

An investigational analysis reveals a potential role for neutrophils in giant-cell arteritis disease progression

Suchita Nadkarni¹, Jesmond Dalli¹, Jane Hollywood², Justin C Mason^{3*}, Bhaskar Dasgupta^{2*}, Mauro Perretti^{1*}

¹William Harvey Research Institute, Barts and the London School of Medicine, UK;

²Department of Rheumatology, Southend University Hospital, UK; ³Vascular Sciences and Rheumatology, Imperial College London, UK

* share senior authorship

Running Title: Neutrophils in GCA

Address for correspondence: Mauro Perretti, The William Harvey Research Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK.

Telephone: +44-207-8828782; Fax: +44-207-8826076; Email: m.perretti@qmul.ac.uk

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 AnxA1^{hi}CD62L^{lo}CD11b^{hi} phenotype, whereas week-1 neutrophils were AnxA1^{hi}CD62L^{lo}CD11b^{lo} and displayed minimal adhesion to endothelial monolayers under flow, and week 24 (i.e. lowest glucocorticoid dose) neutrophils were AnxA1^{hi}CD62L^{hi}CD11b^{hi} 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.

Key Words: Neutrophils, giant cell arteritis, T cells, suppression.

Abbreviations: GCA giant cell arteritis,; OA, osteoarthritis,

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 months¹ 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 sensitive². 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^{3, 4}.

Materials and 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.

78

79 *Flow chamber assay*

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

84

85 *Determination of plasma cytokine levels*

86 Plasma prepared from blood of patients was tested for CXCL5, IL-8, IL-6, sIL-6R, IL-
87 17 and IFN-gamma by ELISA.

88

89 *Statistical analyses*

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

93

94 **Results**95 *Longitudinal changes in GCA neutrophil phenotype and circulating numbers*

96 Neutrophilia was observed within 48h of prednisone commencement ($\sim 5 \times 10^6$
97 neutrophils/ml) and at 1 and 24-weeks post-steroid ($\sim 4 \times 10^6$ /ml; Table 1) when
98 compared to both patient control groups ($\sim 1.2 \times 10^6$ /ml; $P < 0.05$). Longitudinal
99 expression of the glucocorticoid-regulated protein Annexin A1 (AnxA1) revealed high
100 neutrophil surface expression as early as 48h post-therapy and at week-1,
101 approximately 3-4-fold increase above OA and high-dose steroid controls (Figure
102 1A); this peak declined steadily by week-4. However, an increase was detected
103 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 AnxA1^{hi}CD62L^{lo}CD11b^{hi} phenotype, week-1 CD16⁺ neutrophils displayed an AnxA1^{hi}CD62L^{lo}CD11b^{lo} phenotype, and week-24 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 CD16^{bright}CD62L^{dim} population of neutrophils that suppress T-cell proliferation⁷. Re-analysis of our data revealed a significant reduction of CD16^{hi}CD62L^{lo} events (equivalent to CD16^{bright}CD62L^{dim} 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 patients² suggests a potential role for neutrophils, since this cytokine promotes bone marrow mobilization as well as activation and trafficking of neutrophils into perivascular tissue⁸, 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 CD16^{hi}AnxA1^{hi}CD62L^{lo}CD11b^{hi} phenotype at 48 h. This phenotype comes under rapid control within 1-week of treatment, in spite of stable neutrophilia, with a CD16^{hi}AnxA1^{hi}CD62L^{lo}CD11b^{lo} signature. These neutrophils were hyporeactive as confirmed by minimal interaction with an inflamed endothelial monolayer under flow conditions, similar to the CD16^{bright}CD62L^{dim} neutrophil reactivity previously described⁹. This neutrophil phenotype is similar to that reported following oestrogen treatment⁵. In stark contrast, neutrophils at 24-weeks post-glucocorticoids exhibited a CD16^{hi}AnxA1^{hi}CD62L^{hi}CD11b^{hi} 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

regulated¹⁰, and there is evidence for glucocorticoid-induced CD62L shedding¹¹. 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^{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 chemoattractants and IFN- γ 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^{bright}CD62L^{dim}CD11b^{bright}, able to dampen T-cell activation⁷. 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 al⁷, 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 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^{12, 13}. Indeed, CXCR3⁺ T-cells have been identified in the temporal arteries of GCA patients¹⁴.

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.

Supported by British Heart Foundation (PG/09/060), and partly by The Wellcome Trust (086867/Z/08) and Imperial College Biomedical Resource Centre. We thank Dr Neil Dufton for Figure 4.

Disclosures: None.

Table 1. Patient demographics

Ratio (F:M)	Age (Yrs)	Time post Steroid	Neutrophil		
			count	CRP	Steroid dose
			(X10 ⁶ /ml blood)	(mg/L)	(mg/day)
GCA Patients					
5:0		48hr (n=5)	5.9±1.1	43.0±10.5	63.3±3.3
11:3	72±2.0	1 week (n=14)	4.4±0.9 ^{*#}	72.8±23.0	57.8±2.2
6:3		4 weeks (n=9)	2.6±0.4	1.61±0.4	40.0±2.8
6:3		24 weeks (n=9)	4.3±0.6 ^{*#}	11.9±4.8	13.4±2.2
OA controls					
8:2	70±3.3	N/A	1.3±0.2	2.8±0.9	N/A
High dose steroid controls					
5:1	47±9.8	≤ 1 week	2.2±0.3	18.5±5.6	48.0±3.7

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.

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, IFN γ 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 CD16^{hi}CD62L^{hi} (white bars) or CD16^{hi}CD62L^{lo} (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 analysed 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.

References

1. Dasgupta B, Borg F, Hassan N, Alexander L, Barraclough K, Bourke B, Fulcher J, Hollywood J, Hutchings A, James P, Kyle V, Nott J, Power M, Samanta A. Bsr and bhpr guidelines for the management of giantT-cell arteritis. *Rheumatology*. 2010;49:1594–1597
2. Deng J, Younge B, Olshen R, Goronzy J, Weyand C. Th17 and th1 t-cell responses in giantT-cell arteritis. *Circulation*. 2010;121:906-915
3. Chatelain D, Duhaut P, Schmidt J, Loire R, Bosshard S, Guernou M, Pellet H, Piette JC, Sevestre H, P. DJ. Pathological features of temporal arteries in patients with giantT-cell arteritis presenting with permanent visual loss. *Ann Rheum Dis*. 2009;61:84-88
4. Maugeri N, Rovere-Querini P, al e. An intense and short-lasting burst of neutrophil activation differentiates early acute myocardial infarction from systemic inflammatory syndromes. *PLoS ONE*. 2012;7:e39484
5. Nadkarni S, Cooper D, Brancalone V, Bena S, Perretti M. Activation of the annexin a1 pathway underlies the protective effects exerted by estrogen in polymorphonuclear leukocytes. *Arterioscler Thromb Vasc Biol*. 2011;31:2749-2759
6. Hayhoe R, Kamal A, Solito E, Flower R, Cooper D, Perretti M. Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: Indication of distinct receptor involvement. *Blood*. 2006;107:2123-2130
7. Pillay J, Kamp V, van Hoffen E, Visser T, Tak T, Lammers J, Ulfman L, Leenen L, Pickkers P, Koenderman L. A subset of neutrophils in human systemic inflammation inhibits T-cell responses through mac-1. *J Clin Invest*. 2012;122:327-336
8. Stark M, Huo Y, Burcin T, Morris M, Olson T, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via il-23 and il-17. *Immunity*. 2005;22:285-294
9. Kamp VM, Pillay J, Lammers JW, Pickkers P, Ulfman LH, L. K. Human suppressive neutrophils cd16bright/cd62ldim exhibit decreased adhesion. *J leukoc Biol*. 2012;92:1011-1020
10. Perretti M, D'Acquisto F. Annexin a1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol*. 2009;9:62-70
11. Burton J, Kehrli MJ, Kapil S, Horst R. Regulation of I-selectin and cd18 on bovine neutrophils by glucocorticoids: Effects of cortisol and dexamethasone. *J Leukoc Biol*. 1995;57:317-325
12. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, F. S. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 t helper cells (th1s) and th2s. *J Exp Med*. 1998;187:129-134
13. Oo YH, Banz V, Kavanagh D, Liaskou E, Withers DR, Humphreys E, Reynolds GM, Lee-Turner L, Kalia N, Hubscher SG, Klenerman P, Eksteen B, DH A. Cxcr3-dependent recruitment and ccr6-mediated positioning of th-17 cells in the inflamed liver. *J Hepatol*. 2012;57:1044-1051
14. Brühl H, Vielhauer V, Weiss M, Mack M, Schlöndorff D, S. S. Expression of darc, cxcr3 and ccr5 in giantT-cell arteritis. *Rheumatology*. 2005;44:309-313

Novelty and Significance

What is known?

- Giant Cell Arteritis (GCA) is a large vessel vasculatides characterized by inflammation of the temporal artery, burdened by a risk of blindness.
- On suspicion of GCA, large dose therapy with glucocorticoids is immediately commenced, which is tapered over 6 months, provided the patient is showing clinical signs of improvement.
- Mechanistically, T cell recruitment and activation into the temporal artery is considered responsible for pathogenesis.

What New Information Does this Article Contributes?

- Analyses of circulating neutrophils revealed cell activation during the early stage of the disease which then became controlled by large dose glucocorticoids.
- Indication for two neutrophil phenotypes could be found, including one able to suppress T cell proliferation.
- At the end of the 6-month glucocorticoid regimen, this 'suppressor phenotype' is less expressed, and there is re-emergence of signs of neutrophil activation coupled to higher levels of pro-inflammatory cytokines.

An involvement of T cells in the pathogenesis of GCA is accepted, however, mechanisms regulating their hyper-reactivity are poorly understood. We examined, longitudinally over a 6-month glucocorticoid regimen period, the profile of circulating leukocytes, observing selective modulation of neutrophil phenotype and reactivity. When high dose steroids were given, a non-inflamed phenotype of the circulating neutrophil was determined with a larger proportion of the suppressor type, since these neutrophils inhibited T cell proliferation. However, when steroids were tapered, there were clear signs of neutrophil activation associated with high levels of two pro-inflammatory cytokines, interleukin-6 and interleukin-17A. This was associated with a reduction of the 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 establish an effective control on these interactions, however when doses are tapered, there may be still subliminal signs of vascular inflammation possibly conducive to subsequent disease relapse and associated risk of vascular complications. Monitoring neutrophil phenotype can provide novel information on disease status, risk of relapse and steroid dosage.