Effects of IL-6 and IL-6 blockade on neutrophil function in vitro and in vivo

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Abstract

Objectives. Reports on the regulation of neutrophil function by IL-6 are often conflicting. Therapeutic inhibition of IL-6 in RA is associated with occasional neutropenia, but the mechanisms underlying this observation are poorly understood. This study investigated interactions between IL-6, the anti-IL-6 receptor agent tocilizumab (TCZ) and neutrophils in vitro and in vivo.

Methods. Neutrophils were isolated from healthy controls and incubated in vitro with pharmacologically relevant concentrations of IL-6 or TCZ. Neutrophils were also isolated from RA patients, including a cohort following TCZ therapy. Apoptosis was measured by annexin V/propidium iodide (PI) flow cytometry; phagocytosis was measured by incubating apoptotic neutrophils with THP-1-derived macrophages; chemotaxis was measured using cell migration through hanging-cell inserts towards IL-8 and cell surface proteins, including adhesion molecules CD11b (αMβ2 integrin) and CD62L (L-selectin) were measured by flow cytometry.

Results. IL-6 (10–100 ng/ml) did not affect the rate of neutrophil apoptosis, priming of the respiratory burst or adhesion molecule expression nor act as a neutrophil chemoattractant. However, IL-6 enhanced signal transducer and activator of transcription 3 (STAT3) activation and neutrophil migration towards IL-8. TCZ in vitro did not induce apoptosis or phagocytosis of neutrophils, nor did it have a significant effect upon apoptosis or cell surface molecule expression. Neutrophil functions in ex vivo neutrophils from RA patients receiving TCZ treatment were unaffected.

Conclusion. Therapeutic blockade of IL-6, while inducing a transient neutropenia, does not directly affect neutrophil functions associated with host defence. TCZ-associated neutropenia cannot be explained by direct induction of apoptosis by TCZ, induction of apoptosis following depletion of IL-6, nor increased phagocytosis of neutrophils.

Key words: neutrophil, neutropenia, tocilizumab, IL-6, rheumatoid arthritis.

Introduction

IL-6 is a cytokine implicated in many aspects of inflammation associated with RA, including the acute phase response, B cell differentiation, antibody production, T cell proliferation, endothelial cell activation, receptor factor of nuclear factor κB ligand (RANKL) expression and osteoclast activation [1, 2]. Many immune cells do not constitutively express IL-6 receptor (IL-6R) but can bind and respond to IL-6 via trans-signalling, which occurs when soluble IL-6 receptor (sIL-6R) forms a dimer with the ubiquitously expressed gp130 co-receptor to initiate IL-6 signalling [3]. Neutrophils are one of only a few cell types to express sIL-6R, and in RA they may secrete sIL-6R (in response to chemotactic factors such as IL-8) to initiate IL-6 trans-signalling in cells that do not constitutively express this receptor [4, 5]. The direct effect of IL-6 on neutrophil functions remains poorly understood, with conflicting evidence in the literature reporting that IL-6 can either delay, accelerate or have no effect on neutrophil apoptosis [6–9].
The important role of IL-6 in RA pathology is confirmed by the efficacy of IL-6 blockade on disease progression. One such drug, tocilizumab (TCZ) is a humanised anti-IL-6R monoclonal antibody that has shown efficacy in clinical trials [10–14] and is approved for RA treatment. However, a significantly higher than expected proportion of RA patients experience transient neutropenia (compared with MTX alone) in clinical trials with TCZ [10–14]. This neutropenia is rapid (1–2 days) but recovers several days post-infusion, returning to normal levels by 4–6 weeks [15]. The causes of this neutropenia, and the consequences of TCZ-induced neutropenia on host defence, are unknown.

One explanation for this neutropenia is that IL-6 blockade, directly or indirectly, may affect neutrophil apoptosis and removal from the circulation. Neutrophils express IL-6R, which could directly bind IL-6 blockers such as TCZ, to act as an opsonin and promote neutrophil phagocytosis by FcγR-expressing phagocytic cells. Alternatively, TCZ bound to cell surface neutrophil IL-6R could trigger intracellular signalling pathways, leading to apoptosis. If neutrophils are dependent upon IL-6 for cell survival, then serum depletion by IL-6 blockers may increase their apoptosis in vivo, leading to decreased circulating levels. However, alternative explanations to explain TCZ-induced neutropenia are possible, e.g. by induction of margination. IL-6 administration in animals induces neutrophilia, via rapid mobilisation of neutrophils from the marginated pool into the circulating pool, followed by accelerated release of neutrophils from the bone marrow [16–18]. Serum levels of IL-6 are elevated in RA patients [19], therefore decreases in circulating IL-6 following TCZ infusion may induce margination of neutrophils.

The aims of this research were (i) to determine the effects of IL-6, at concentrations relevant to disease, on neutrophils from both healthy controls and RA patients in order to determine if inflammatory disease alters their responsiveness to IL-6 and (ii) to investigate the effects of IL-6 blockade on healthy and RA neutrophils in vitro and in vivo to determine if TCZ has any adverse effects on neutrophil function that, apart from the transient neutropenia, could impair host defence.

**Patients and methods**

**Patients and controls**

Venous blood was collected into lithium-heparin vacutainers from healthy controls and RA patients. Participants gave written informed consent according to the Declaration of Helsinki. Ethics approval was obtained from the University of Liverpool Committee on Research Ethics and Sefton Adults Research Ethics Committee. Two cohorts of RA patients were examined. The first (patient group 1; see supplementary Table S1, available at Rheumatology Online) were RA patients attending blood-monitoring clinics and receiving combination DMARD therapy. The second (patient group 2) were part of a 52-week, single-centre, open-label study to evaluate the effects of TCZ on neutrophil function in patients with active RA. These patients had an inadequate response to non-biologic DMARD and/or anti-TNF therapy. The clinical characteristics of these patients are shown in supplementary Table S2, available at Rheumatology Online. Patients who failed to respond to anti-TNF therapy (either as monotherapy or with DMARDs) were administered TCZ after five half-life washouts of the anti-TNF. Patients received an i.v. infusion of 8 mg/kg TCZ every 4 weeks, for up to a total of 13 infusions (to week 48), with the final visit at 52 weeks. All patients in group 2 received concurrent MTX throughout the clinical trial period. Supplementary Table S3, available at Rheumatology Online, describes the visit/dosing schedule and the clinical and laboratory sampling regimens. Demographics for the healthy controls are shown in supplementary Table S4, available at Rheumatology Online.

**Isolation of neutrophils**

Neutrophils (purity >97%, viability >98%) were isolated using Polymorphprep (Axis-Shield, Oslo, Norway) and contaminating erythrocytes were removed by hypotonic lysis as described previously [20]. Freshly isolated neutrophils were suspended at 10⁶ or 5 x 10⁵ cells/ml in Roswell Park Memorial Institute (RPMI) media [containing 10% human AB serum (Sigma, Gillingham, UK)] and incubated at 37°C in 5% CO₂ for up to 20 h. Cytokines were added at 0 h: IL-6 (0.1–100 ng/ml; Sigma), IL-8 (100 ng/ml; Sigma), GM-CSF (5 ng/ml; Roche, Welwyn Garden City, UK) and TNF–α (10 ng/ml; Calbiochem, Nottingham, UK).

**In vitro incubation with TCZ**

Neutrophils and THP-1 monocytes were incubated at 10⁶ cells/ml for up to 20 h with physiologically relevant concentrations of TCZ (10–200 µg/ml; Roche) [21] or IgG isotype control antibody (10–200 µg/ml; Sigma).

**Measurement of apoptosis**

Neutrophils (2.5 x 10⁶) were diluted in 50 µl of Hanks’ balanced salt solution (HBSS; Life Technologies, Paisley, UK) containing 0.5 µl of annexin V-fluorescein isothiocyanate (FITC) (Life Technologies) and incubated in the dark at room temperature for 15 min. The total volume was then made up to 250 µl with HBSS containing propidium iodide (PI; 1 µg/ml) before analysis by flow cytometry (>5000 events analysed).

**Antibody staining and flow cytometry**

Neutrophils (5 x 10⁶) were resuspended in PBS (plus 0.2% BSA) and antibody binding was carried out at 4°C in the dark for 30 min with conjugated antibodies as follows: CD62L-FITC, CD16-PE, CD18-PE, CD64-FITC, IL-6R-FITC, mTNF–α-FITC (all R&D Systems, Abingdon, UK), CD11b-PE (Miltenyi Biotec, Bisley, UK), CD63-APC (BioLegend, London, UK), isotype controls [Santa Cruz Biotechnology, (Insight Biotechnology), Wembley, UK]. Cells were fixed with 2% paraformaldehyde and fluorescence was measured by flow cytometry (>5000 events analysed).
Neutrophil function in vitro and in vivo

Neutrophils were rapidly lysed in boiling Laemml buffer containing phosphatase inhibitors (Calbiochem). Proteins were separated by SDS-PAGE on a 12% gel and transferred onto polyvinyldene fluoride (PVDF) membrane (Millipore, Watford, UK). Primary antibodies were Mcl-1, caspase-8, caspase-9, caspase-3, phospho-STAT-3 (Tyr705) (all 1:1000; Cell Signalling Technology, NPB, Hitchin, UK), total-STAT-3 (1:2000; Cell Signaling) and actin (1:10 000; Abcam, Cambridge, UK). Secondary antibodies were anti-rabbit IgG (GE Healthcare, Little Chalfont, UK) and anti-mouse IgG (Sigma) horseradish peroxidase (HRP)-linked antibodies (1:10 000). Bound antibodies were detected using the ECL system (Millipore) on carefully exposed film (Amersham, Amersham, UK).

Chemotaxis assay
The chemotaxis assay was carried out in poly-hema-coated tissue culture plates using hanging cell inserts (Millipore) with a 3 µm porous membrane. Chemotactic agents were added to RPMI media in the bottom chamber as follows: formyl-methionyl-leucyl-phenylalanine (fMLP, 10⁻⁸ M; Sigma), IL-8 (100 ng/ml; Sigma) and IL-6 (10–100 ng/ml; Sigma). Neutrophils (10⁵) were added to the top chamber and incubated for 90 min at 37°C and 5% CO₂. The number of migrated neutrophils (in the bottom chamber) after 90 min incubation was measured using a Coulter Counter Multisizer 3 (Beckman Coulter, Hitchin, UK).

Measurement of the respiratory burst
Neutrophils (5 × 10⁶/ml) were incubated for 30 min with IL-6 (1, 10 or 100 ng/ml) or GM-CSF (5 ng/ml) or for 15 min with IL-8 (100 ng/ml) in the absence or presence of IL-6 (10 ng/ml) or TNF-α (10 ng/ml). Cells (2 × 10⁵) were diluted in HBSS containing luminol (10 μM) and the respiratory burst was stimulated with fMLP (1 μM) or phorbol 12-myristate 13-acetate (PMA, 100 ng/ml). Freshly isolated neutrophils (2 × 10⁵) from patients undergoing TCZ therapy (patient group 2) were also analysed for both endogenous-, fMLP- and PMA-stimulated respiratory burst activity. Luminescence was measured continuously, in duplicate, for 30 min.

Phagocytosis assays using apoptotic neutrophils and THP-1-derived macrophages
THP-1 monocytes were maintained in culture in RPMI media [containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% non-essential amino acids (NEAA)] at 37°C in 5% CO₂. Differentiation into macrophages was achieved by addition of PMA (80 nM) to 4 × 10⁵ cells/ml. Media were changed after 48 h and the cells incubated for a further 24 h. Apoptotic neutrophils (10⁵) were washed twice in THP-1 media and incubated with THP-1-derived macrophages for 2 h. After this, medium containing non-phagocytosed neutrophils was aspirated and the cells washed twice with PBS. Cells were fixed for 20 min using 2.5% glutaraldehyde (Sigma) in PBS and washed with PBS before staining MPO-positive neutrophils for 1 h using o-dianisidine dihydrochloride (0.1 mg/ml; Sigma) and 0.03% hydrogen peroxide in PBS [22]. Cells were washed with PBS and the level of phagocytosis was determined by light microscopy, counting the number of phagocytosed neutrophils per THP-1 macrophage in three fields per well (typically 100 THP-1-derived macrophages per field).

Phagocytosis and respiratory burst activity of neutrophils following TCZ therapy
Staphylococcus aureus were heat-killed at 60°C for 30 min, washed twice, incubated in the dark at 4°C for 2 h in PBS containing 30 μM Pl, washed three times in HBSS containing 0.1% gelatin, opsonized by incubation (5 × 10⁸ bacteria/ml) with 10% heat-inactivated human serum for 30 min at 37°C and washed three times in PBS. Freshly isolated neutrophils (10⁵/ml) from patients undergoing TCZ therapy (patient group 2) were incubated for 30 min at 37°C with PI-labelled, opsonised heat-killed S. aureus (SAPI), at a ratio of 1:10. After incubation the neutrophils were washed twice and suspended in PBS containing 5 mM EDTA, 3 mM sodium azide and 1% paraformaldehyde. Cells were analysed immediately by flow cytometry. Reactive oxygen species (ROS) production was measured by adding dihydrorhodamine-123 (2 μM) to neutrophils (10⁵/ml) that were incubated in the dark for 15 min at 37°C with gentle agitation before analysis by flow cytometry.

Statistical analysis
Statistical analysis was carried out using SPSS version 20 (IBM, Armonk, NY, USA), using the Student’s t-test unless otherwise stated.

Results
Effects of IL-6 and IL-6 blockade on neutrophil apoptosis or phagocytosis
To investigate the effects of IL-6 and IL-6 blockade on neutrophil apoptosis, we incubated neutrophils from healthy controls and RA patients (patient group 1) with pathophysiological concentrations of IL-6 (0.1–100 ng/ml) [19] and TCZ (10–200 μg/ml) [21] (data not shown). TCZ did not significantly affect the constitutive rate of apoptosis of neutrophils isolated from the blood of either healthy controls or RA patients (Fig. 1A). We then investigated whether TCZ enhanced the phagocytosis of apoptotic neutrophils by macrophages. Following 20 h of incubation with TCZ (or isotype control antibody), apoptotic neutrophils from healthy controls and RA patients were incubated with THP-1-derived macrophages for 2 h and the percentage of phagocytosed neutrophils was determined by light microscopy. TCZ did not significantly increase the rate of phagocytosis of apoptotic neutrophils from either healthy controls or RA patients (Fig. 1A).
IL-6 does not affect the rate of neutrophil apoptosis

There is considerable controversy in the literature regarding the effects of IL-6 on neutrophil apoptosis [6–9]. Neutrophils from healthy controls were incubated with IL-6 over the concentration range of 0.1–100 ng/ml (data not shown), levels measurable in the serum of patients with inflammatory conditions [19]. The rate of apoptosis of IL-6-treated cells at 20 h was not significantly different from untreated controls (Fig. 1B), whereas GM-CSF protected against cell death. We also determined the effects of IL-6 on apoptosis of RA neutrophils, in the event that RA neutrophils exhibited altered responsiveness to this cytokine, but again, IL-6 had no effect on apoptosis (Fig. 1B). The levels of expression of proteins regulating apoptosis (anti-apoptotic: Mcl-1; pro-apoptotic: caspase-3, -8 and -9) were unchanged in healthy controls (data not shown) or RA neutrophils following incubation with IL-6 (10 or 100 ng/ml) for 4 and 20 h (Fig. 1C). Previous work [6] has suggested that the effects of IL-6 on neutrophil apoptosis may be dependent on cell concentration, so we repeated experiments on neutrophil suspensions of $10^6$ and $5 \times 10^6$ cells/ml (it was not possible to use neutrophils at concentrations $>5 \times 10^6$ cells/ml because of excessive cell clumping). Rates of constitutive apoptosis in RA neutrophils were greater at the higher cell concentration (Fig. 1D), but IL-6 was again without effect.

Fig. 1 Effect of IL-6 and IL-6 blockade on neutrophil apoptosis in vitro

(A) TCZ did not significantly enhance the rate of apoptosis or phagocytosis in healthy ($n = 5$, closed bar) or RA ($n = 6$, open bar) neutrophils. (B) IL-6 did not alter the rate of apoptosis in healthy ($n = 4$, closed bar) or RA ($n = 10$, open bar) neutrophils (GM-CSF, positive control; **$P < 0.01$). (C) The level of Mcl-1 and caspase-3, -8 and -9 was not affected by IL-6 (GM-CSF, positive control). (D) Apoptosis was significantly higher in RA neutrophils at cell concentrations of $5 \times 10^6$/ml (open bar) compared with $10^6$/ml (closed bar) ($n = 4$; *$P < 0.05$), but was unaffected by IL-6. TCZ: tocilizumab; UT: untreated.
These data clearly indicate that IL-6 exerts no effect on neutrophil apoptosis under these conditions and RA neutrophils do not have altered responsiveness to this cytokine. Therefore it is doubtful that IL-6 can delay neutrophil apoptosis in vivo in RA, so increased neutrophil apoptosis following IL-6 depletion (by TCZ) is unlikely to account for the neutropenia observed in RA patients.

**IL-6 does not prime the neutrophil respiratory burst, but activates STAT-3**

We next investigated the ability of IL-6 to prime the respiratory burst, a key component of host defence and bacterial killing. Neutrophils from healthy controls and RA patients (patient group 1) were treated for 15–120 min with IL-6 (1, 10 or 100 ng/ml) or with GM-CSF (5 ng/ml) as a positive control. IL-6 did not prime neutrophils for a respiratory burst stimulated with either fMLP (receptor mediated) (Fig. 2A) or PMA (receptor independent) (Fig. 2B) over the entire period of 120 min. Data shown represent 30 min priming incubation. In order to determine whether intracellular signalling pathways in neutrophils were activated in response to IL-6, we stimulated healthy control neutrophils with IL-6 (10 ng/ml) for 10–60 min and measured activation (by phosphorylation) of STAT-3. Rapid STAT-3 phosphorylation was detected by western blotting of protein lysates from neutrophils and THP-1 cells (as a positive control) (Fig. 2C and D). Pre-incubation of neutrophils and THP-1 cells with TCZ (100 ng/ml) for 30 min prior to addition of IL-6 prevented phosphorylation of STAT-3. These experiments confirm that neutrophils are responsive to IL-6, but this IL-6 signalling does not affect the rates of apoptosis nor prime the respiratory burst. They also confirm that TCZ blocks the IL-6 R in vitro.

**Effects of IL-6 on neutrophil chemotaxis and adhesion molecule expression**

We next investigated the effects of IL-6 on the expression of neutrophil adhesion molecules and chemotaxis, as changes in adhesive properties in response to IL-6 blockade may increase the numbers of margined neutrophils, thereby decreasing the number of circulating neutrophils. Neutrophils from healthy controls and RA patients (patient group 1) were allowed to migrate towards IL-6 (10 or 100 ng/ml) for 90 min. In contrast to the effects of fMLP (10⁻⁸ M) and IL-8 (100 ng/ml), IL-6 did not enhance neutrophil chemotaxis compared with random migration (Fig. 3A). Next, neutrophils from healthy controls and RA patients (patient group 1) were pre-incubated with IL-6 (10 or 100 ng/ml) for 1 h, then allowed to migrate towards IL-8 (100 ng/ml) for 90 min. The rate of chemotaxis towards IL-8 in neutrophils that were pre-incubated in media alone for 1 h was lower than that observed in freshly isolated neutrophils. However, pre-incubation with IL-6 (10 or 100 ng/ml) significantly enhanced chemotaxis towards IL-8 in healthy control neutrophils (Fig. 3B; P < 0.05). This enhanced effect was also observed in RA neutrophils, but did not reach statistical significance due to higher constitutive levels of migration towards IL-8.

Finally, we investigated the effects of IL-6 on neutrophil adhesion molecule expression. Neutrophils from healthy controls and RA patients (patient group 1) were treated for 1 h with IL-6 (10 or 100 ng/ml) or GM-CSF (5 ng/ml) as a positive control. Surface molecule expression was measured by flow cytometry. Unlike the effects observed after GM-CSF treatment, IL-6 did not induce up-regulation of \( \alpha_M \beta_2 \) integrin (CD11b) or shedding of L-selectin (CD62L) adhesion molecules (Fig. 3C; P < 0.01). Similarly there were no effects of IL-6 on surface expression of FcγRIIb (CD16), FcγRIIa (CD32), IL-6R (CD126) and β2 integrin (CD18) (data not shown). Together, these data show that IL-6 is not a neutrophil chemoattractant, but can enhance IL-8-induced chemotaxis, although this effect does not appear to be mediated by changes in adhesive properties of neutrophils in response to IL-6 alone.

**Effects of IL-6 and IL-8 in combination on neutrophil function**

The above experiments indicated that IL-6 used alone did not affect any of the neutrophil functions that were tested (although it did induce STAT3 activation), but acted synergistically with IL-8 to enhance chemotaxis. Therefore cells from healthy controls and RA patients (patient group 1) were incubated for 20 h with IL-6 (10 ng/ml) or IL-8 alone and the effects on apoptosis and priming of the respiratory burst were measured. IL-8 significantly delayed the rate of neutrophil apoptosis (Fig. 4A; P < 0.01) in both control and RA neutrophils. The rate of apoptosis in neutrophils incubated with both IL-6 and IL-8 was significantly different from untreated cells (P < 0.01). However, the rate of apoptosis was not significantly different from IL-8 treated cells, suggesting that IL-8 alone mediates this effect. A possible synergistic effect of IL-6 and IL-8 on the respiratory burst was also investigated. Incubation of healthy control (Fig. 4B) or RA (data not shown) neutrophils with IL-8 (100 ng/ml) for 15 min primed the respiratory burst upon stimulation with fMLP or PMA, but there was no additional response when IL-6 (10 ng/ml) was added to IL-8 during priming.

**Effects of therapeutic IL-6 blockade on RA neutrophil function ex vivo**

A cohort of 20 RA patients (patient group 2) who were previously non-responsive to DMARD or anti-TNF therapy were recruited to a clinical trial to determine the effects of TCZ on neutrophil function. Blood neutrophils were isolated at baseline and at 4 and 12 weeks post-therapy. A number of clinical parameters, including blood neutrophil counts and clinical markers of disease activity, were also recorded (see supplementary Table S2, available at Rheumatology Online). Neutrophil functions were then measured in ex vivo neutrophils.

**Disease activity and neutrophil count**

TCZ therapy resulted in significant improvement in the 28-joint DAS (DAS28) from baseline (week 0). DAS28 scores decreased significantly between visits (baseline–week 4,
Neutrophils from healthy controls (n = 5) and RA patients (n = 6, data not shown) were incubated for 30 min in media or with IL-6 (1, 10 or 100 ng/ml) or GM-CSF (5 ng/ml). IL-6 was not found to prime the respiratory burst when stimulated with (A) fMLP (receptor mediated) or (B) PMA (receptor independent) (ROS production following GM-CSF priming; *P < 0.05, **P < 0.01). (C) IL-6 (10 ng/ml) induced rapid phosphorylation of STAT3 in healthy control neutrophils (n = 3) and THP-1 cells (n = 3), which was inhibited by pre-incubation with TCZ (100 μg/ml) for 30 min prior to addition of IL-6. (D) Densitometry analysis of phosphorylated STAT3 protein, normalized to total STAT3. STAT: signal transducer and activator of transcription; fMLP: formyl-methionyl-leucyl-phenylalanine; PMA: phorbol 12-myristate 13-acetate; ROS: reactive oxygen species; TCZ: tocilizumab; UT: untreated.

P = 0.002; week 4–week 12, P < 0.001), indicating an improvement in disease (see supplementary Table S3, available at Rheumatology Online, and Fig. 5A). Over this period, neutrophil counts decreased from baseline to week 4 and recovered by week 12, but overall the mean neutrophil counts for the entire cohort showed no significant changes post-therapy. During the course of therapy, four patients developed a transient neutropenia, but this did not require any intervention and neutrophil counts returned to baseline levels, on average, by 8 weeks after neutropenia was identified.

**Apoptosis**

Freshly isolated neutrophils were either analysed immediately (0 h) or incubated for 4 h and 20 h in the presence or absence of GM-CSF (5 ng/ml). There was no significant
**Fig. 3** Effect of IL-6 on neutrophil chemotaxis and adhesion

**A** IL-6 did not enhance neutrophil chemotaxis compared with random migration in healthy control ($n=4$, closed bar) and RA ($n=6$, open bar) neutrophils (fMLP, IL-8, positive controls; **$P<0.01$**).

**B** IL-6 significantly enhanced chemotaxis towards IL-8 in healthy control neutrophils ($n=4$, closed bar; *$P<0.05$). This effect was not statistically significant in the entire population of RA neutrophils ($n=6$, open bar; $P>0.05$).

**C** IL-6 did not induce shedding of CD62L (L-selectin) or up-regulation of CD11b adhesion molecules, which was observed with GM-CSF (**$P<0.01$** in both healthy control ($n=4$) or RA ($n=6$) neutrophils. fMLP: formyl-methionyl-leucyl-phenylalanine; UT: untreated.
difference in the levels of neutrophil apoptosis following TCZ therapy (Fig. 5B) measured by flow cytometry.

Respiratory burst activity
Freshly isolated neutrophils were stimulated with fMLP or PMA to measure receptor-dependent and independent respiratory burst activity, respectively. There was no significant difference in chemiluminescence response following therapy (Fig. 5C). There was a trend towards increased fMLP-stimulated oxidase activity, perhaps indicative of in vivo priming, by 12 weeks post-therapy, but this did not reach statistical significance.
Phagocytosis and intraphagosomal oxidase activity
We next investigated if TCZ treatment altered neutrophil phagocytic ability. Intracellular reactive oxidant production was measured by incubating neutrophils with dihydrorhodamine-123 (2 μM) and phagocytosis was determined after incubation with SAPI. Both the respiratory burst and phagocytosis were unaltered after TCZ therapy (Fig. 5D), suggesting that therapeutic IL-6 blockade does not impair host defence mechanisms.

Neutrophil surface molecule expression
Immediately after isolation, the expression levels of neutrophil cell surface markers were measured by flow cytometry. Expression levels of CD11b, CD18, CD16, L-selectin (CD62L) (Fig. 6), CD32, CD64, CD63, IL-6R and membrane-bound TNF (data not shown) were unaltered during the time course of TCZ blockade. There was a trend towards decreased surface expression of CD11b and increased expression of CD16, but these small changes in expression levels did not reach statistical significance.

Discussion
Our study provides new insights into the role of IL-6 on the function of human neutrophils and shows that therapeutic blockade of IL-6, while inducing a transient neutropenia, does not affect neutrophil functions associated with effective protection against bacterial infections. Our data indicate that, in contrast to some reports in the literature [6-9], IL-6 (at pathophysiological concentrations) does not affect neutrophil apoptosis, nor does TCZ enhance the rate of clearance of apoptotic neutrophils by macrophages. Therefore the transient neutropenia observed in RA patients following IL-6 blockade is unlikely to be a direct effect of the drug on the rate of neutrophil apoptosis.

Neutropenia has been reported in a proportion of RA patients receiving anti-TNF therapy [23]. While developing neutropenia appears to be a greater risk to patients receiving TCZ therapy, the number of serious infections in these patients does not appear to be increased, suggesting that while peripheral blood neutrophil numbers are lower, the normal function of the remaining neutrophils is not impaired. Our study confirms that neutrophil functions associated with effective host protection against bacterial infections are unaffected by therapeutic IL-6 blockade.

Our data strongly suggest that TCZ-induced neutropenia is not caused by an increase in neutrophil apoptosis, nor by increased phagocytosis of neutrophils.
Given this, there must be an alternative explanation for the decrease in circulating neutrophil numbers following administration of the drug. One explanation is that TCZ may influence the distribution of neutrophils between circulating and marginated pools. IL-6 has previously been shown to induce rapid (2-6 h) mobilisation of neutrophils from the marginated to the circulating pools in animal models [16, 24, 25]. It is therefore possible that IL-6 blockade could enhance margination and thereby result in a decrease in the circulating pool of neutrophils. The decrease in L-selectin levels on circulating neutrophils in animal models exposed to exogenous IL-6 would appear to support this hypothesis. However, we did not observe IL-6-induced L-selectin shedding in our in vitro study, an observation that has also been made by others in humans and monkeys [25]. IL-6 has been shown to induce shedding of P-selectin glycoprotein ligand-1 (CD162) in monkeys in vivo, which was associated with an increase in neutrophil counts [25]. However, CD162 shedding was not observed in purified human neutrophils, but was seen in human whole blood in vitro, but we did not measure this in our study.

While most of our experiments failed to detect a direct effect of IL-6 on neutrophil function (i.e. it did not delay apoptosis, prime the respiration burst, trigger chemotaxis or affect expression of adhesion molecules), two surprising results were obtained. First, IL-6 triggered the rapid and transient activation of STAT3, indicating that human neutrophils detect and respond to this cytokine. Second, we showed that IL-6-treated neutrophils displayed enhanced chemotaxis to IL-8, even though this treatment did not have any enhancing effect on other measures of neutrophil function tested. This effect on chemotaxis may suggest that in the presence of IL-6 there may be a greater egress of neutrophils into inflamed tissues. It is not possible to predict what effects this IL-6/IL-8 synergy has on the marginated pool.

An additional explanation for the neutropenia observed with IL-6 inhibition in RA may be an increase in transit time through the bone marrow. IL-6 has been shown to accelerate the release of neutrophils from the bone marrow in animal models [16, 17], with the IL-6/IL-6 R complex being critical for emergency granulopoiesis responses in mice [18]. Given the requirement for the bone marrow to produce $10^{10}$ neutrophils per day, this might explain the transient nature of the neutropenia with TCZ therapy. Importantly, our data show that while the total neutrophil count may drop after administration of TCZ, the remaining neutrophils are fully functional and show no impairment in their ability to phagocytose bacteria or mount a respiratory burst. The transient nature of the neutropenia also means that neutrophil counts begin to recover within days, thus minimising the risk of serious infection.

In summary, we have shown that IL-6, at concentrations reported in vivo, has no direct effect on a number of neutrophil functions associated with host protection against infections. Further, in vivo therapeutic blockade of IL-6 in RA, while inducing a transient neutropenia, does not affect these important antibacterial neutrophil functions.
Neutrophil function in vitro and in vivo

Rheumatology key messages

- IL-6 has no direct effect on human neutrophil functions associated with host defence.
- Tocilizumab induces transient neutropenia but does not impair antibacterial neutrophil functions.
- Neutropenia is not caused by tocilizumab-induced apoptosis or phagocytosis of apoptotic neutrophils.

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Supplementary data

Supplementary data are available at Rheumatology Online.

References


