a daily basis.

Results

In total, 30 healthy adults were enrolled; all of them were randomized, and 27 (90%) received all the scheduled doses of the CXCR2 antagonist AZD5069 and completed the study. All were non-smoking, and the women were of non-childbearing potential, aged 18–45 years at screening (Supplementary Table S1). All were Caucasian, to avoid the possibility of ethnic differences in neutrophil kinetics affecting the study results. Baseline arithmetic mean circulating neutrophil counts were 3.43×10^9 l^{-1} [standard deviation (SD 0.89)] and 3.21×10^9 l^{-1} (SD 0.80) for the AZD5069 and placebo treatment groups, respectively (Supplementary Table S2). As expected, circulating neutrophil counts were already reduced after 1 day of treatment (Figure 2).

Exercise and G-CSF induced mobilization of neutrophils during treatment with AZD5069 in human volunteers

Compared with pre-exercise levels, the blood neutrophil count increased rapidly by a similar percentage in
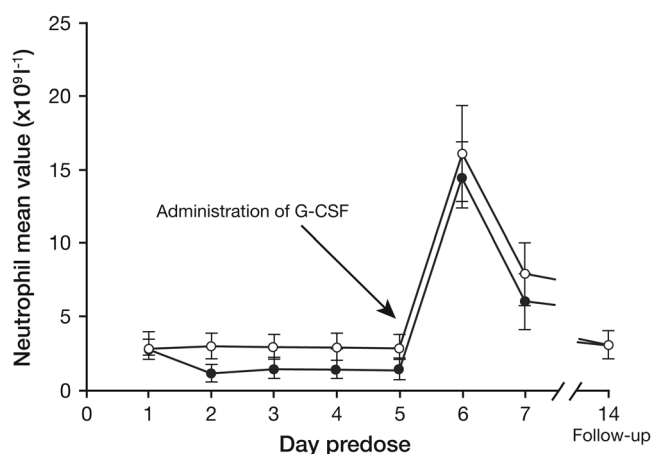
Figure 2

Arithmetic mean (± standard deviation) morning predose circulating neutrophil count *vs.* time profiles for subjects receiving AZD5069 and placebo. The lowest mean predose neutrophil value was observed prior to the day 2 morning dose ($1.10 \times 10^9 \, l^{-1}$). G-CSF, granulocyte-colony stimulating factor. —●— AZD5069 100 mg *bid*, —○— Placebo *bid*

AZD5069-treated and placebo-treated subjects (Figure 3A). The absolute increase in the neutrophil count during treatment with AZD5069 was about half that seen during placebo treatment (Figure 3B). The blood neutrophil count was increased throughout a 4-h measurement period in the placebo group. Notably, the increase was not sustained over the whole period in the AZD5069-treated subjects.

We also explored whether AZD5069 treatment affected G-CSF-induced mobilization of human neutrophils. After subcutaneous G-CSF injection, there was a substantial increase in blood neutrophil count with both placebo and AZD5069, peaking at 12 h post-G-CSF and sustained over the 36-h measurement period in both the placebo- and AZD5069-treated subjects (Figure 4A). The absolute increases in neutrophil counts were similar during treatment with AZD5069 and placebo (Figure 4B). By the follow-up visit 7 days after the last dose of AZD5069, morning mean values for blood neutrophil counts for both the AZD5069- and placebo-treated subjects had returned to near baseline.

As a secondary assessment, the 24-h diurnal variation in neutrophil count was explored at steady state (day 3). AZD5069 reduced neutrophil counts throughout the 24-h observation period *vs.* placebo. The blood neutrophil count was lower with AZD5069 after both morning and evening doses, and throughout the 12 h after dosing. The mean lowest count over the 24 h was observed at a median time of 5 h after the morning dose (Supplementary Figure S1). Mean blood neutrophil counts remained below pretreatment baseline in subjects receiving AZD5069 throughout the sampling period. However, the pattern of response over time after treatment indicates that a diurnal variation was maintained in the presence of AZD5069 (Supplementary Figure S1).
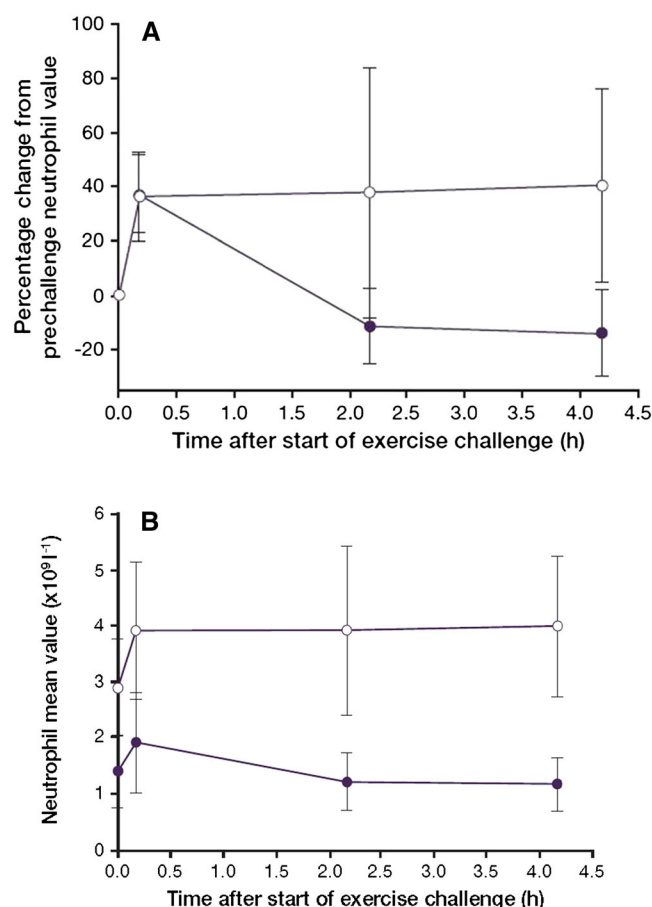

Figure 3

Increases in peripheral blood neutrophil counts (**A** – percentage change, **B** – absolute numbers) over time in response to exercise challenge in human volunteers. Subjects were administered either AZD5069 (100 mg *bid*) or placebo orally (*bid*) prior to physical exercise. Peripheral blood samples were collected at the indicated time points. Each point is presented as mean ± standard deviation of $n = 28$ subjects per arm. —●— AZD5069 100 mg *bid* —○— Placebo *bid*

Phagocytosis of E. coli and oxidative burst activity ex vivo are preserved in human volunteers during treatment with AZD5069

To examine the effects of AZD5069 on neutrophil effector functions, we measured phagocytic and oxidative burst capacity *ex vivo*. Neutrophils in whole-blood samples from AZD5069-treated volunteers displayed normal uptake of opsonized FITC-labelled *E. coli* (Supplementary Table S3) with the percentage of phagocytic neutrophils matching that observed in cells from placebo-treated patients. At all time points measured, in both treatment groups, >95% of neutrophils were able to phagocytose *E. coli*. Similarly, superoxide anion production in *E. coli*-stimulated human neutrophils was not affected by AZD5069 treatment in healthy volunteers (Supplementary Table S3). As the percentages of phagocytic and oxidizing neutrophils represent a qualitative measure of neutrophil function, we also analysed the quantitative data (MFI values). In phagocytic assays, the MFI values reflect the number of
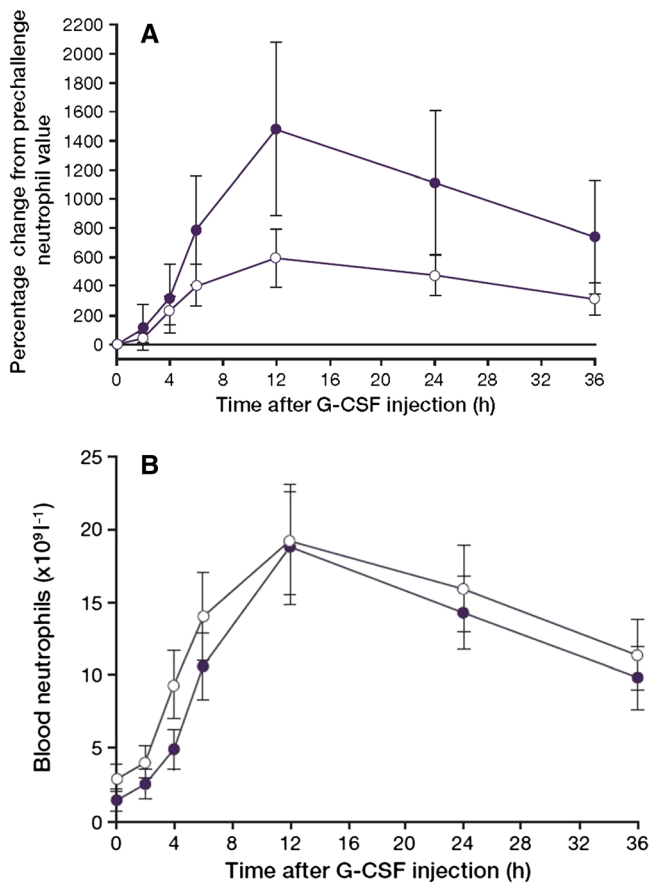
Figure 4

Increases in peripheral blood neutrophil counts (**A** – percentage change, **B** – absolute numbers) over time in response to granulocyte-colony stimulating factor (G-CSF) stimulation *in vivo*. Subjects were administered either AZD5069 (100 mg *bid*) or placebo orally (*bid*) following stimulation with G-CSF given by subcutaneous injection (300 μg). Peripheral blood samples were collected at the indicated time points. The data represent mean ± standard deviation (SD) of $n = 28$ subjects per arm. ⬤— AZD5069 100 mg *bid* —○— Placebo *bid*

Table 1

Statistical analysis on effects of AZD 5069 on neutrophil function and activation markers

Variable	Day 4 (compensated for baseline)		Day 14 (compensated for baseline)	
	Effect (log scale)	*P* value	Effect (log scale)	*P* value
Phagocytosis	0.025	0.721	0.067	0.275
Oxidative burst	0.068	0.375	−0.078	0.128
CD16	0.007	0.912	−0.044	0.282
CD11b	−0.137	0.182	−0.038	0.470
CD62L	0.132	0.136	−0.062	0.078

The analysis was done by using a linear mixed model with either Day 4 or Day 14 measurements, log-transformed, as the response variable. The effect and *p*-values were extracted from the linear model fit (coefficient and *P*-value for treatment A).

Activation state of neutrophils ex vivo is unaffected by treatment with AZD5069 in human volunteers

To ascertain whether CXCR2 antagonism could also affect the neutrophil activation state, we measured the surface expression of the neutrophil activation-associated markers, CD11b and CD62L, using flow cytometry [33, 34]. There were no significant differences between neutrophils derived from AZD5069-treated subjects versus placebo, regarding CD11b or CD62L expression (Figures 7A,B). Similarly, no differences were observed in the surface expression of CD16 (Figure 7C), a marker of viable, non-apoptotic neutrophils [35]. The expression of CD16 also changes in activated and immature neutrophils [45]. Hence, antagonism of CXCR2 on neutrophils by AZD5069 *in vivo* does not modulate surface expression of neutrophil activation, maturity and apoptosis markers.

Effects of AZD5069 treatment on blood inflammatory mediators in human volunteers

The serum concentrations of the CXCR2 ligands IL-8 and GROα significantly increased during AZD5069 treatment (day 4), but were stable during placebo treatment (Figures 8A,B). In contrast, there was only a modest increase in concentrations of the CXCR2 ligand ENA-78 with AZD5069, compared with placebo (Figure 8C). Serum concentrations of G-CSF increased significantly during the AZD5069 treatment period (day 4), but remained stable during the placebo treatment period (Figure 8D). Notably, this increase in G-CSF concentrations was not sufficient to reverse the AZD5069-mediated decrease in the number of neutrophils in circulation. The range of IL-8 values was 2.4727 pg ml^{-1} to 158.27 pg ml^{-1}. Day 1/baseline and day 14/follow-up values for IL-8 were within a relatively narrow range (2.47–32.00 pg ml^{-1}) in all subjects studied. ENA-78 values ranged from 245 pg ml^{-1} to 4144 pg ml^{-1}, whereas G-CSF values ranged from 2.02 pg ml^{-1} to 140.01 pg ml^{-1}. The range of GROα values was from below the limit of detection (31.25 pg ml^{-1}) to 744.12 pg ml^{-1}. The majority of day

ingested/phagocytosed bacteria (FITC-labelled *E. coli*). The MFI data clearly show that the phagocytic activity of neutrophils is similar in the presence of AZD5069 or placebo (Table 1, Figure 5).

To measure the level of oxidative burst activity, neutrophils were incubated with *E. coli*, in the presence of fluorogenic substrate DHR 123. The resulting change in MFI represents a quantitative measure of superoxide anion production by neutrophils. As with phagocytic activity, the MFI values for oxidative burst activity were similar in the AZD5069- and placebo-treated subjects (Table 1, Figure 6). These data reveal for the first time that, in human volunteers, neutrophils show no functional abnormalities in their antimicrobial immune functions during CXCR2 antagonism.

Neutrophil phagocytic function was also visualized using a method that focuses on single nucleated cells and excludes cell agglomerates and doublets (Supplementary Figure S2). Visualization confirms the absence of effects of AZD5069 on neutrophil function.
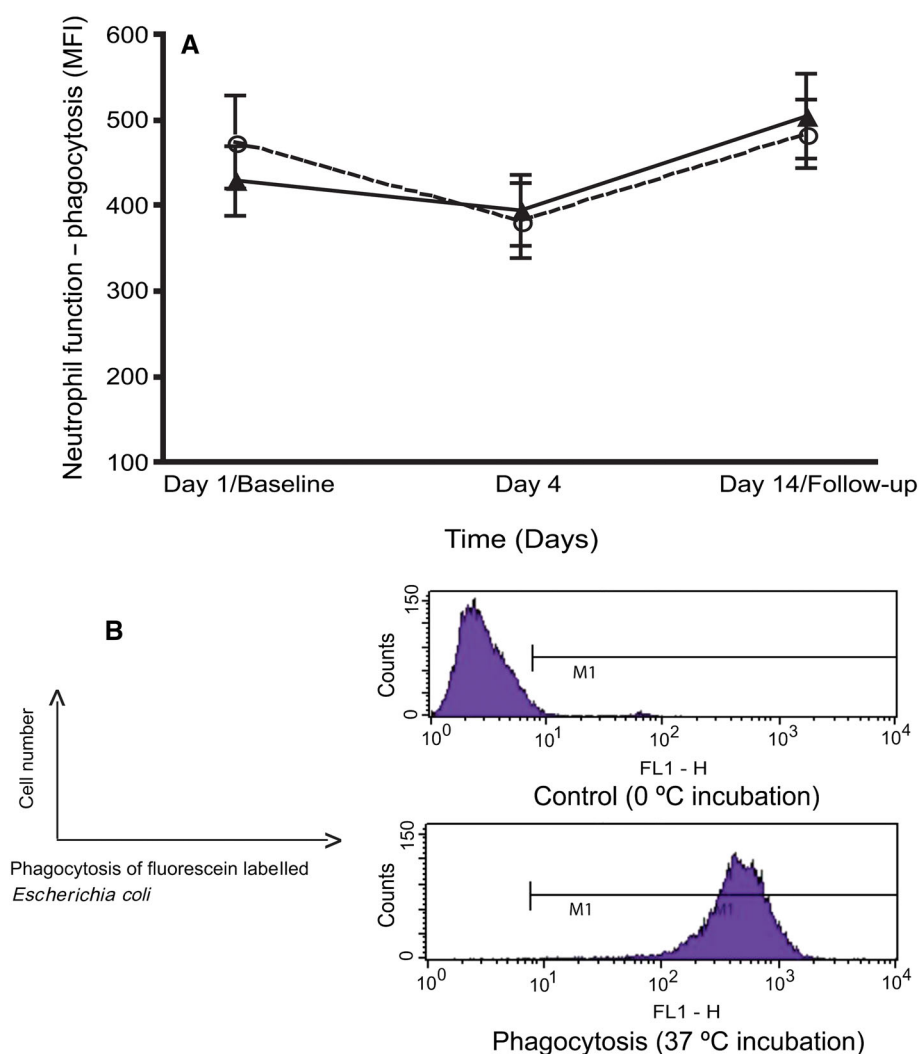
Figure 5

Neutrophil phagocytosis in the presence of AZD5069. Neutrophils were incubated with fluorescein-labelled *Escherichia coli* at 0°C as a negative control or at 37°C to stimulate phagocytosis. The number of ingested/phagocytosed fluorescein–*E. coli* bacteria is measured as mean fluorescence intensity (MFI) values. **A**. The data are shown as mean and 95% confidence interval of MFI values for all subjects at baseline, in the presence of AZD5069 or placebo (day 4 of treatment) and at follow up (day 14). ▲ AZD5069, ○ Placebo. **B**. Representative flow cytometry graphs for negative control (incubation with fluorescein–*E. coli* at 0°C) and phagocytosis of fluorescein–*E. coli* at 37°C are shown

1/baseline and day 14/follow-up values for GROα were below the detection limits of the assay. Serum concentrations of all ligands had returned to baseline levels at the follow-up visit 2 weeks after the end of treatment (Figures 8A–D). Serum hsCRP, a systemic marker of inflammation, remained at low levels during both treatment periods (Supplementary Figure S3).

Safety and tolerability

The number of subjects reporting at least one adverse event (AE) was similar for AZD5069 (55.2%) and placebo (58.6%). However, the number of subjects with at least one AE considered to be related to treatment was higher with AZD5069 than placebo (27.6% *vs*. 17.2%). The AEs reported were generally mild in intensity; the most common were back pain (n =11, 37.9% with both AZD5069 and placebo)

and headache (n = 9, 31% with both AZD5069 and placebo). Most of the subjects who reported back pain did so within 24 h after the G-CSF injection.

Two subjects withdrew from the study during the first treatment period owing to infection – one in the AZD5069 treatment group (tonsillitis on the second day of treatment) and one in the placebo group (orchitis). The subject with tonsillitis reported symptoms at only 36 h after his first dose of AZD5069, and the illness resolved quickly without antibiotics; the investigator therefore reported it as being unrelated to study treatment.

One subject withdrew from the study for personal reasons during the second treatment period, when receiving placebo.

With the exception of the expected reductions in white blood cell and blood neutrophil counts (Supplementary Table S2), no clinically relevant changes in clinical
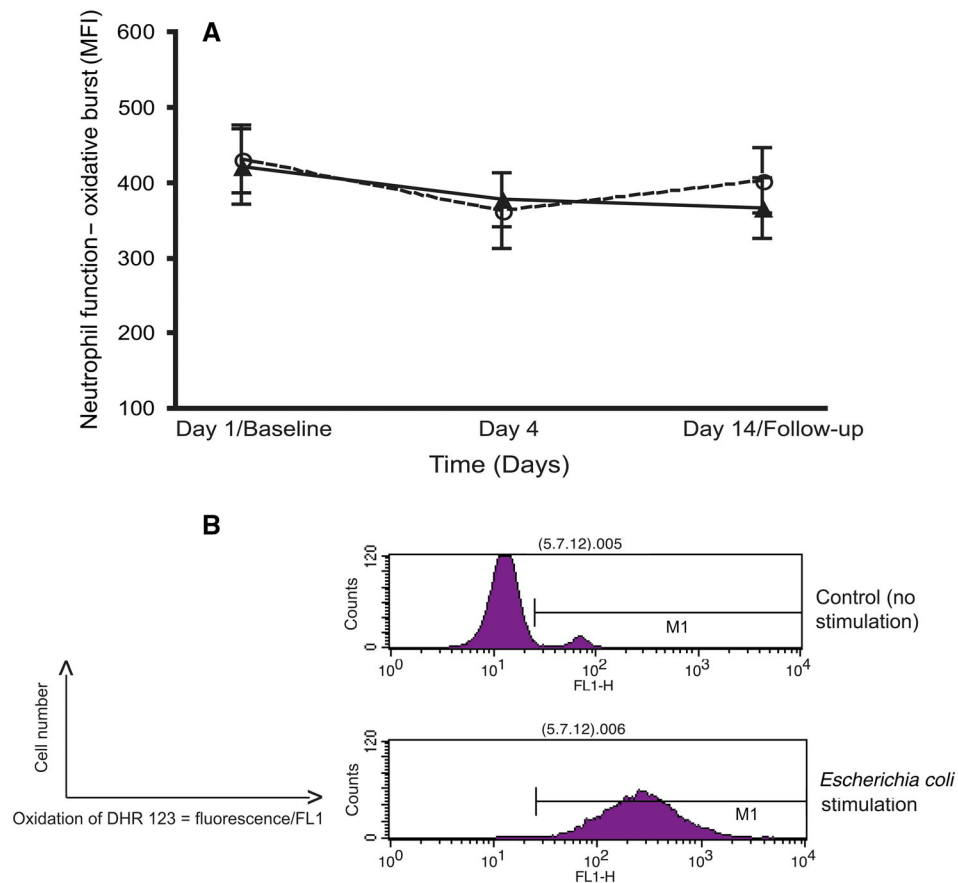
Figure 6

Neutrophil oxidative burst in the presence of AZD5069. Neutrophils were incubated with *Escherichia coli* to stimulate the oxidative burst or were left unstimulated as a control. The oxidative burst is measured quantitatively by flow cytometric detection of green fluorescence generated by the oxidation of the fluorogenic substrate dihydrorhodamine (DHR) 123. **A**. The data are shown as mean and 95% confidence interval of mean fluorescence intensity (MFI) values for all subjects at baseline, in the presence of AZD5069 or placebo (day 4 of treatment) and at follow-up (day 14). ▲ AZD5069, ⊖ Placebo. **B**. Representative flow cytometry graphs for control (no stimulation) and the oxidative burst in the presence of *E. coli* stimulation are shown

chemistry, haematology, urinalysis, liver function, vital signs or 12-lead ECG were observed.

Discussion

The present study was the first to examine prospectively neutrophil effector functions within the same human volunteers before and during treatment with a CXCR2 antagonist (AZD5069) in a placebo-controlled clinical setting. We made two novel and important observations. First, the ability of neutrophils to mobilize from the bone marrow into the peripheral circulation after G-CSF induction was largely unaffected by CXCR2 antagonism. The duration and the absolute size of the neutrophil response to physical exercise were reduced by CXCR2 antagonism; after AZD5069, an increase in neutrophil count was seen at 10-min after the start of the exercise challenge, but no increase was evident at 2-h or 4-h time points. Although the absolute size of the neutrophil response was reduced by CXCR2 antagonism, the percentage increase was almost identical to that seen during placebo treatment because the pre-exercise neutrophil

count was lower during AZD5069 treatment than during placebo. Thus, mobilization continued to occur in response to an exercise challenge during CXCR2 antagonism, but the effect was attenuated and transient. Secondly, treatment with AZD5069 did not interfere with the key antimicrobial effector functions of neutrophils, namely phagocytosis and oxidative burst activity, indicating that these granulocytes remained functionally active.

A CXCR2 antagonist mediates its effects through reducing the homing of neutrophils to inflamed tissues, and chemotaxis is therefore impeded. The bone marrow and other compartments, such as the marginal pool, serve as reservoirs for mature neutrophils that can be mobilized rapidly in response to physiological or pathological stimuli. In the present study, we demonstrated that the ability of neutrophils to respond to G-CSF stimulation remained functionally intact during CXCR2 antagonism, and that the response to physical exercise, although attenuated, continued to occur. Recent data showed similar effects on blood neutrophils after inhalation of LPS during treatment with another CXCR2 antagonist (AZD8309) [21]. The 10-min exercise challenge in the present study significantly increased
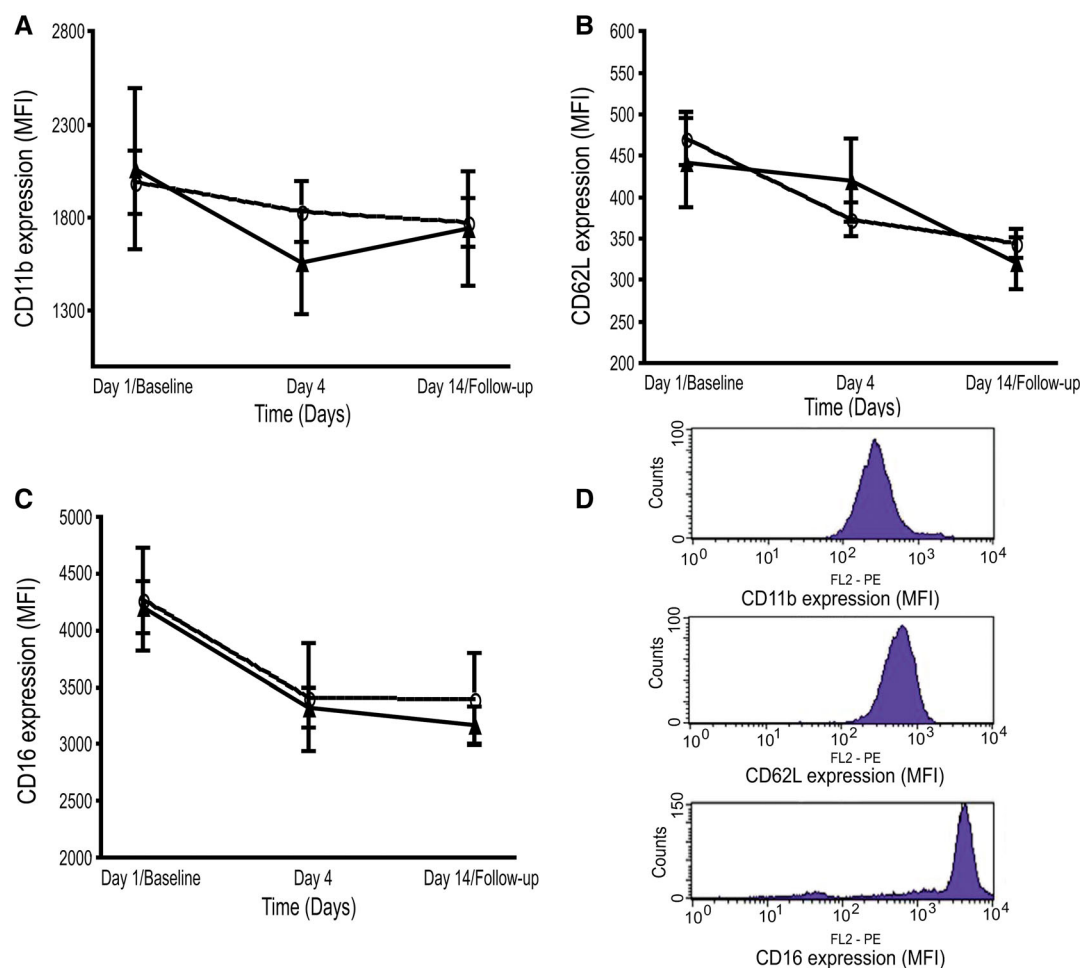
Figure 7

Neutrophil activation marker expression in the presence of AZD5069 or placebo. The cell surface expression of CD11b (**A**), CD62L (**B**) and CD16 (**C**) are presented as mean fluorescence intensity (MFI) units (mean and 95% confidence interval) at pretreatment baseline (day 1), treatment day 4 and day 14/follow-up. ▲ AZD5069, ⊸ Placebo. Representative flow cytometry histograms for each of the neutrophil cell surface markers are shown (**D**). The general appearance of these flow cytometry histograms was not affected by the treatment or the time point in the study.

mean blood neutrophil counts in both placebo and AZD5069 treatment groups. Blood neutrophil counts increased by the same percentage in both groups; however, the absolute increase in blood neutrophil count was lower in the AZD5069 treatment group. Although the neutrophil count remained above normal for at least 4 h after exercise during placebo treatment, the mean blood neutrophil count in individuals receiving AZD5069 returned to baseline within 2 h. The underlying mechanisms behind this require further study.

Challenge with G-CSF led to a large increase in the circulating neutrophil count during both placebo and AZD5069 treatment, which peaked at 12 h and remained above baseline over the 36-h observation period (Figure 4). The absolute increases in the neutrophil count were similar after AZD5069 and placebo; however, because the pre-G-CSF neutrophil count during AZD5069 treatment was about half that on placebo treatment, the relative increase after AZD5069 was roughly double that after placebo.

This large and rapid increase in blood neutrophils indicates a redistribution of neutrophils from the bone marrow to the blood. Similar increases in blood neutrophil counts have been found using other stimuli (ozone or LPS) in previous clinical studies with CXCR2 antagonists [19–21, 27]. In addition, we have shown that a diurnal variation in blood neutrophils is maintained during treatment with AZD5069 (Supplementary Figure S1). These results in human volunteers indicate that bone marrow neutrophil mobilization into the peripheral circulation is not solely dependent on CXCR2 signalling pathways. This is supported by other data suggesting that CXCR2 and CXCR4 interact to regulate neutrophil release from the bone marrow [46, 47].

Neutrophil activation is associated with the upregulation of cell surface CD11b expression and CD62L shedding [33, 34]. We analysed the expression of these cell surface markers of human neutrophils prior to (baseline) and after placebo or AZD5069 treatment, using flow cytometry. No significant difference in the expression of either of these
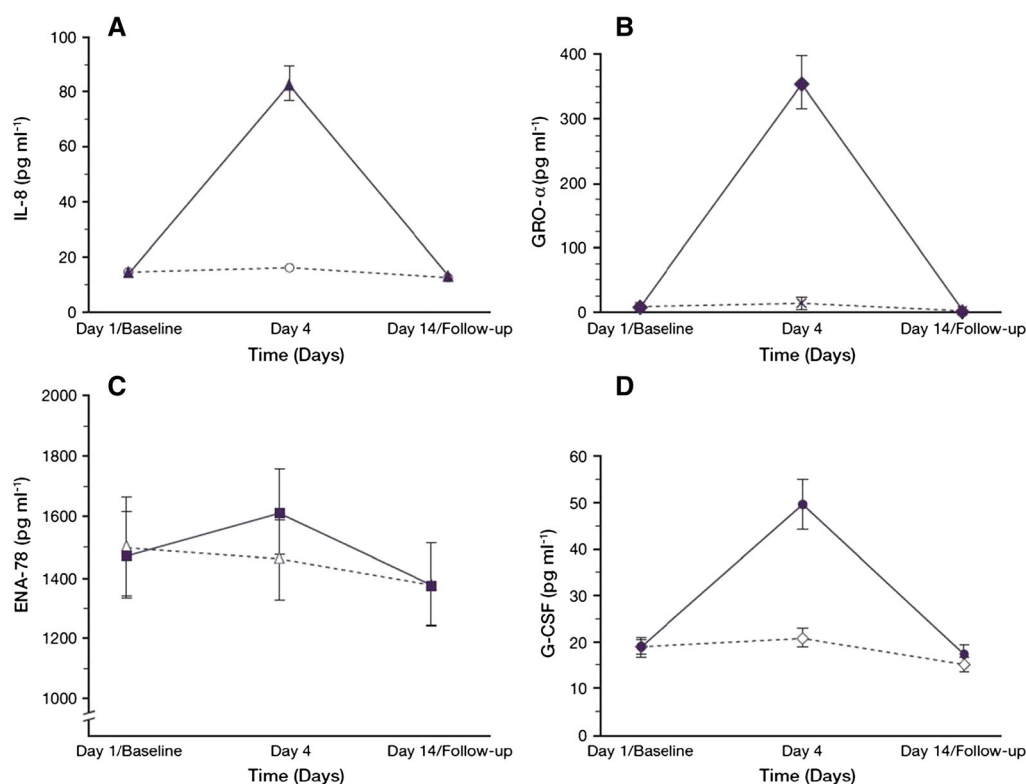
Figure 8

Changes in serum concentrations of the CXCR2 ligands **A** interleukin 8 (IL-8), **B** growth-related oncogene α (GROα), **C** epithelial-derived neutrophil-activating protein 78 (ENA-78) and **D** granulocyte-colony stimulating factor (G-CSF) observed during treatment with AZD5069, compared with placebo, in human volunteers. The baseline, day 1 pretreatment concentrations and post-treatment day14/follow-up concentrations are also shown for each analyte. Subjects were administered either AZD5069 (100 mg *bid*) or placebo orally (*bid*). Peripheral blood samples were collected for mediator analysis at the indicated time points. The data represent the mean ± standard deviation of $n = 28$ subjects per arm. **A.** ▲ AZD5069 (100 mg *bid*), -○- Placebo. **B.** ◆ AZD5069 (100 mg *bid*), -✳- Placebo. **C.** ■ AZD5069 (100 mg *bid*), -△- Placebo. **D.** ● AZD5069 (100 mg *bid*), -◇- Placebo

markers of activation was seen on circulating neutrophils during either treatment.

When neutrophils undergo constitutive apoptosis, they lose expression of the cell surface receptor CD16, and this could serve as a potential marker of viable, non-apoptotic neutrophils [35]. Thus, we also examined the expression of CD16 on circulating neutrophils during both placebo and AZD5069 treatment, and found that it was not significantly affected by AZD5069 treatment. These findings suggest that the regulation of neutrophil activation and survival are unaffected during CXCR2 antagonism.

Interestingly, treatment with AZD5069 increased circulating concentrations of the high-affinity CXCR2 ligands IL-8 and GROα (but not ENA-78) as well as of G-CSF, compared with placebo, but these effects were completely reversible upon cessation of treatment. This is consistent with findings in an *in vivo* mouse model of cigarette smoke-induced lung inflammation, in which SCH527123, a CXCR2 antagonist, significantly increased concentrations of the murine IL-8 homologues Keratinocyte Chemoattractant (KC) and Macrophage Inflammatory Protein 2 (MIP-2) [48]. Moreover, these neutrophilic chemokines have been shown to be significantly elevated in the blood of CXCR2 knockout mice compared with wild-type littermates [49]. There is also recent clinical evidence that blood concentrations of IL-8 were significantly increased in a dose-dependent manner in patients with cystic fibrosis receiving a CXCR2 antagonist (SB-656933) [23], and in patients with bronchiectasis who received AZD5069 [24], further indicating a potential compensatory upregulation of CXCR2-specific ligands. Although the specific mechanisms regulating this systemic mediator release have not been investigated, a possible explanation of this observed adaptive increase could be a limited physiological compensatory response following blockade of CXCR2 receptors.

The increase in IL-8 and GROα in human volunteers, although statistically significant, was not sufficient to reverse the CXCR2 antagonist-mediated decrease in number of neutrophils in circulation. The increased concentrations of the CXCR2 ligands did not appear to cause systemic inflammation as the levels of hsCRP remained low (Supplementary Figure S3), nor did they reach the concentrations shown in previous *in vitro* studies to be necessary to induce neutrophil effector functions [34, 50, 51]. For example, the potency of IL-8 to mediate neutrophil polarization and chemotaxis in order to produce 50% of maximal effect (EC_{50}) was reported as 6.3 ± 1.0 nM [51] and 2.79 nM, respectively [34].

With respect to inducing neutrophil oxidative burst activity, the estimated EC_{50} for IL-8 has been reported to be higher than 100 nM *in vitro* [50]. In the present clinical study, the serum concentration of the CXCR2 ligand IL-8 was 80 pg ml^{-1} (=10 pM; mean value) and for GROα was 846 pg ml^{-1} (=106 pM; mean value). On average, the observed serum concentrations of IL-8 and GROα were approximately 1000-fold and 100-fold lower, respectively, compared with the concentrations required to induce neutrophil effector functions in the *in vitro* studies. By extrapolation, it is unlikely that such relatively low levels of CXCR2 ligands (i.e. the pg ml^{-1} range) in circulation will be able to contribute to the neutrophil-mediated inflammation observed in chronic inflammatory diseases.

Our clinical study provides evidence that CXCR2 receptor antagonism has no discernible effects on neutrophil function in terms of their ability to engage in antimicrobial activities – namely, phagocytosis and oxidative burst activity. However, this would need further validation in a large-scale clinical trial, involving a large cohort of patients. AZD5069 has been shown to be generally well tolerated both by healthy humans, as reported in the present study, and by patients with COPD or bronchiectasis in Phase IIa studies [24]. Finally, it is important to note that there is clinical evidence confirming the long-term safety and tolerability of a CXCR2 antagonist over a 6-month treatment period in COPD patients, without impairing their host defences against infections [25].

This was the first demonstration in healthy volunteers that in the presence of a CXCR2 antagonist, AZD5069, neutrophils retain their key innate effector functions with regard to their capacity to i) mobilize from the marginating pool or bone marrow into the circulation in response to physiological stimuli; ii) effectively phagocytose bacterial pathogens and iii) mount an oxidative burst adequately in response to phagocytosed pathogens, such as *E coli*. Although CXCR2 antagonism with AZD5069 reduced circulating neutrophil counts, as expected from previous data, the pattern of response over time after treatment indicated that diurnal variation was maintained. We conclude that CXCR2 antagonists could potentially serve as novel anti-inflammatory therapeutics without compromising the immune vigilance of neutrophils against microbial pathogens.

In the present study, AZD5069, administered as 100 mg twice daily, was considered to be well tolerated and no safety concerns were raised. However, further large-scale clinical studies with AZD5069 are needed, to explore its therapeutic potential, safety and tolerability in the treatment of chronic inflammatory conditions that are characterized by neutrophil-driven inflammation.

Competing Interests

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: SJ and SW had support from AstraZeneca for the submitted work; BL, CH and CK are employees of AstraZeneca and hold shares in the company; MU is an employee of AstraZeneca; no other relationships or activities that could appear to have influenced the submitted work.

Contributors

All authors were involved at all stages in the development of the manuscript. BL was involved in all aspects of the study and manuscript development. CH was involved at all stages in the development of the manuscript. CK was responsible for human study: study design, study protocol, study set-up, interpretation and review of study report. MU was responsible for interpretation of biomarker data and manuscript development. SJ was the principal investigator and responsible for study set-up, analysis of the functional assays and biomarkers in the clinical study. and review of study report. SW was responsible for conduct of the human study and review of study report.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1
Reduction in blood neutrophil count on day 3 of the treatment period. Each point is shown as the arithmetic mean (± standard deviation) neutrophil percentage change from naïve (pretreatment) baseline *vs.* time by treatment, on a linear scale

Figure S2
Images of neutrophils from representative subjects treated with either placebo or AZD5069

Figure S3
Serum levels of high-sensitivity C-reactive protein remained low during both treatment periods. Figure shows mean values (standard deviation)

Table S1
Demographics of human volunteer study population

Table S2
Circulating neutrophil counts over time in human volunteers

Table S3
Human volunteer neutrophil functional assays at the indicated time points