An Investigational Analysis Reveals a Potential Role for Neutrophils in Giant-Cell Arteritis Disease Progression

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ABSTRACT

**Rationale:** Giant-cell arteritis (GCA) is a large vessel vasculitis characterized by immune cell infiltration, yet the potential involvement of neutrophils has been rarely studied.

**Objective:** We investigated whether alterations in neutrophil reactivity occurred in the pathogenesis of GCA, or during its clinical management with a canonical glucocorticoid dose regimen over a 6-month period.

**Methods and Results:** Blood samples were taken within 48h of therapy commencement and at week-1, 4 and 24 post-glucocorticoid. Flow-cytometric analysis revealed three distinct neutrophils populations and phenotypes. Within 48hrs of steroid treatment, neutrophils displayed an AnxA1hiCD62LloCD11bhi phenotype, whereas week-1 neutrophils were AnxA1hiCD62LloCD11blo and displayed minimal adhesion to endothelial monolayers under flow, and week 24 (i.e. lowest glucocorticoid dose) neutrophils were AnxA1hiCD62LhiCD11bhi with increased endothelial adhesion under flow. Week 24 plasma analyses showed high levels of CXCL5, IL-8, IL-17 and IL-6. Importantly, comparison of week-1 and 24 samples revealed a suppressive neutrophil effect on T-cell proliferation at the former time point only. Finally, in vitro incubation of naïve neutrophils with concentrations of IL-6 and IL-17 quantified in GCA plasma at week-1 and 24 replicated this differential modulation of lymphocyte proliferation.

**Conclusion:** This translational study highlights a novel clinical manifestations of GCA, with evidence for a neutrophil component and an ‘escaped’ pro-inflammatory phenotype when glucocorticoid therapy is tapered. These results indicate potential involvement of neutrophils in GCA pathogenesis.

**Keywords:** Neutrophils, giant cell arteritis, T cells, suppression.

**Nonstandard Abbreviations and Acronyms:**
- GCA  giant cell arteritis
- OA  osteroarthritis

INTRODUCTION

Giant-cell arteritis (GCA) is a systemic inflammatory disease associated with focal granulomatous panarteritis predominantly involving extracranial branches of the aorta. The most feared complication is critical ischemia leading to anterior ischemic optic neuropathy and permanent sight loss (~20% of cases). Clinical management of GCA is with immediate high-dose glucocorticoids started on suspicion, with tapering over several months depending on the patients’ clinical response to treatment.

GCA is considered a Th1 and Th17 T-cell mediated disease. IFN-gamma secreting Th1 cells are relatively glucocorticoid-resistant and largely responsible for chronic disease activity. In contrast, raised plasma IL-17 levels and Th17 cell arterial wall infiltration is steroid sensitive. It is noteworthy that a pivotal property of IL-17 (referred to herein as IL-17) is neutrophil activation, yet studies on neutrophil phenotype in GCA pathology are scant.
METHODS

Extended details in Supplemental Materials.

Patients.
This study is conducted in accordance with the Declaration of Helsinki. Patients gave informed consent and samples were collected from Southend University and Hammersmith Hospitals (protocol approved by the East London & The City Local Research Ethics Committee; Table 1 for patient demographics).

Flow-cytometry.
A whole blood staining protocol was performed as described5.

Flow chamber assay.
Human umbilical vein endothelial cells (HUVEC) (ethics as above) were stimulated with TNF-α (10 ng/mL, 4h). Blood neutrophils were isolated via density gradient6 and analysis of total cell capture, rolling and firmly adherent neutrophils was performed off-line6.

Determination of plasma cytokine levels,
Plasma prepared from blood of patients was tested for CXCL5, IL-8, IL-6, sIL-6R, IL-17 and IFN-gamma by ELISA.

Statistical analyses.
Either paired Student’s T test (for 48 h and week-1 post-steroid samples) or one-way repeated measures ANOVA for longitudinal analyses were carried out. Statistical differences were accepted if P<0.05.

RESULTS

Longitudinal changes in GCA neutrophil phenotype and circulating numbers.

Neutrophilia was observed within 48h of prednisone commencement (~5x10^6 neutrophils/ml) and at 1 and 24-weeks post-steroid (~4x10^6/ml; Table 1) when compared to both patient control groups (~1.2x10^6/ml; P<0.05). Longitudinal expression of the glucocorticoid-regulated protein Annexin A1 (AnxA1) revealed high neutrophil surface expression as early as 48h post-therapy and at week-1, approximately 3-4-fold increase above OA and high-dose steroid controls (Figure 1A); this peak declined steadily by week-4. However, an increase was detected again at week 24 corresponding to glucocorticoid tapering (Figure 1A). This is a non-genomic response, since no significant difference in AnxA1 mRNA was observed across the groups (Online Figure II). Expression of the AnxA1 receptor ALX/FPR2 did not change at any time point (Figure 1B).

We next analyzed the longitudinal expression of CD62L and CD11b. GCA neutrophils expressed low levels of CD62L when compared to the two control groups, with reduction evident as early as 48h post-therapy commencement. Values began to increase from week-4, with higher cellular expression by week 24 (Figure 1A,B; P<0.05). Although CD11b expression was high at 48h, it rapidly decreased by week-1 (Figure 1A) with no significant difference from controls. Again there was a 3-fold selective increase in CD11b on GCA neutrophils at week 24 (Figure 1A).

Therefore, within 48hrs of steroid treatment, neutrophils displayed an AnxA1hiCD62LloCD11bhi phenotype, week-1 CD16+ neutrophils displayed an AnxA1hiCD62LloCD11bhi phenotype, and week-24

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Online Figure III). These phenotypes correlated with neutrophil-endothelial cell interactions under flow. There was a 3-fold decrease in GCA-neutrophil interactions at week-1 (as compared to OA), due to marked attenuation in rolling and adhesion (Figure 1C). In contrast, week-24 GCA neutrophils exhibited significantly increased capture and adhesion (Figure 1C).

**Suppressor neutrophil reduction at 24-weeks post-glucocorticoid.**

Plasma analyses of the chemokines IL-8 and CXCL5 indicated a significant increase within 48hrs of steroid commencement, declining at 1 and 4 weeks post-steroid. However, there was a significant augmentation of both neutrophil chemoattractants at 24 weeks post steroid, with levels similar to that observed at 48hrs (Figure 2A). Plasma IFN-γ levels were significantly high 48hrs post therapy, compared to controls groups and 1 week post steroid, but there was no further significant changes in IFN-γ levels was observed during the rest of the time course (Figure 2A). In contrast to IFN-γ, IL-6 levels increased in GCA patients 24-weeks post-steroid, which coincided with an increase in IL-17 (Figure 2A).

A recent study described a novel CD16brightCD62Ldim population of neutrophils that suppress T-cell proliferation 7. Re-analysis of our data revealed a significant reduction of CD16hiCD62Llo events (equivalent to CD16brightCD62Ldim suppressors) at week 24 when compared to week-1 (Figure 2B), with higher levels of AnxA1 compared to week-1 (Figure 2B). Incubation of neutrophils from healthy donors with concentrations of IL-17 and IL-6 measured in GCA patient plasma at week-1 and 24 replicated the difference in the neutrophil suppressor population (Figure 3A) and their ability to suppress T cell proliferation. neutrophils treated with week-1 levels of the two cytokines were able to effectively suppress T cell proliferation, but not when treated with week-24 levels of IL-6 and IL-17 (Figure 3B).

Finally, we quantified chemokine receptor expression on T-cells following co-culture with neutrophils treated with IL-6 and IL-17 in combination. Whereas CXCR4 expression did not significantly change (data not shown), a 2-3-fold increase in T-cell CXCR3 expression was observed upon co-culture with week 24, but not week-1 concentrations of IL-6 and IL-17 (Figure 3C).

**DISCUSSION**

The recent identification of IL-17-producing T-cells in GCA patients suggests a potential role for neutrophils, since this cytokine promotes bone marrow mobilization as well as activation and trafficking of neutrophils into perivascular tissue, yet there is scant evidence for a role for neutrophils in GCA 3, 4. We monitored neutrophil function and phenotypes during a canonical 6-month glucocorticoid treatment, and provide evidence for a role for neutrophil phenotypic changes in GCA pathology.

Our initial interest in neutrophils and GCA stemmed from the neutrophilia typically seen in patients on steroid therapy (Table 1). Persistent neutrophilia observed at 24-weeks (a time when most patients have achieved clinical remission), suggested existence of a sub-clinical vascular inflammatory state that might explain disease reemergence. To test this hypothesis, we analyzed neutrophil phenotypes as early as 48h post-steroid and at 1, 4 and 24-weeks post-therapy. GCA neutrophils display a classically activated CD16hiAnxA1hiCD62LloCD11bhi phenotype at 48 h. This phenotype comes under rapid control within 1-week of treatment, in spite of stable neutrophilia, with a CD16hiAnxA1hiCD62LloCD11blo signature. These neutrophils were hyporeactive as confirmed by minimal interaction with an inflamed endothelial monolayer under flow conditions, similar to the CD16brightCD62Ldim neutrophil reactivity previously described. This neutrophil phenotype is similar to that reported following oestrogen treatment 5. In stark contrast, neutrophils at 24-weeks post-glucocorticoids exhibited a CD16hiAnxA1hiCD62LhiCD11bhi phenotype correlating with marked adhesion to endothelial monolayers.
We initially postulated the neutrophil phenotype observed at week-1 was a direct consequence of high-dose steroid therapy, since AnxA1 is glucocorticoid regulated\textsuperscript{10}, and there is evidence for glucocorticoid-induced CD62L shedding\textsuperscript{11}. However this protective neutrophil phenotype was specific to steroid-treated GCA, since cells from the high-dose steroid control group did not display the same hyporeactive phenotype. Furthermore, despite the high AnxA1 levels on week 24 neutrophils, there was still significantly increased firm adhesion, suggesting either a defective protein\textsuperscript{5, 6} or inability to counteract the cellular hyperactivity. The molecular mechanisms behind AnxA1 mobilization at week-1 and 24 warrant further investigation.

The emerging hypothesis of a neutrophil component in GCA was confirmed by cytokine measurements: the highest circulating levels of CXCL5 and IL-8, together with IL-6 and IL-17, were observed at week 24 (Figure 2). It should also be noted that levels of both neutrophil chemotactants and IFN-\(\gamma\) were significantly augmented within 48hrs of steroid commencement, when compared to high dose steroid controls who had been on steroid therapy for a similar length of time. Therefore, taken together, this neutrophil component appears to be specific to GCA. Increased IL-17 expression following therapeutic control is congruent with a model whereby T-cell/neutrophil crosstalk is key to GCA progression, possibly exacerbating vascular inflammation.

Near completion of this study, two neutrophil phenotypes were reported in the blood of volunteers infused with lipopolysaccharide, with a novel suppressor pool, identified as CD16\textsuperscript{bright}CD62L\textsuperscript{dim}CD11b\textsuperscript{bright}, able to dampen T-cell activation\textsuperscript{7}. This suppressor pool was detected in our week-1 samples, and almost halved by at week 24 post-steroid, making the present GCA study the first to identify this neutrophil subset in disease. Combining our functional data with those of Pillay et al\textsuperscript{7}, we hypothesize that week-24 GCA neutrophils are unable to suppress T-cell responses, favoring loss of glucocorticoid control and, in time, re-emergence of vascular inflammation.

Intriguingly, we could reproduce \textit{in vitro} the neutrophil dichotomy using concentrations of IL-6 and IL-17 equivalent to those measured in GCA plasma samples. The reduction in suppressor neutrophils, after treatment with these cytokines correlated with attenuated inhibition of lymphocyte proliferation. Furthermore, analysis of T-cells co-cultured with these neutrophils demonstrated high levels of the chemokine receptor CXCR3, an important determinant for Th1 and Th17 cell trafficking to inflamed tissues\textsuperscript{12, 13}. Indeed, CXCR3\textsuperscript{+} T-cells have been identified in the temporal arteries of GCA patients\textsuperscript{14}.

In conclusion, we report potential involvement of neutrophils in GCA pathogenesis and/or relapse. Our data support the concept that the disease process is incompletely controlled by glucocorticoid therapy, since tapering leads to loss of the neutrophil suppressor subset. This, in turn, may be the prelude to lymphocyte proliferation and disease relapse with an associated increased risk of vascular complications (Figure 4). Thus, monitoring neutrophil phenotype might inform on disease status, predict risk of relapse and facilitate steroid tapering in individual patient.

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DISCLOSURES
None.
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FIGURE LEGENDS

Figure 1. Longitudinal neutrophil phenotypic and reactivity changes in GCA. A. Analysis of neutrophil phenotype with respect to AnxA1, FPR2/ALX, CD62L and CD11b in GCA blood 48hrs post steroid (n=5) and at 1, 4 and 24 weeks post steroid (n=9; open circles), compared to OA (n=10; white bars) and high dose steroid (n=6; hatched bars) controls. B. GCA neutrophils at 1 and 24-weeks post-steroid were flowed over activated HUVEC monolayers under shear. GCA neutrophil reactivity was compared with OA neutrophils. Representative images are shown. P<0.05 ‡ compared to 48hr, §compared to OA, §compared to high-dose steroid, *compared to GCA week-1.

Figure 2. Suppressor neutrophil population in GCA. A. Plasma samples collected from GCA 48hrs post steroid (n=5) and at 1, 4 and 24 weeks post steroid (n=9; open circles) were analyzed for IL-6, IL-17, CXCL5, IL-8, IFNγ and sIL-6R and compared to OA (n=10; white bars) or high dose steroid controls (n=6, hatched bars). B. Neutrophils from GCA patients gated as CD16hiCD62Lhi (white bars) or CD16hiCD62Llo (hatched bars) populations (left panel) were further analyzed for AnxA1 (middle panel) and CD11b (right panel) expression. Representative plots of CD16 and CD62L distribution on neutrophils are shown. P<0.05, *compared to week 1, #compared to OA control, §compared to high dose steroids, +compared to 48hr.

Figure 3. In vitro GCA suppressor neutrophils. A. Neutrophils from healthy donors were treated in vitro with vehicle (white bars), week-1 (light grey bars) or week 24 (dark grey bars) concentrations of IL-6 and IL-17 measured in GCA plasma (see Figure 2). Representative plots are shown. B. Proliferation assay. Neutrophils were washed and co-cultured with autologous CFSE-labelled T-cells stimulated with anti-CD3 and anti-CD28. Proliferation was assessed 5 days post co-culture. C. CXCR3 expression on T-cells following in vitro co-culture. Data are from three separate experiments, P<0.05; *compared to week-1; §compared to vehicle.

Figure 4. Potential role for neutrophils in GCA disease progression. Hypothetical role(s) of neutrophils in GCA (see Discussion). Blue neutrophil depicts neutrophil at 1 week post-steroid, which is capable of tempering T cell responses and limiting inflammation. Red neutrophil depicts neutrophil at week 24 post-steroid, where we hypothesis this cell is now unable to control T cell responses, hence leaving unchecked T cell migration to the affected vessel possibly via CXCR3 upregulation.
Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Ratio (F:M)</th>
<th>Age (Yrs)</th>
<th>Time post Steroid</th>
<th>Neutrophil count (X10⁶/ml blood)</th>
<th>CRP (mg/L)</th>
<th>Steroid dose (mg/day)</th>
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<tbody>
<tr>
<td>GCA Patients</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>5:0</td>
<td></td>
<td>48hr (n=5)</td>
<td>5.9±1.1</td>
<td>43.0±10.5</td>
<td>63.3±3.3</td>
</tr>
<tr>
<td>11:3</td>
<td>72±2.0</td>
<td>1 week (n=14)</td>
<td>4.4±0.9*#</td>
<td>72.8±23.0</td>
<td>57.8±2.2</td>
</tr>
<tr>
<td>6:3</td>
<td></td>
<td>4 weeks (n=9)</td>
<td>2.6±0.4</td>
<td>1.61±0.4</td>
<td>40.0±2.8</td>
</tr>
<tr>
<td>6:3</td>
<td></td>
<td>24 weeks (n=9)</td>
<td>4.3±0.6*#</td>
<td>11.9±4.8</td>
<td>13.4±2.2</td>
</tr>
<tr>
<td>OA controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>8:2</td>
<td>70±3.3</td>
<td>N/A</td>
<td>1.3±0.2</td>
<td>2.8±0.9</td>
<td>N/A</td>
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<tr>
<td>High dose steroid controls</td>
<td></td>
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<tr>
<td>5:1</td>
<td>47±9.8</td>
<td>≤1 week</td>
<td>2.2±0.3</td>
<td>18.5±5.6</td>
<td>48.0±3.7</td>
</tr>
</tbody>
</table>

Reported here are the patient demographics for GCA and two control groups: age-matched osteoarthritis (OA) patients and high dose steroid controls (heterogeneous: Takayasu’s arteritis, n=3, and ANCA vasculitis patients, n=3). Data are expressed as mean±SEM (n). *P<0.05 compared to OA controls; #P<0.05 compared to high dose steroids (One-way ANOVA). No statistical difference emerged between the age range of GCA and OA patients.
Novelty and Significance

What Is Known?

- Giant Cell Arteritis (GCA) is a large vessel disease characterized by inflammation of the large and medium size arteries, commonly the temporal artery.
- GCA is an important cause of acute vision loss.
- GCA is currently treated with large dose glucocorticoid therapy, which upon improvement in clinical symptoms is tapered over 6 months.
- T cell activation and recruitment into the involved artery is implicated in the pathogenesis of GCA.

What New Information Does this Article Contribute?

- Analyses of circulating neutrophils showed activation during the early stage of the disease.
- Administration of large dose glucocorticoids controls neutrophil activation.
- Within 48 hours after treatment with a high dose steroids neutrophils showed minimal adhesion to endothelial monolayers.
- However, upon tapering of steroids after six months of therapy, neutrophils showed increased endothelial adhesion under flow conditions.
- Likewise, after six months of therapy the ‘suppressor phenotype’ of neutrophil is less expressed, and there is re-emergence of signs of neutrophil activation coupled to higher levels of pro-inflammatory cytokines.

Previous studies have shown that T cells are involved in the pathogenesis of GCA. However, mechanisms regulating their hyper-reactivity are poorly understood. We examined the profile and activity of the circulating leukocytes in patients with GCA longitudinally over a 6-month glucocorticoid therapy period. Administration of high dose steroids was associated with a low inflammatory profile of the circulating neutrophil, indicated by the presence of a larger proportion of the suppressor neutrophils, which inhibited T cell proliferation. However, tapering of steroids was associated with clear signs of neutrophil activation and high levels of two pro-inflammatory cytokines, interleukin-6 and interleukin-17A. These changes were associated with reduced proportion of the neutrophils with the suppressor phenotype. Collectively, these results suggest a potential involvement of neutrophils in the pathogenesis of GCA and might indicate novel interactions between neutrophils and T cells. High doses of glucocorticoids control these interactions favorably. However, tapering of the steroid dose is associated with subliminal signs of vascular inflammation, possibly conducive to subsequent disease relapse and the associated vascular complications. Monitoring neutrophil phenotype might provide novel information on disease status, risk of relapse and steroid dosage.
Figure 1

A

AnxA1

ALX/FPR2

CD62L

CD11b

Weeks post steroid

Weeks post steroid

Weeks post steroid

Weeks post steroid

B

Capture

Adhesion

No. of Neutrophils (no. per field)

OA ctrl

Week 1 GCA

Week 24 GCA
**Figure 2**

A. Levels of cytokines and chemokines over time in response to steroid therapy:

- **IL-6**
- **IL-17**
- **CXCL5**
- **IFNγ**
- **sIL-6R**

Weeks post steroid:

B. Changes in neutrophil subpopulations and cell surface protein expression:

- **CD16+CD62Lhi**
- **CD16+CD62Llo**
- **AnxA1**
- **CD11b**

Weeks post steroid:

- **Week 1**
- **Week 24**

*Statistical significance indicators: #, §, **, ⏰, ♯, ♯♯, ***, +*
Figure 3

A

CD16/CD62L (%)

Vehicle
Week 1 Cytokines
Week 24 Cytokines

B

Cell Division (% precursor frequency)

Vehicle
Week 1 IL-6/IL-17A
Week 24 IL-6/IL-17A
Lymphocytes only

C

CXCR3 Expression (MFI Units)

Vehicle
Week 1 levels of IL-6/IL-17
Week 24 levels of IL-6/IL-17
**Week 1: Steroid high**
- ↓ IL-6
- ↓ IL-17

**Week 24: Steroid low**
- ↑ IL-6
- ↑ IL-17

**Regulated T cell proliferation**

**Dysregulated T cell proliferation**

**Key:**
- AnxA1
- CD11b
- CXCL3
- CD16
- CD62L

**Temporal Artery**
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Supplemental Materials

Patients

Patients gave informed consent and samples were collected from Southend University Hospital approved by the East London & The City Local Research Ethics Committee (Rec Ref. 05/Q0603/34 ELCHA, London, United Kingdom). Briefly, blood was collected between 9 and 10am from biopsy positive patients at weeks 1, 4, and 24-post glucocorticoid commencement. Each patient was followed at the time points indicated. According to BSR BHPR guidelines, steroid therapy is started as soon as GCA is suspected. Consequently, no samples prior to glucocorticoid therapy were available.

We have used a heterogeneous population of patients on high dose steroids as steroid controls. These patients included Takayasu’s arteritis (X3) and ANCA vasculitis (x3). We also used osteoarthritis (OA) patients as age-matched control. In all cases, C reactive protein (CRP) levels, glucocorticoid dose and tapering and absolute PMN cell numbers are presented in Table 1 for the two patient groups.

Flow cytometry detection of surface proteins on human PMN

All conjugated antibodies were obtained from eBioscience (Hatfield, UK), unless otherwise stated. Briefly, whole blood aliquots (50μL) were subjected to a three-step staining protocol, starting with an incubation for 1 h at 4°C with mouse anti-human AnxA1 (monoclonal 1B, produced in-house) or anti-human FPR2/ALX (Genovac, Freiburg, Germany); both at a final concentration of 20μg/mL plus IgG1k isotype control. Blood was then washed twice in PBS containing 10mM CaCl₂ and 1.5%
BSA, followed by 30 min incubation at 4°C with a rabbit anti-mouse IgG FITC-conjugated secondary Ab (AbD Serotec, Oxford, UK). Blood was then washed as above and incubated for a further 30 min with conjugated antibodies to CD16 (PE, clone eBioCB16) and L-selectin (PE-Cy5, clone DREG-56) or, in some samples, with CD11b (APC, clone ICRF44). Following two washes, blood was lysed using a whole blood lysis kit (as per manufacturer’s protocol, Beckman Coulter, High Wycombe, UK).

In all cases, 20,000 events were acquired by using a FACSCalibur flow cytometer (Becton Dickinson), and analysed using FlowJo analysis software (Version 9.4.1.1, Treestar Inc, Stanford, CA). See Online Figure IA for whole blood gating strategy

*Flow chamber assay*

Use of human umbilical vein endothelial cells (HUVEC) was approved by the East London & The City Local Research Ethics Committee (REC Ref 06/Q0605/40 ELCHA, London, United Kingdom). HUVEC were cultured until confluence and stimulated with 10 ng/mL tumor necrosis factor-α for 4 hours (Sigma-Aldrich)³. Patient PMN were isolated via density gradient as previously described³. Isolated cells were incubated with vehicle or 10nM rhAnxA1 for 10 min at 37°C, before flow over HUVEC monolayers at a rate of 1 dyn/cm², for 8 min, as previously described³. PMN/HUVEC interaction in the flow chamber was monitored on 6 random fields recorded for 10 seconds. Analysis of total cell capture, rolling and firmly adherent PMN was carried out off-line by manual quantification using Image-Pro Plus software (Media Cybernetics, Inc. Bethesda, MD USA).
Determination of plasma of CXCL5, IL-8, IL-6, sIL-6R, IL-17A and IFN-gamma concentrations

Plasma (blood taken between 9 and 10am from each patient at each time point) prepared from blood of patients was tested for CXCL5, IL-8, IL-6, sIL-6R, IL-17A and IFN-gamma using specific enzyme-immunoassays according to manufacturer’s instructions (CXCL5 from R&D Systems, Oxford, UK; IL-6 and sIL-6R from eBioscience Hatfield, UK; IL-8, IL-17A and IFN-gamma from Peprotech, NJ, USA)

Real-Time PCR

Patient PMN were isolated via density gradient as described\(^3\). Isolated PMN were then suspended in TRIzol\(^\text{®}\) and stored at -80°C until required. RNA was extracted according to manufacturer’s instruction (Invitrogen, Paisley, UK). cDNA was synthesised using 1µg of RNA from 3 or more replicates with the SuperScript III Reverse Transcriptase (Invitrogen, UK). Real time-PCR was performed in duplicates or triplicates, with 200ng of cDNA per well, 1µl primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), using the ABI Prism 7900HT Sequence Detection System. Quantitect\(^\text{®}\) primers (QIAGEN, Crawley, UK) used are the following: HsGAPDH (QT01192646), HsANXA1 (QT00078197) and HsFPR2 (QT00204295). A dissociation step was always included to confirm the absence of un-specific products. Gene Expression Master Mix (Applied Biosystems, Warrington, UK). Fold change was calculated as \(2^{-\Delta\Delta C_{t}}\) using Hs GAPDH as endogenous control.

In vitro PMN culture and lymphocyte proliferation assay
PMN, isolated from healthy volunteers as described above, were treated with human IL-6 and IL-17A (Peprotech, NJ, USA; concentrations based on circulating levels measured in GCA plasma at 1 and 24 weeks post-glucocorticoid: week 1 doses were 300pg/ml IL-17 + 30pg/ml IL-6; week 24 doses were 400pg/ml IL-17 + 70pg/ml IL-6) for 30 min, before flow cytometry analysis for surface expression of CD16 and CD62L. For coculture experiments, PMN were treated with IL-6/IL-17A as described, washed and co-incubated (at a 2:1 ratio) with CSFE-labelled lymphocytes onto plates where anti-CD3 (1 µg/mL; clone HIT3a, eBioscience) and anti-CD28 (5µg/mL clone CD28.2, eBioscience). Following 5 day culture, lymphocyte proliferation was analysed by flow cytometry and quantified by using the Proliferation Platform in FlowJo™. See Online Figure IB and IC for lymphocyte purity and gating strategy, respectively.
References


Online Figure I. Gating strategy for granulocytes (A) used in all whole blood FACS analyses. Purity of CD14 removal following adhesion of PBMC for 1hr at 37°C in RPMI-1640 (without FCS). Left panel indicates CD14 and CD3 percentage prior to adhesion. Right panel indicates CD14 and CD3 percentages following adhesion (A). B. Gating strategy on lymphocytes following 5 day culture with PMN (B).
Online Figure II. Dynamics of Annexin A1 (AnxA1) mRNA expression in purified neutrophils.

Real-time PCR analysis of AnxA1 gene expression from isolated neutrophils of GCA. Neutrophils were isolated at time points indicated in A and B. Results are fold-change as $2^{-\Delta\Delta CT}$ using GAPDH as endogenous control.

* $P<0.05$ vs. OA control,
§ $P<0.05$ compared to GCA week 1. (1-way ANOVA, Bonferroni post-correction).
Online Figure III. Histograms for cell phenotyping.
Representative histogram plots denoting neutrophil phenotypes of GCA patients 1 and 24 weeks post steroid commencement, with respect to AnxA1, CD62L and CD11b cell surface expression.