New Hypotheses in Clinical Medicine

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

Suchita Nadkarni, Jesmond Dalli, Jane Hollywood, Justin C. Mason,* Bhaskar Dasgupta,* Mauro Perretti*

Rationale: Giant-cell arteritis (GCA) is a large-vessel vasculitis characterized by immune cell infiltration, yet the potential involvement of neutrophils has rarely been 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 during a 6-month period.

Methods and Results: Blood samples were taken within 48 hours of therapy commencement and at weeks 1, 4, and 24 after glucocorticoid dose. Flow cytometric analysis revealed 3 distinct neutrophil populations and phenotypes. Within 48 hours of steroid treatment, neutrophils displayed an AnxA1hiCD62LloCD11blo phenotype, whereas week 1 neutrophils were AnxA1hiCD62LloCD11blo and displayed minimal adhesion to endothelial monolayers under flow, and week 24 (ie, lowest glucocorticoid dose) neutrophils were AnxA1hiCD62LhiCD11blo with increased endothelial adhesion under flow. Week 24 plasma analyses showed high levels of C-X-C motif chemokine ligand 5, interleukin (IL)-8, IL-17, and IL-6. Importantly, comparison of week 1 and week 24 samples revealed a suppressive neutrophil effect on T-cell proliferation at the former time point only. Finally, in vitro incubation of naive neutrophils with concentrations of IL-6 and IL-17 quantified in GCA plasma at weeks 1 and 24 replicated this differential modulation of lymphocyte proliferation.

Conclusions: This translational study highlights a novel clinical manifestation of GCA, with evidence for a neutrophil component and an escaped proinflammatory phenotype when glucocorticoid therapy is tapered. These results indicate potential involvement of neutrophils in GCA pathogenesis. (Circ Res. 2014;114:242-248.)

Key Words: giant-cell arteritis ■ neutrophils ■ T-lymphocytes

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 ischonic 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 months1 depending on the clinical response of patients to treatment. GCA is considered a T helper (Th)-1 and Th17 T-cell-mediated disease. Interferon-γ (IFN-γ)-secreting Th1 cells are relatively glucocorticoid-resistant and largely responsible for chronic disease activity. In contrast, increased plasma interleukin (IL) 17 levels and Th17 cell arterial wall infiltration are steroid-sensitive.2 It is noteworthy that a pivotal property of IL-17 (referred to herein as IL-17) is neutrophil activation, yet studies of neutrophil phenotype in GCA pathology are scant.3,4

Methods

Detailed Methods section is given in the online-only Data Supplement.

Patients

This study was 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 and the City Local Research Ethics Committee; see Table for patient demographics).

Flow Cytometry

A whole-blood staining protocol was performed as described.5

Flow Chamber Assay

Human umbilical vein endothelial cells (ethics as stated) were stimulated with tumor necrosis factor-α (10 ng/mL, 4 hours). Blood neutrophils were isolated via density gradient,6 and analysis of total cell capture and rolling and firmly adherent neutrophils was performed off-line.6

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Neutrophilia was observed within 48 hours of prednisone commencement ($\approx 5 \times 10^6$ neutrophils/mL) and at 1 and 24 weeks after steroids ($\approx 4 \times 10^6$/mL; Table) compared with both patient control groups ($\approx 1.2 \times 10^6$/mL; $P<0.05$). Longitudinal expression of the glucocorticoid-regulated protein annexin A1 (AnxA1) revealed high neutrophil surface expression as early as 48 hours after therapy. At week 1, = 3- to 4-fold increase higher than that for osteoarthritis and high-dose steroid control group patients (Figure 1A) was found; 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 nongenomic response because no significant difference in AnxA1 mRNA was observed across the 4 groups (Online Figure II). Expression of the AnxA1 receptor lipoxin A4 receptor/formyl-peptide receptor 2 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 compared with the 2 control groups, with reduction evident as early as 48 hours after therapy commencement. Values began to increase from week 4, with higher cellular expression by week 24 (Figure 1A and 1B; $P<0.05$). Although CD11b expression was high at 48 hours, it rapidly decreased by week 1 (Figure 1A), with no significant difference from control group patients. Again, there was a 3-fold selective increase in CD11b on GCA neutrophils at week 24 (Figure 1A).

Therefore, 48 hours post steroid treatment, neutrophils displayed an AnxA1$^{\text{hi}}$CD62L$^{\text{lo}}$CD11b$^{\text{hi}}$ phenotype, 1 week post steroid CD16$^+$ neutrophils displayed an AnxA1$^{\text{lo}}$CD62L$^{\text{hi}}$CD11b$^{\text{lo}}$ phenotype, and 24 weeks post steroid, CD16$^+$ neutrophils displayed an AnxA1$^{\text{hi}}$CD62L$^{\text{hi}}$CD11b$^{\text{hi}}$ phenotype (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 (compared with osteoarthritis) as a result of marked attenuation in rolling and adhesion (Figure 1C). In contrast, week 24 GCA neutrophils exhibited significantly increased capture and adhesion (Figure 1C).

### Table. Patient Demographics

<table>
<thead>
<tr>
<th>Ratio (F:M)</th>
<th>Age, y</th>
<th>Time After Steroid</th>
<th>Neutrophil Count ($\times 10^6$/mL Blood)</th>
<th>CRP, mg/L</th>
<th>Steroid Dose, mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with GCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:0</td>
<td>48 h (n=5)</td>
<td>5.9±1.1</td>
<td>43.0±10.5</td>
<td>63.3±3.3</td>
<td></td>
</tr>
<tr>
<td>11:3</td>
<td>72±2.0</td>
<td>1 wk (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>4 wk (n=9)</td>
<td>2.6±0.4</td>
<td>1.61±0.4</td>
<td>40.0±2.8</td>
<td></td>
</tr>
<tr>
<td>6:3</td>
<td>24 wk (n=9)</td>
<td>4.3±0.6†</td>
<td>11.9±4.8</td>
<td>13.4±2.2</td>
<td></td>
</tr>
<tr>
<td>OA control group</td>
<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>
</tr>
<tr>
<td>High-dose steroid control group</td>
<td>5:1</td>
<td>47±9.8</td>
<td>≤1 wk</td>
<td>2.2±0.3</td>
<td>18.5±5.6</td>
</tr>
</tbody>
</table>

Patient demographics for GCA and 2 control groups: age-matched osteoarthritis (OA) patients and high-dose steroid control group patients (heterogeneous: Takayasu arteritis [n=3] and patients with anti-neutrophil cytoplasmic antibodies vasculitis [n=3]). Data are expressed as mean±SEM (n). CRP indicates C-reactive protein; GCA, giant-cell arteritis; and N/A, not applicable.

* $P<0.05$ compared with OA control group.
† $P<0.05$ compared with high-dose steroids (1-way ANOVA). No statistical difference emerged between the age ranges of patients with GCA and with OA.
Incubation of neutrophils from healthy donors with concentrations of IL-17 and IL-6 measured in the plasma of patients with GCA at weeks 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 2 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 after coculture with neutrophils treated with IL-6 and IL-17 in combination. Whereas C-X-C motif chemokine receptor-4 expression did not significantly change (data not shown),
A 2-fold to 3-fold increase in T-cell C-X-C motif chemokine receptor-3 expression was observed on coculture 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 patients with GCA suggests a potential role for neutrophils because this
cytokine promotes bone marrow mobilization as well as activation and trafficking of neutrophils into perivascular tissue, yet there is scant evidence for the role of neutrophils in GCA. We monitored neutrophil function and phenotypes during a canonical 6-month glucocorticoid treatment and provide evidence for the role of neutrophil phenotypic changes in GCA pathology.

Our initial interest in neutrophils and GCA stemmed from the neutrophilia typically seen in patients using steroid therapy (Table). Persistent neutrophilia observed at 24 weeks (a time when most patients have achieved clinical remission) suggested existence of a subclinical vascular inflammatory state that might explain disease reemergence. To test this hypothesis, we analyzed neutrophil phenotypes as early as 48 hours after steroids and at 1, 4, and 24 weeks after therapy. GCA neutrophils display a classically activated CD16hiAnxA1hiCD62LloCD11bhi phenotype at 48 hours. This phenotype comes under rapid control within 1 week of treatment, despite stable neutrophilia, with a CD16hiAnxA1hiCD62LloCD11bhi 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 after estrogen treatment.

We initially postulated that the neutrophil phenotype observed at week 1 was a direct consequence of high-dose steroid therapy because AnxA1 is glucocorticoid regulated and there is evidence for glucocorticoid-induced CD62L shedding. However, this protective neutrophil phenotype was specific to steroid-treated GCA because cells from the high-dose steroid control group did not display the same hyporeactive phenotype. Furthermore, despite the high AnxA1 levels...
In week 24 neutrophils, there was still significantly increased firm adhesion, suggesting either a defective protein\(^5,6\) or an inability to counteract the cellular hyperactivity. The molecular mechanisms behind AnxA1 mobilization at weeks 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 C-X-C motif chemokine ligand 5 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 chemoattractants and IFN-\(\gamma\) were significantly augmented within 48 hours of steroid commencement compared with high-dose steroid control group patients who had been using steroid therapy for a similar length of time. Therefore, taken together, this neutrophil component seems to be specific to GCA. Increased IL-17 expression after 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, 2 neutrophil phenotypes were reported in the blood of volunteers infused with lipopolysaccharide with a novel suppressor pool, identified as CD16\(^{bright}\)CD62L\(^{dim}\)CD11b\(^{bright}\), able to dampen T-cell activation.\(^7\) This suppressor pool was detected in our week 1 samples and almost halved by week 24 after steroids, making the present GCA study the first to identify this neutrophil subset in disease. Combining our functional data with those of Pillay et al,\(^7\) 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 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 cocultured with these neutrophils demonstrated high levels of the chemokine receptor C-X-C motif chemokine receptor-3, an important determinant of Th1 and Th17 cell trafficking to inflamed tissues.\(^12,13\)

C-X-C motif chemokine receptor-3\(^+\) T cells have been identified in the temporal arteries of patients with GCA.\(^14\)

In conclusion, we report potential involvement of neutrophils in GCA pathogenesis and relapse. Our data support the concept that the disease process is incompletely controlled by glucocorticoid therapy because 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 disease status, predict risk of relapse, and facilitate steroid tapering in individual patients.

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**Disclosures**

None.

**References**


5. Nadkarni S, Cooper D, Brancialeone V, Bena S, Perretti M. Activation of the annexin A1 pathway underlies the protective effects exerted by...

**Novelty and Significance**

**What Is Known?**

- Giant-cell arteritis (GCA) is a large-vessel disease characterized by inflammation of large and medium arteries, commonly the temporal artery.
- GCA is an important cause of acute vision loss.
- GCA is currently treated with high-dose glucocorticoid therapy, which, on improvement in clinical symptoms, tapers over 6 months.
- T-cell activation and recruitment into the involved artery are 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 high-dose glucocorticoids controls neutrophil activation.
- Within 48 hours after treatment with high-dose steroids, neutrophils showed minimal adhesion to endothelial monolayers.
- However, on tapering of steroids after 6 months of therapy, neutrophils showed increased endothelial adhesion under flow conditions.
- Similarly, after 6 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 proinflammatory 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 during 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 suppressor neutrophils that inhibited T-cell proliferation. However, tapering of steroids was associated with clear signs of neutrophil activation and high levels of 2 proinflammatory 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.
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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\(^1\), 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 (50\( \mu \)L) were subjected to a three-step staining protocol, starting with an incubation for 1 h at 4\(^\circ\)C with mouse anti-human AnxA1 (monoclonal 1B, produced in-house\(^2\)) or anti-human FPR2/ALX (Genovac, Freiburg, Germany); both at a final concentration of 20\( \mu \)g/mL plus IgG1k isotype control. Blood was then washed twice in PBS containing 10mM CaCl\(_2\) 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 described\(^3\). 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 dyne/cm\(^2\), for 8 min, as previously described\(^3\). 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 10 am 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. Isolated PMN were then suspended in TRIzol® and stored at -80°C until required. RNA was extracted according to manufacturer’s instruction (Invitrogen, Paisley, UK). cDNA was synthesised using 1ug 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® 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 H13a, 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 1 hr 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 $2^{-\Delta\Delta C_t}$ 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.