

WestminsterResearch

<http://www.westminster.ac.uk/westminsterresearch>

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

Jurcevic, S.

This is the peer reviewed version of the following article: Jurcevic, S. (2015) The effect of a selective CXCR2 antagonist (AZD5069) on human blood neutrophil count and innate immune functions. *Br J Clin Pharmacol.* 80 (6) 1324-36 1365-2125 , which has been published in final form at <https://dx.doi.org/10.1111/bcp.12724>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

The WestminsterResearch online digital archive at the University of Westminster aims to make the research output of the University available to a wider audience. Copyright and Moral Rights remain with the authors and/or copyright owners.

Whilst further distribution of specific materials from within this archive is forbidden, you may freely distribute the URL of WestminsterResearch: (<http://westminsterresearch.wmin.ac.uk/>).

In case of abuse or copyright appearing without permission e-mail repository@westminster.ac.uk

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

Stipo Jurcevic¹; Charles Humfrey²; Mohib Uddin³; Steve Warrington⁴; Bengt Larsson³;
Christina Keen³

¹Division of Transplantation Immunology & Mucosal Biology, King's College London,
London, UK*; ²AstraZeneca, R&D Alderley Park, UK; ³AstraZeneca, R&D Mölndal,
Sweden; ⁴Hammersmith Medicines Research Ltd (HMR), London, UK

Corresponding author

Dr Christina Keen. AstraZeneca R&D Mölndal, Pepparedsleden 1, Mölndal, Sweden,
SE-431 83

Tel: +4631 7761000; Fax: +4631 7763806; Email: christina.keen@astrazeneca.com

Running title: Effects of CXCR2 antagonism on human neutrophils

Keywords: neutrophil recruitment, oxidative burst, phagocytosis, CXCR2 antagonist,
asthma, COPD

Number of references: 51; **Number of Figures:** 8

Number of Tables: 1; **Current word count:** 4673

**At the time the work was carried out. Current affiliation: Department of Biomedical Sciences, University of
Westminster, London, UK.*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bcp.12724

Abstract

Aims: To investigate whether selective antagonism of the 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, cross-over study, 30 healthy volunteers were randomised to treatment with the CXCR2 antagonist AZD5069 (100 mg) or placebo, twice daily orally for 6 days. 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 oxidative burst response to *E. coli*.

Results: AZD5069 treatment reversibly reduced circulating neutrophil count from baseline by a mean (SD) of $-1.67 (0.67) \times 10^9/L$ vs $0.19 (0.78) \times 10^9/L$ 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* was unaffected by AZD5069 ($p=0.375$, $p=0.721$ respectively vs baseline, Day 4). AZD5069 was well tolerated.

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

Study highlights

What is 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 does this study add to our knowledge?

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

BJCP heading for original research articles: **Translational Research**

Introduction

Neutrophils regulate innate immune responses but can also contribute to tissue damage through 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 characterised 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 1s (FEV₁) [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 1 (CXCL1 or Growth-related oncogene α [GRO α]), CXCL5 (epithelial-derived neutrophil-activating protein 78 [ENA-78]) and CXCL8 (interleukin-8 [IL-8]), have been reported in the airways of patients with COPD, cystic fibrosis or 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 pre-clinical and clinical data support the development of selective CXCR2 antagonists for the treatment of inflammatory lung diseases characterised by neutrophilic inflammation [1, 16]. Pre-clinical evidence has shown that genetic and pharmacological inactivation of the 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 have 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 in both 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 Ph2b clinical trial has shown 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 indicate that treatment with AZD5069 50 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 mobilisation from 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 mobilise neutrophils from 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].

While previous studies using isolated primary cells have demonstrated that selective antagonism of CXCR2 *per se* does not interfere with the key neutrophil anti-microbial activities *in vitro* [16, 31, 32], this has not been fully explored in a clinical setting. The primary aim of this study, therefore, was to investigate whether neutrophils from healthy volunteers demonstrate any functional abnormalities in their key innate effector functions (*e.g.* neutrophil mobilisation into 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 (MAC-1) and CD62L (L-selectin), using flow cytometry [33, 34]. CD16 (Fc γ RIIIb), a potential marker of viable non-apoptotic neutrophils [35], was assessed at the same time-points. Serum concentrations of CXCR2 ligands IL-8, GRO α and ENA-78 were also assessed before, during and after treatment with AZD5069.

Methods

Clinical data

A single-centre, randomised, double-blind, placebo-controlled, cross-over study

(Clinicaltrials.gov Identifier NCT01480739) was conducted in 30 healthy subjects (Figure 1)

at the Hammersmith Medicines Research (HMR) Medical Laboratory, London, United

Kingdom. First subject was enrolled on 4 May 2012; last subject visit was 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 cross-over 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 active and placebo. Pre-dose blood sampling was also done at the same

time point on each day.

Study drugs were administered orally twice daily (*bid*) for 6 d, followed by a washout period

of at least 21 d and then a second 6 d treatment period. Subjects were randomised, 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 d 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.

This 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-policiesstandards/Our-global-policies>).

Neutrophil effector function assays

Neutrophil function (phagocytosis and oxidative burst) was considered to be normal in the clinical study if more than 80% of neutrophils were observed phagocytosing and oxidising 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 opsonised FITC-labelled *E. coli* (4×10^7 bacteria) was measured *ex vivo* as described previously [38-40]. Neutrophil phagocytic function was visualised 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 pre-study assay qualification experiments we determined intra-assay (within assay) coefficient of variation (CV%) for neutrophil function assays. The CV% for the phagocytosis and superoxide anion production assays were 12.54% and 8.67%, respectively.

Activation state of 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 magnitude and direction of changes in the expression of surface markers: >30% changes in the level of expression (mean fluorescence intensity [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 neutrophil surface were measured as mean fluorescence intensity (MFI) units and analysed independently for each of these activation markers. The CV% for these assays were determined in pre-study 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 mobilisation of neutrophils

To determine whether treatment with AZD5069 affects exercise-induced mobilisation of human neutrophils *in vivo*, AZD5069-treated volunteers were challenged with a standardised 10 min sub-maximal exercise test. On Day 4, subjects performed a standardised 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 mobilising neutrophils from other compartments, such as the marginating pool, into circulation [43]. Blood samples were collected before and at 10 min (directly after the challenge), 2 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 release of neutrophils into the circulation. 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 mobilised 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, GRO α and ENA-78) and G-CSF were measured using Luminex Fluorokine MAP reagents and ELISA kit for GRO α (both R&D Systems, Abingdon, UK). The lower detection limits for Luminex assays were 2.47 pg/mL for IL-8, 4.00 pg/mL for ENA-78 and 2.02 pg/mL for G-CSF. The lower detection limit for ELISA assay for GRO α was 31.25 pg/mL. The CV% for assays measuring inflammatory mediators determined in pre-study assay qualification tests were 12.01% for IL-8, 14.48% for GRO α , 4.42% for ENA-78 and 14.93% for G-CSF. The within-assay CV% for the 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. (Drug/molecular target nomenclature conforms to the *Brit J Pharmacol. Guide to Receptors and Channels* [GRAC])[44]

Statistical methods

Statistical analyses were performed by Quintiles, Overland Park, Kansas (USA) and Quintiles (Bangalore, India), using SAS[®] Version 9.2. Continuous variables were summarised 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 Operation Procedures (SOPs) and Work Instructions. All PD computations were performed using SAS Version 9.2. Graphics were prepared with SAS Version 9.2 or SigmaPlot 9.0.

A linear model (on the log scale) was used to analyse the data from the cross-over 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 cross-over analysis was performed with R version 3.1.2 using the nlme package version 3.1.

Safety and tolerability

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; 30 were randomised and of those 27 (90.0%) received all the scheduled doses of the CXCR2 antagonist AZD5069 and completed the study. All were healthy, non-smoking men, or women of non-childbearing potential, aged 18–45 years at screening (Supplementary Table 1). All were Caucasian, to avoid the possibility of ethnic differences in neutrophil kinetics affecting the study results. Baseline arithmetic mean circulating neutrophil counts were $3.43 \times 10^9/L$ (SD 0.89) and $3.21 \times 10^9/L$ (SD 0.80) for the AZD5069 and placebo treatment groups, respectively (Supplementary Table 2). As expected, circulating neutrophil counts were already reduced after one day of treatment. (Figure 2)

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

Compared with pre-exercise levels, blood neutrophil count increased rapidly by a similar percentage in AZD5069-treated and in placebo-treated subjects (Figure 3A). The absolute increase in neutrophil count during treatment with AZD5069 was about half that seen during placebo treatment (Figure 3B). 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 mobilisation 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 last dose of AZD5069, morning mean values for blood neutrophil counts for both 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. 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 1). Mean blood neutrophil counts remained below pre-treatment 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 1).

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 opsonised fluorescein isothiocyanate-labeled (FITC) *E. coli* (Supplementary Table 3) with the percentage of phagocytic neutrophils matching that observed in cells from placebo-treated patients. At all time-points measured in both treatment groups, AZD5069 and placebo, >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 3). As the percentages of phagocytic and oxidising neutrophils

represent a qualitative measure of neutrophil function, we also analysed the quantitative data, mean fluorescence intensity (MFI) values. In phagocytic assays, the MFI values reflect the number of ingested/phagocytosed bacteria (fluorescein-labelled *E. coli*). The MFI data clearly show that 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 (dihydrorhodamine) 123. The resulting change in mean fluorescence intensity (MFI) represents a quantitative measure of superoxide anion production by neutrophils. As with phagocytic activity, the MFI values for oxidative burst activity are similar in both AZD5069 treatment and placebo (Table 1, Figure 6). These data reveal for the first time that, in human volunteers, neutrophils show no functional abnormalities in their anti-microbial immune functions during CXCR2 antagonism.

Neutrophil phagocytic function was also visualised using a method that focuses on single nucleated cells and excludes cell agglomerates and doublets (Supplementary Figure 2).

Visualisation confirms the absence of effects of AZD5069 on neutrophil function.

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 & 7B). Similarly, no differences were observed in 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

Significant increases in serum concentrations of the CXCR2 ligands, IL-8 and GRO α , were observed during AZD5069 treatment (Day 4), whereas concentrations of these chemokines were stable during placebo treatment (Figures 8A & 8B). In contrast, we observed only modest changes in concentrations of the CXCR2 ligand ENA-78 (Figure 8C). Serum concentrations of G-CSF increased significantly during AZD5069 treatment period (Day 4), whereas the concentrations of G-CSF 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 numbers of neutrophils in circulation. The range of IL-8 values was 2.47 to 158.27 pg/mL. Day 1/baseline and Day 14/follow up values for IL-8 were within a relatively narrow range of 2.47-32.00 pg/mL in all subjects studied. ENA-78 values ranged from 245 to 4144 pg/mL, whereas G-CSF values ranged from 2.02 to 140.01 pg/mL. The range of GRO α values was from below the limit of detection to 744.12 pg/mL. The majority of Day 1/baseline and Day 14/follow up values for GRO α were below detection limits of the assay. Serum concentrations of all ligands had returned to baseline levels at the follow-up visit two weeks after end of treatment (Figures 8A–D). Serum high sensitivity C-reactive protein (hsCRP), a systemic marker of inflammation, remained at low levels during both treatment periods (Supplementary Figure 3).

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 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 reporting back pain did so within 24h after the G-CSF injection.

Two subjects withdrew from the study in 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. Because the tonsillitis began so soon after the start of dosing with AZD5069 and resolved rapidly without antibiotic treatment, the investigator reported it as 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 cells and blood neutrophil counts (Supplementary Table 2), no clinically relevant changes in clinical chemistry, haematology, urinalysis, liver function, vital signs or 12-lead ECG were observed.

Discussion

Our study is 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 have made two novel and important observations. First, the ability of neutrophils to mobilise from bone marrow into 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 timepoints. Although the absolute size of the neutrophil response was reduced by CXCR2 antagonism, the percentage increase was almost identical to the increase seen during placebo treatment because the pre-exercise neutrophil count was lower during AZD5069 treatment than during placebo. Thus mobilisation 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 anti-microbial 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 together with other compartments such as the marginal pool serve as reservoirs for mature neutrophils that can be mobilised rapidly in response to physiological or pathological stimuli. In this study, we have demonstrated that the ability of neutrophils to respond to G-CSF stimulation remained functionally intact during CXCR2 antagonism, and 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 our study significantly increased 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. While the neutrophil count remained above normal for at least 4 h after exercise during placebo treatment, 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 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 neutrophils were similar after AZD5069 and placebo; however, because the pre-GCSF neutrophil count on AZD5069 was about half that on placebo treatment, the relative increase after AZD5069 was roughly double the relative increase after placebo.

This large and rapid increase in blood neutrophils indicates a redistribution of neutrophils from bone marrow to 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 1). These results in human volunteers indicate that bone marrow neutrophil mobilisation 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 upregulation of cell surface CD11b 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 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. The expression of CD16 on neutrophils was not significantly affected by AZD5069 treatment. These findings suggest that 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 GRO α 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 KC and MIP-2 [48]. These neutrophilic chemokines have also been shown to be significantly elevated in the blood of CXCR2 knock-out 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 GRO α in human volunteers, although statistically significant, was not sufficient to reverse the CXCR2 antagonist-mediated decrease in numbers 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 3), 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 polarisation and chemotaxis in order to produce 50% of maximal effect (EC₅₀) was reported as 6.3 \pm 1.0 nM [51] and 2.79 nM, respectively [34].

With respect to inducing neutrophil oxidative burst activity, the estimated EC₅₀ 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 (= 10 pM; mean value) and for GRO α was 846 pg/mL (= 106 pM; mean value). On average, the observed concentrations of IL-8 and GRO α were approximately 1000-fold and 100-fold lower in circulation respectively, compared with the concentrations required to effectively induce neutrophil effector functions in the *in vitro* studies. By extrapolation, it is unlikely that such relatively low levels of CXCR2 ligands (*i.e.* picogram/mL range) in circulation will be able to contribute to the neutrophil-mediated inflammation observed in chronic inflammatory diseases.

Our clinical study provides evidence that antagonism of CXCR2 receptors has no discernible effects on neutrophil function in terms of their ability to engage in anti-microbial 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 is generally well tolerated by both healthy humans as reported in this 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 is 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) mobilise from the marginating pool or bone marrow into the circulation in response to physiological stimuli, ii) effectively phagocytose bacterial pathogens and iii) adequately mount an oxidative burst in response to phagocytosed pathogens, such as *E coli*. Although antagonism of CXCR2 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.

AZD5069, administered as 100 mg twice daily, was considered to be well tolerated and no safety concerns were raised in this study. 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 characterised by neutrophil-driven inflammation.

Acknowledgements

The authors wish to thank the nursing and technical staff of Hammersmith Medicines Research, and the volunteer subjects, for their contribution to this research, Professor Jan Palmbald for his expert advice on the design and interpretation of the clinical study and Maria Nordberg and Katherine Wiley for technical assistance with the pre-clinical experiments. The research in Dr Jurcevic's laboratory was supported by the MRC Centre for Transplantation. We are grateful for the expert contribution of King's College London scientists, Drs Roseanna Greenlaw, Christine Wong and Simon Freeley. For statistical support we acknowledge Margareta Puu and Alexandra Jauhiainen, AstraZeneca. Medical writing assistance was provided by David Candlish of inScience Communications, Springer Healthcare, funded by AstraZeneca.

Conflict of interest/Disclosure

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.

Author contributions

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.

Accepted Article

References

1. Barnes PJ. New molecular targets for the treatment of neutrophilic diseases. *J Allergy Clin Immunol*. 2007;119(5):1055-62.
2. Chollet-Martin S, Montravers P, Gilbert C, Elbim C, Desmots JM, Fagon JY, Gougerot-Pocidal MA. Subpopulation of hyperresponsive polymorphonuclear neutrophils in patients with adult respiratory distress syndrome. Role of cytokine production. *Am Rev Respir Dis*. 1992;146(4):990-96.
3. Quint JK, Wedzicha JA. The neutrophil in chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 2007;119(5):1065-71.
4. Jatakanon A, Uasaf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med*. 1999;160(5):1532-39.
5. Wenzel SE, Schwartz LB, Langmack E, Halliday JL, Trudeau B, Gibbs RL, Chu HW. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med*. 1999;160(3):1001-08.
6. Uddin M, Nong G, Ward J, Seumois G, Prince LR, Wilson SJ, Cornelius V, Dent G, Djukanovic R. Prosurvival activity for airway neutrophils in severe asthma. *Thorax*. 2010;65(8):684-89.
7. Stanescu D, Sanna A, Veniter C, Kostianev S, Calcagni PG, Fabbri LM, Maestrelli P. Airways obstruction, chronic expectoration, and rapid decline of FEV₁ in smokers are associated with increased levels of sputum neutrophils. *Thorax*. 1996;51:267-71.
8. Shaw DE, Berry MA, Hargadon B, McKenna S, Shelley MJ, Green RH, Brightling CE, Wardlaw AJ, Pavord ID. Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest*. 2007;132(6):1871-75.
9. Qiu Y, Zhu J, Bandi V, Atmar RL, Hattotuwa K, Guntupalli KK, Jeffery PK. Biopsy Neutrophilia, Neutrophil Chemokine and Receptor Gene Expression in Severe Exacerbations of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med*. 2003;168(8):968-75.
10. MacDowall AL, Peters SP. Neutrophils in asthma. *Curr Allergy Asthma Rep*. 2007;7(6):464-68.
11. Richman-Eisenstat JB, Jorens PG, Hebert CA, Ueki I, Nadel JA. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Am J Physiol Lung cellular and Molecular Physiology*. 1993;264:L413-18.
12. Través S, Culpitt S, Russell R, Barnes P, Donnelly L. Increased levels of the chemokines GRO α and MCP-1 in sputum samples from patients with COPD. *Thorax*. 2002;57(7):590-95.
13. Mackerness KJ, Jenkins GR, Bush A, Jose PJ. Characterisation of the range of neutrophil stimulating mediators in cystic fibrosis sputum. *Thorax*. 2008;63(7):614-20.
14. Beeh K, Kornmann O, Buhl R, Culpitt SV, Giembycz MA, Barnes PJ. Neutrophil Chemotactic Activity of Sputum From Patients With COPD*: Role of Interleukin 8 and Leukotriene B₄. *Chest*. 2003;123(4):1240-47.
15. Donnelly LE, Barnes PJ. Chemokine receptors as therapeutic targets in chronic obstructive pulmonary disease. *Trends Pharmacol Sci*. 2006;27(10):546-53.
16. Chapman RW, Phillips JE, Hipkin RW, Curran AK, Lundell D, Fine JS. CXCR2 antagonists for the treatment of pulmonary disease. *Pharmacol Ther*. 2009;121:55-68.
17. Goncalves A-S, Appelberg R. The Involvement of the chemokine receptor CXCR2 in neutrophil recruitment in LPS-induced inflammation and in *Mycobacterium avium* infection. *Scand J Immunol*. 2002;55(6):585-91.
18. Chapman RW, Minnicozzi M, C.S. C, Phillips JE, Kung TT, R.W. H, Fan X, Rindgen D, Deno G, Bond R, Gonsiorek W, Billah MM, Fine JS, Hey JA. A novel, orally active CXCR1/2 receptor antagonist, SCH527123, inhibits neutrophil recruitment, mucus production, and goblet cell hyperplasia in animal models of pulmonary inflammation. *J Pharmacol Exp Ther*. 2007;322(2):486-93.
19. Holz O, Khalilieh S, Ludwig-Sengpiel A, Watz H, Stryszak P, Soni P, Tsai M, Sadeh J, Magnussen H. SCH527123, a novel CXCR2 antagonist, inhibits ozone-induced neutrophilia in healthy subjects. *Eur Respir J*. 2010 Mar;35(3):564-70.

20. Lazaar AL, Sweeney LE, MacDonald AJ, Alexis NE, Chen C, Tal-Singer R. SB-656933, a novel CXCR2 selective antagonist, inhibits ex vivo neutrophil activation and ozone-induced airway inflammation in humans. *Brit J Clin Pharmacol*. 2011 Aug;72(2):282-93.
21. Leaker B, Barnes PJ, O'Connor BJ. Inhibition of LPS-induced airway neutrophilic inflammation in healthy volunteers with an oral CXCR2 antagonist. *Respir Res*. 2013;14:137.
22. Virtala R, Ekman AK, Janson L, Westin U, Cardell LO. Airway inflammation evaluated in a human nasal lipopolysaccharide challenge model by investigating the effect of a CXCR2 inhibitor. *Clin Exp Allergy*. 2012;42(4):590-96.
23. Moss RB, Mistry SJ, Konstan MW, Pilewski JM, Kerem E, Tal-Singer R, Lazaar AL. Safety and early treatment effects of the CXCR2 antagonist SB-656933 in patients with cystic fibrosis. *J Cystic Fibrosis*. 2013;12(3):241-48.
24. De Soyza A, Pavord I, Elborn JS, Smith D, Wray H, Puu M, Larsson B, Stockley R. A randomised, placebo-controlled study of the CXCR2 antagonist AZD5069 in bronchiectasis. *Eur Respir J*. 2015(In press).
25. Rennard SI, Dale DC, Donohue JF, Kanniss F, Magnussen H, Sutherland ER, Watz H, Lu S, Stryszak P, Rosenberg E, Staudinger H. CXCR2 Antagonist MK-7123-A Phase 2 proof-of-concept trial for chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2015.
26. Kirsten AM, Förster K, Radechky E, Linnhoff A, Balint B, Watz H, Wray H, Salkeld L, Cullberg M, Larsson B. The safety and tolerability of oral AZD5069, a selective CXCR2 antagonist, in patients with moderate-to-severe COPD. *Pulm Pharmacol Ther*. 2015;31:36-41.
27. Nair P, Gaga M, Zervas, Alagha K, Hargreave FE, O'Byrne PM, Stryszak P, Gann L, Sadeh J, Chanez P. Safety and efficacy of a CXCR2 antagonist in patients with severe asthma and sputum neutrophils: a randomized, placebo-controlled clinical trial. *Clin Exp Allergy*. 2012;42(7):1097-103.
28. Seiberling M, Kamtchoua T, Stryszak P, Ma X, Langdon RB, Khalilieh S. Humoral immunity and delayed-type hypersensitivity in healthy subjects treated for 30days with MK-7123, a selective CXCR2 antagonist. *Int Immunopharmacol*. 2013;17(2):178-83.
29. MacNee W, Selby C. Neutrophil kinetics in the lungs. *Clin Sci*. 1990;79:97-107.
30. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trends Immunol*. 2010;31(8):318-24.
31. Jones SA, Wolf M, Qin S, Mackay CR, Baggiolini M. Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. *Proc Natl Acad Sci USA*. 1996;93:6682-86.
32. Jones SA, Dewald B, Clark-Lewis I, Baggiolini M. Chemokine antagonists that discriminate between interleukin-8 receptors. Selective blockers of CXCR2. *J Biol Chem*. 1997;272(26):16166-69.
33. Mann BS, Chung KF. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. *Respir Res*. 2006;7:59.
34. Uddin M, Seumois G, Lau LC, Ryttila P, Davies DE, Djukanovic R. Enhancement of neutrophil function by the bronchial epithelium stimulated by epidermal growth factor. *Eur Respir J*. 2008;31(4):714-24.
35. Dransfield I, Buckle AM, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J Immunol*. 1994;153(3):1254-63.
36. Data on file. AstraZeneca. 2014.
37. Lorch U, Negi H, Tabata H, Wray H, Cullberg M, Larsson B. The safety, tolerability and pharmacokinetics of AZD5069, a novel CXCR2 antagonist, in healthy Japanese volunteers. *Eur Respir J*. 2012;40(Suppl. 56):4842.
38. Liras G, Carballo F. An impaired phagocytic function is associated with leucocyte activation in the early stages of severe acute pancreatitis. *Gut*. 1996;39:39-42.
39. Shawcross DL, Wright GAK, Stadlbauer V, Hodges SJ, Davies NA, Wheeler-Jones C, Pitsillides AA, Jalan R. Ammonia impairs neutrophil phagocytic function in liver disease. *Hepatology*. 2008;48:1202-12.
40. Taylor NJ, Nishtala A, Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, Wendon JA, Shawcross DL. Circulating neutrophil dysfunction in acute liver failure. *Hepatology*. 2013;57(3):1142-52.
41. Shalekoff S, Tiemessen CT, Gray CM, Martin DJ. Depressed phagocytosis and oxidative burst in polymorphonuclear leukocytes from individuals with pulmonary tuberculosis with or without human

- immunodeficiency virus Type 1 infection. *Clin Vaccine Immunol* 1998;5:41-4.
42. Nicholson GC, Tennant RC, Carpenter DC, Sarau HM, Kon OM, Barnes PJ, Salmon M, Vessey RS, Tal-Singer R, Hansel TT. A novel flow cytometric assay of human whole blood neutrophil and monocyte CD11b levels: Upregulation by chemokines is related to receptor expression, comparison with neutrophil shape change, and effects of a chemokine receptor (CXCR2) antagonist. *Pulmon Pharmacol Ther.* 2007;20(1):52-9.
 43. Phillips D, Rezvani K, Bain BJ. Exercise induced mobilisation of the marginated granulocyte pool in the investigation of ethnic neutropenia. *J Clin Pathol.* 2000;53:481-83.
 44. Alexander SPH, Mathie A, Peters JA. Guide to Receptors and Channels (GRAC) 5th Edition. *Br J Pharmacol.* 2011;164(Suppl 1):S1-S324.
 45. Pillay* J, Ramakers BP, Kamp VK, Loi ALT, Lam SW, Hietbrink F, Leenen LP, Tool AT, Pickkers P, Koenderman L. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia *Journal of Leukocyte Biology.* 2010;88:211-20.
 46. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest.* 2010;120(7):2423-31.
 47. Martin C, Burdon PCE, Bridger G, Gutierrez-Ramos J-C, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity.* 2003;19:583-93.
 48. Thatcher TH, McHugh NA, Egan RW, Chapman RW, Hey JA, Turner CK, Redonnet MR, Seweryniak KE, Sime PJ, Phipps RP. The Role of CXCR2 in Cigarette Smoke-Induced Lung Inflammation. *Am J Physiol Lung Cell Mol Physiol.* 2005;289(2):L322-28.
 49. Cardona AE, Sasse ME, Liu L, Cardona SM, Mizutani M, Savarin C, Hu T, Ransohoff RM. Scavenging roles of chemokine receptors: chemokine receptor deficiency is associated with increased levels of ligand in circulation and tissues. *Blood.* 2008;112:256-63.
 50. Fu H, Bylund J, Karlsson A, Pellmé S, C. D. The mechanism for activation of the neutrophil NADPH-oxidase by the peptides formyl-Met-Leu-Phe and Trp-Lys-Tyr-Met-Val-Met differs from that for interleukin-8. *Immunology.* 2004;112(2):201-10.
 51. Glynn PC, Henney EM, Hall IP. Peripheral blood neutrophils are hyperresponsive to IL-8 and Gro-alpha in cryptogenic fibrosing alveolitis. *Eur Respir J.* 2001;18:522-29.

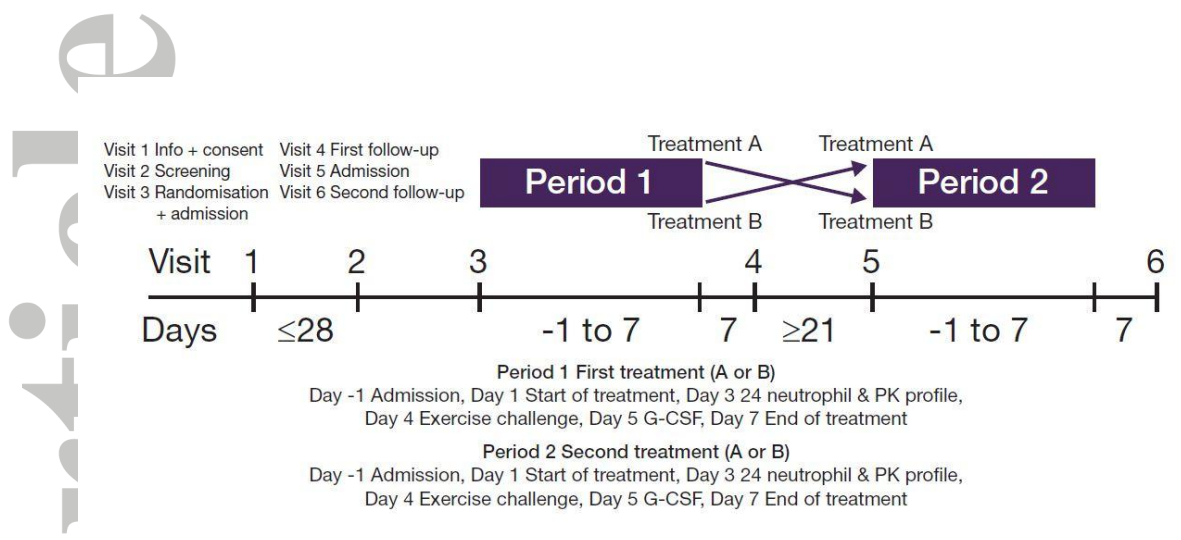


Figure 1. Study plan. Following screening and enrolment, AZD5069 or placebo were administered orally twice daily (*bid*) for 6 d, followed by a washout period of at least 21 d and then a second 6 d treatment period. Subjects were randomised to one of two treatment sequences, AB or BA, where A was 100 mg AZD5069 *bid* and B was matching placebo *bid*. A two-way cross-over design was chosen to counter any inter-individual variation in neutrophil counts in human volunteers.

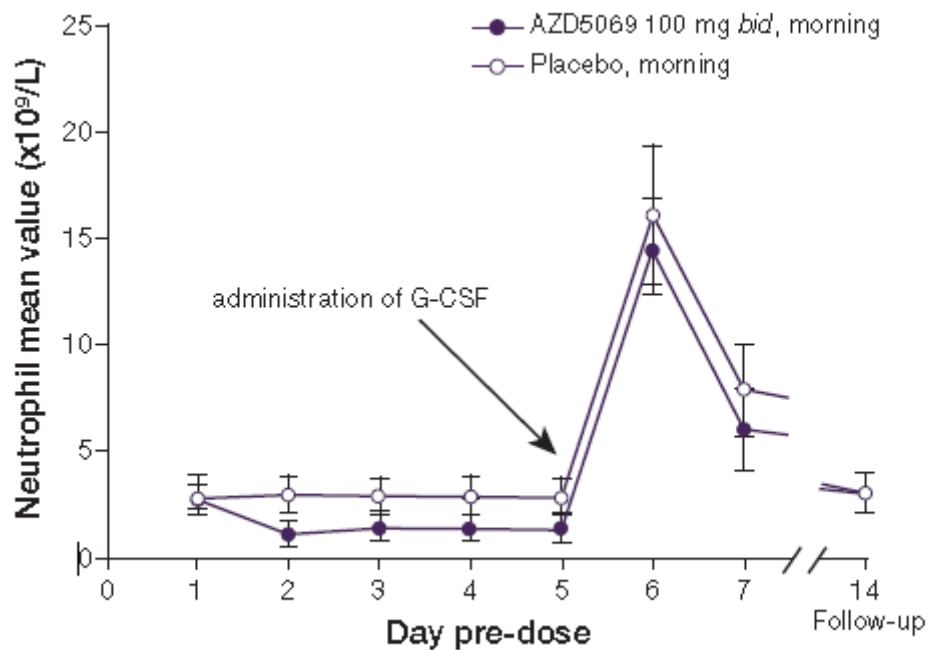
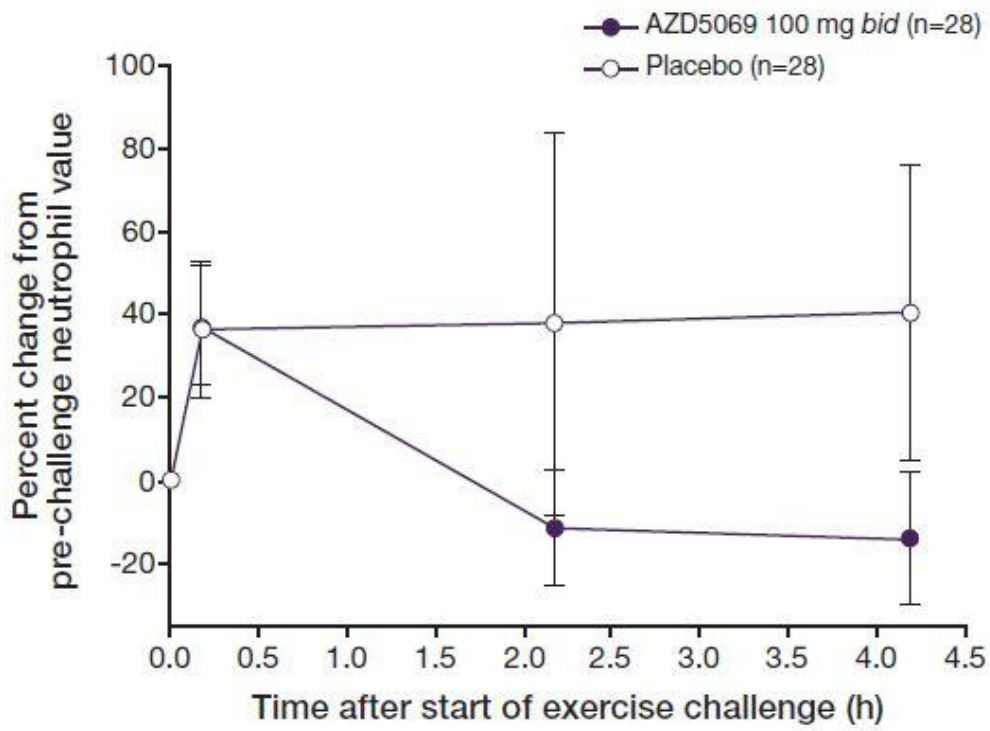


Figure 2. Arithmetic mean (\pm SD) morning pre-dose circulating neutrophil count versus time profiles for subjects receiving AZD5069 and Placebo. The lowest mean pre-dose neutrophil value was observed prior to the Day 2 morning dose ($1.10 \times 10^9/L$)

A



B

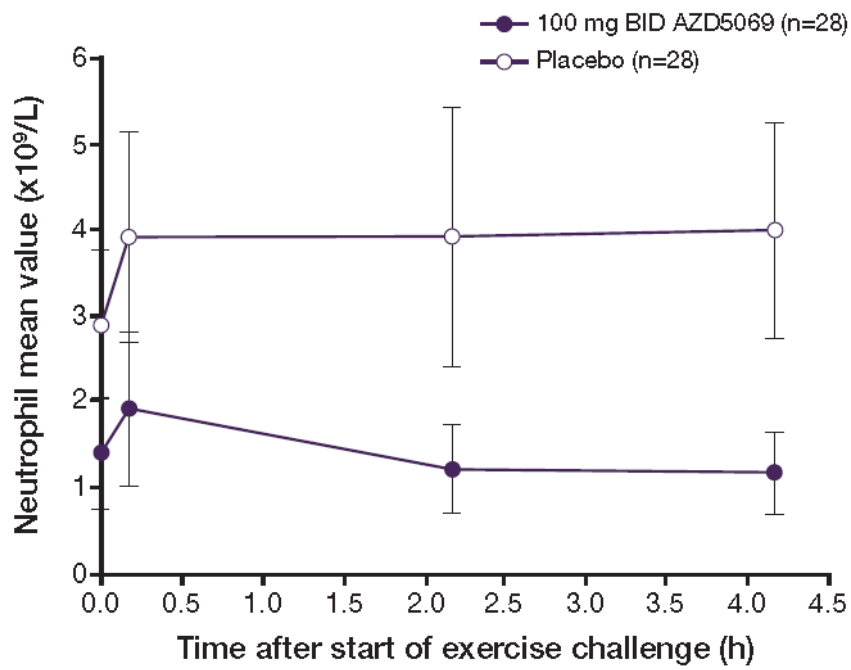
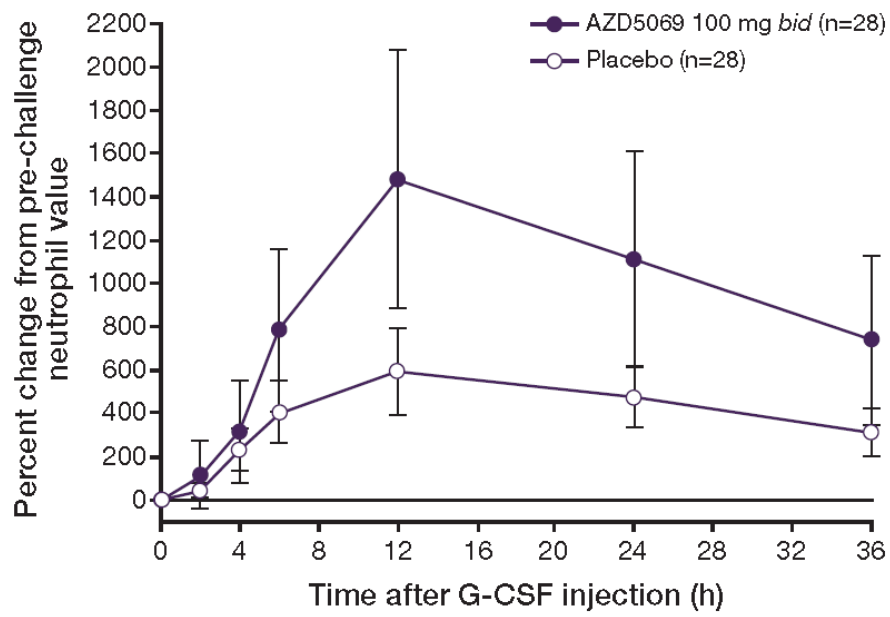


Figure 3. Increases in peripheral blood neutrophil counts (A – percentage change, B – absolute numbers) over time in response to exercise challenge in human volunteers. Subjects were administered either AZD5069 (100 mg *bid*) or placebo orally (*bid*) prior to physical exercise. Peripheral blood samples were collected at the indicated time-points. Each point is presented as mean \pm SD of n = 28 subjects per arm.

Accepted Article

A



B

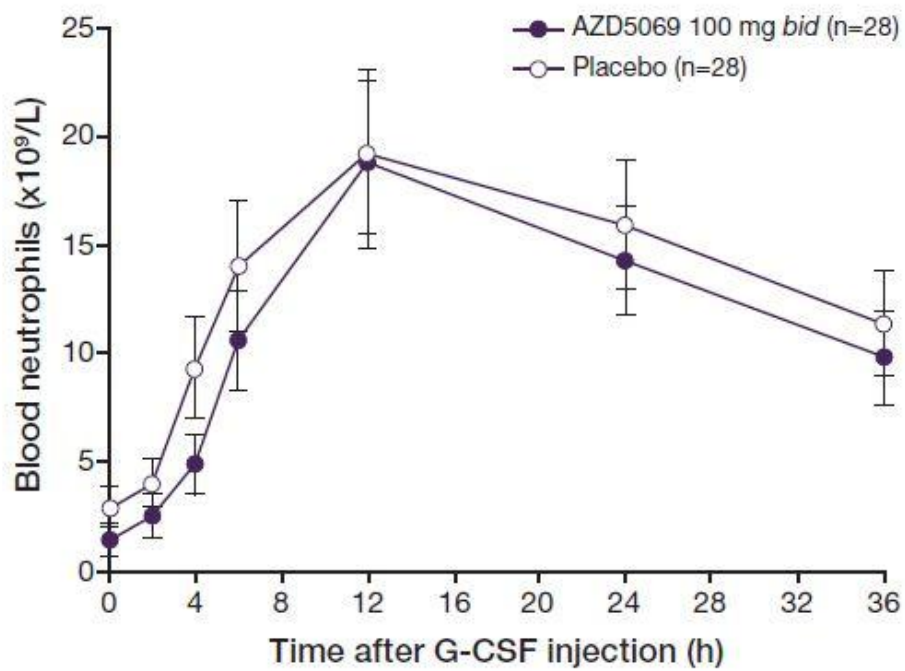


Figure 4. Increases in peripheral blood neutrophil counts (A – percentage change, B – absolute numbers) over time in response to G-CSF stimulation *in vivo*. Subjects were administered either AZD5069 (100 mg *bid*) or placebo orally (*bid*) following stimulation with G-CSF given by subcutaneous injection (300 µg). Peripheral blood samples were collected at the indicated time-points. The data represent mean ± SD of $n = 28$ subjects per arm. Each point is presented as mean ± SD of $n = 28$ subjects per arm.

e

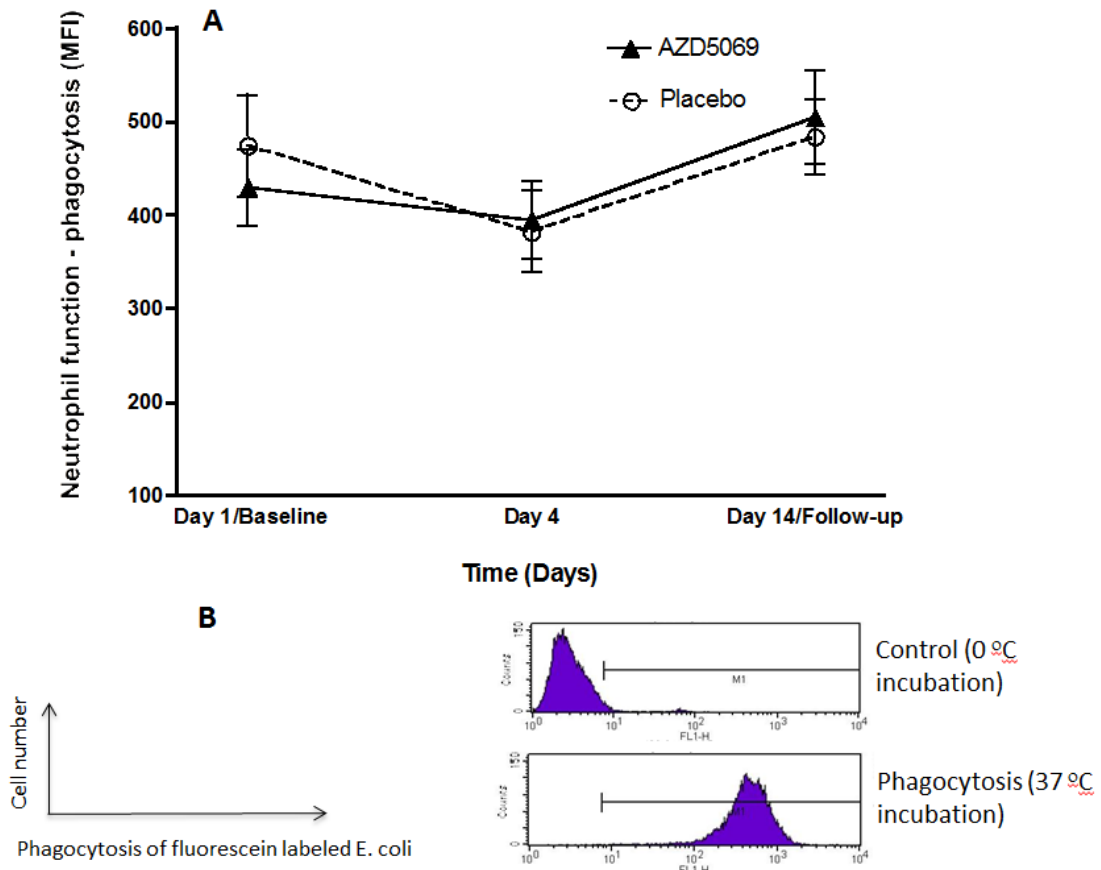


Figure 5. Neutrophil phagocytosis in the presence of AZD5069. Neutrophils were incubated with fluorescein-labeled *E. coli* at 0 °C as a negative control or at 37 °C to stimulate phagocytosis. The number of ingested/phagocytosed fluorescein-*E. coli* bacteria is measured as MFI (mean fluorescence intensity) values. **A.** The data are shown as mean & 95% CI 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). **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.

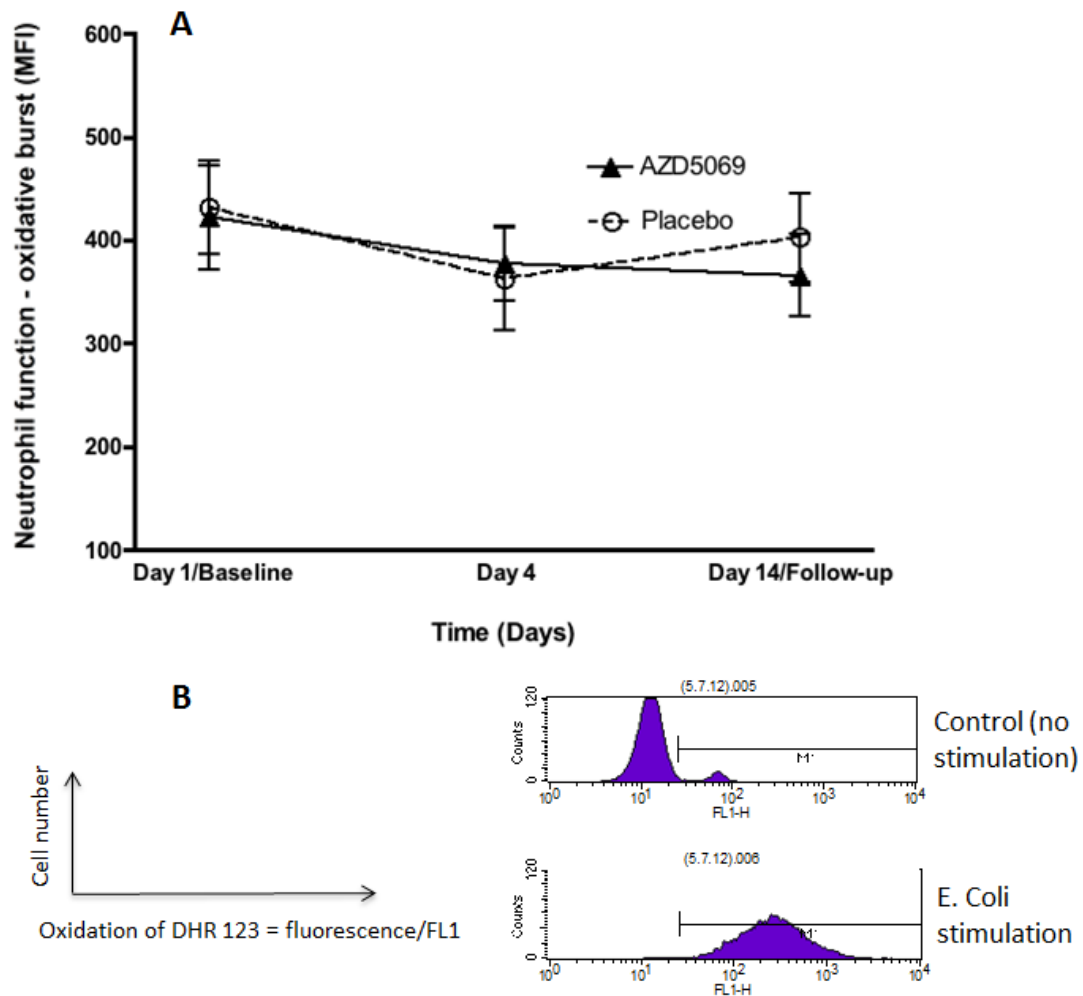


Figure 6. Neutrophil oxidative burst in the presence of AZD5069. Neutrophils were incubated with *E. coli* to stimulate oxidative burst or left unstimulated as a control. The oxidative burst is measured quantitatively by flow cytometric detection of green fluorescence generated due to oxidation of fluorogenic substrate DHR (dihydrorhodamine) 123. **A.** The data are shown as mean & 95% CI of MFI (Mean Fluorescence Intensity) values for all subjects at baseline, in the presence of AZD5069 or placebo (Day 4 of treatment) and at follow up (Day 14). **B.** Representative flow cytometry graphs for control (no stimulation) and oxidative burst in the presence of *E. coli* stimulation are shown.

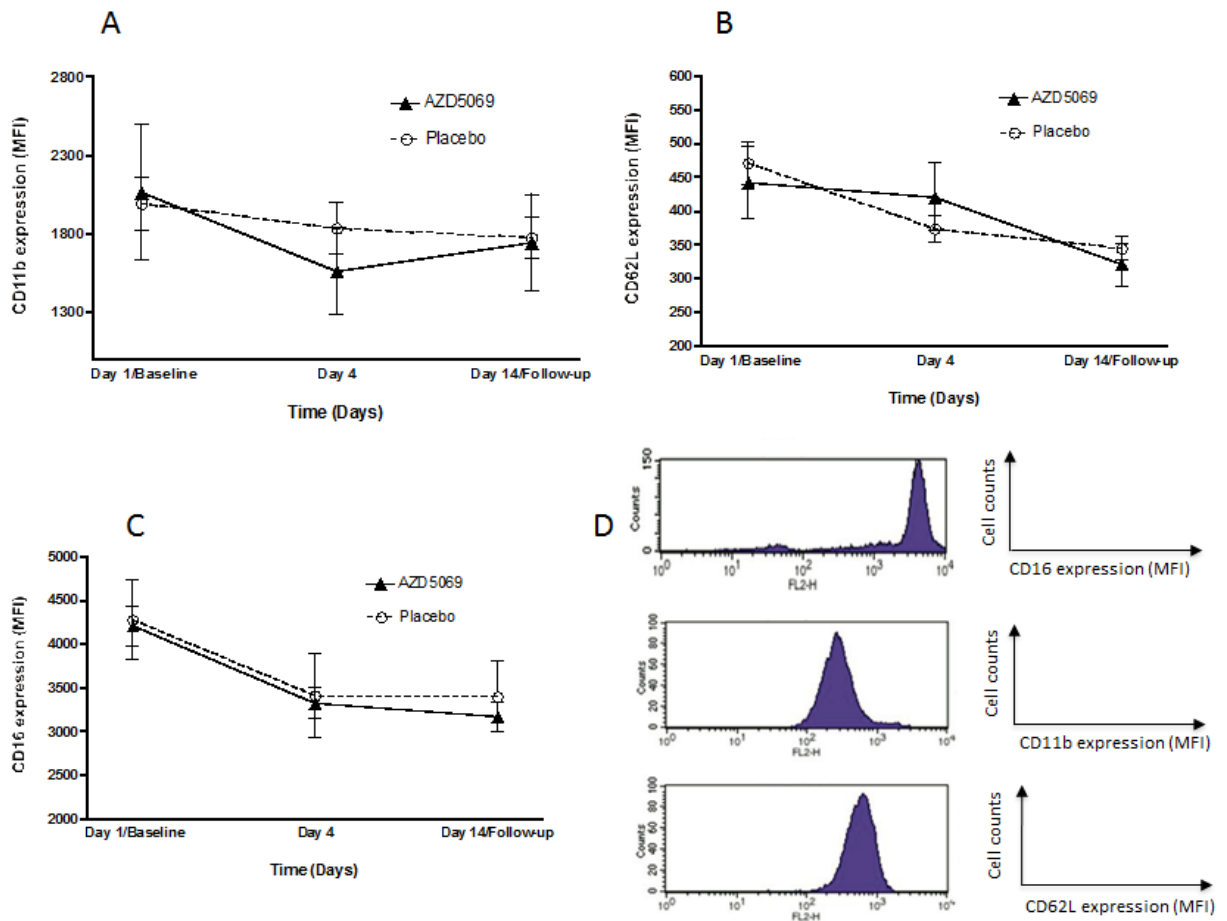


Figure 7. Neutrophil activation markers expression in the presence of AZD5069 or placebo. The cell surface expression of CD16 (A), CD11b (B) and CD62L (C) are presented as Mean Fluorescence Intensity (MFI) Units (mean & 95% CI) at pre-treatment baseline (Day 1), treatment Day 4 and Day 14/Follow-up. 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.

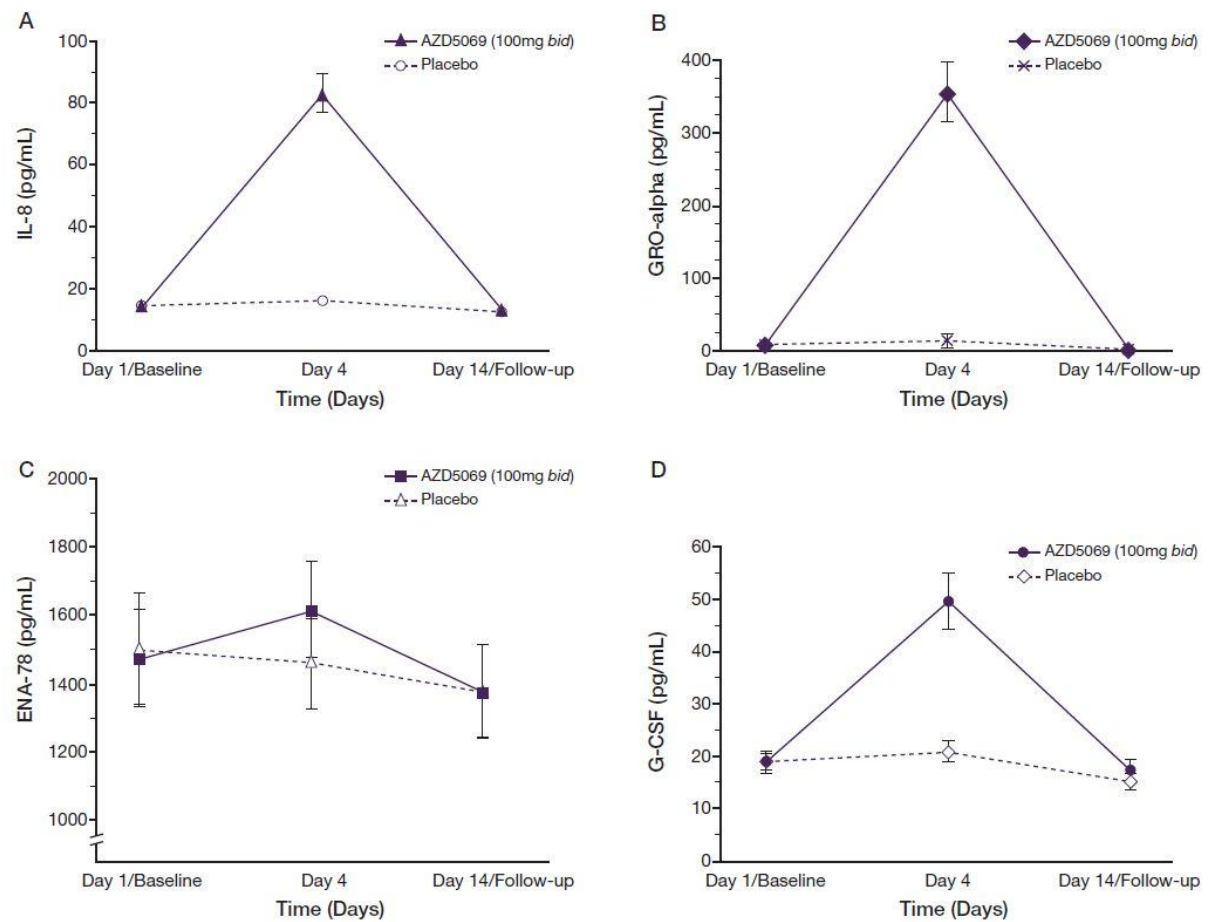


Figure 8. Changes in serum concentrations of the CXCR2 ligands IL-8, GRO α , ENA-78 and G-CSF observed during treatment with AZD5069, compared with placebo, in human volunteers. The baseline, Day 1 pre-treatment concentrations and post-treatment Day14/follow-up concentrations are also shown for each 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 mean \pm SD of $n = 28$ subjects per arm.

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)	<i>p</i> value	Effect (log scale)	<i>p</i> 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).