

Original article

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Difference in the expression of IL-9 and IL-17 correlates with different histological pattern of vascular wall injury in giant cell arteritis

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Abstract

Objective. GCA is a large- and medium-vessel arteritis characterized by a range of histological patterns of vascular wall injury. The aim of this study was to immunologically characterize the various histological patterns of GCA.

Methods. Thirty-five consecutive patients with biopsy-proven GCA and 15 normal controls were studied. IL-8, IL-9, IL-9R, IL-17, IL-4, TGF- β and thymic stromal lymphopoietin expression was evaluated by RT-PCR and immunohistochemistry on artery biopsy specimens. Confocal microscopy was used to characterize the phenotypes of IL-9-producing and IL-9R-expressing cells. Five additional patients who had received prednisone when the temporal artery biopsy was performed were also enrolled to evaluate the effect of glucocorticoids on IL-9 and IL-17 expression.

Results. IL-17 overexpression was observed mainly in arteries with transmural inflammation and vasa vasorum vasculitis. IL-9 overexpression and Th9 polarization predominated in arteries with transmural inflammation and small-vessel vasculitis. The tissue expression of both IL-9 and IL-17 was correlated with the intensity of the systemic inflammatory response. IL-4, TGF- β and thymic stromal lymphopoietin, which are involved in the differentiation of Th9 cells, were overexpressed in arteries with transmural inflammation and small-vessel vasculitis. IL-9R was also overexpressed in GCA arteries with transmural inflammation and was accompanied by increased expression of IL-8.

Conclusion. Herein we provide the first evidence that distinct populations of potentially autoreactive T cells, expressing different cytokines (Th17 vs Th9), characterize patients with particular histological subsets of GCA and may thus contribute to the heterogeneity of tissue lesions observed in these patients.

Key words: Th9, Th17, giant cell arteritis, small vessel vasculitis, vasa vasorum vasculitis.

Rheumatology key messages

- Th9 cells are present in the inflamed arteries of GCA patients.
- Th9 and Th17 cells differently characterize different histological patterns of GCA.
- IL-9 is involved in the regulation of macrophage function in GCA.

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Introduction

GCA is a large- and medium-vessel arteritis characterized by a range of histological patterns of vascular wall injury. The classic histological lesion of GCA is characterized by the presence of transmural inflammation, with or without giant cells [1]. However, in some cases inflammation is restricted to the peri-adventitial small vessels [peri-adventitial small-vessel vasculitis (SVV)] or to the vasa vasorum [vasa vasorum vasculitis (VVV)] [2–4]. The presence of an immunological Th1/Th17 signature in classic GCA has been previously demonstrated [5]; however, a precise immunological characterization of the various histological patterns in GCA is still lacking.

Depending on the cytokine milieu, naïve CD4⁺ T cells differentiate into specialized subsets, such as Th1, Th2, Th9, Th17 or Treg, which are characterized by the expression of lineage-specific transcription factors and produce a repertoire of specific cytokines [6]. Th2-derived cytokines have been previously demonstrated to be consistently absent in GCA [7, 8]. We have recently shown, however, that IL-33 (a cytokine involved in promoting the Th2 response) is overexpressed in the inflamed arteries of GCA patients and is accompanied by strong M2 polarization [9]. Furthermore, IL-33 has recently also been associated with the secretion of IL-9 by human CD4⁺ T cells isolated from peripheral blood [10].

IL-9 is a multifunctional cytokine that modulates various immune cell types, such as Treg cells, Th17 cells and antigen-presenting cells [11]. IL-9 is produced by several subsets of CD4⁺ T effector cells (e.g. Th17, Th2 and Treg cells), but only Th9 cells seem to be characterized by specific and potent production of IL-9 [11]. Th9 cells develop from naïve T cells in the presence of TGF β , IL-4 and thymic stromal lymphopoietin (TSLP) and require PU-1 as a specific transcription factor for their differentiation [11, 12]. Although the functions of Th9 cells have not been completely clarified, they seem to be involved in several types of inflammatory disease in both mice and humans [11]. Whether IL-9 and Th9 cells are involved in the pathogenesis of GCA is not known. The aim of this study was to evaluate the expression of IL-9 and Th9 cells and IL-17 in patients with GCA in relation to the various histological patterns. Here we report that a distinct immunological polarization appears to characterize each specific histological pattern of GCA. IL-9 and Th9, but not Th17, polarization predominates in inflamed arteries of GCA patients with inflammation restricted to the peri-adventitial small vessels. Conversely, a more intense Th17 polarization with weak IL-9 expression predominates in GCA arteries with inflammation restricted to the vasa vasorum. Finally, a concomitant strong Th9 and Th17 response was observed in the more inflamed arteries displaying transmural inflammation and especially in those arteries with a granulomatous reaction. Our results might imply certain pathogenic aspects for particular histological subsets of GCA.

Patients and methods

Patients

Forty consecutive patients (25 women, 15 men) with biopsy-proven GCA were studied. Of these, 35 patients were untreated at the time of artery biopsy. To assess the effect of glucocorticoids on IL-9 and IL-17 expression, artery samples from five patients who had received prednisone (1 mg/kg per day) for an average of 21 days (range 8–33) when the temporal artery biopsy was excised were also considered for this study. Patients with prior or current diagnosis of cancer, autoimmune disease other than GCA, or chronic infection were carefully excluded. The median age of GCA patients was 71 years (range: 59–85 years) and median ESR was 51.8 mm/h (range: 10–110). Headache and artery tenderness and/or decreased or absent temporal artery pulsation were present in 31 of 40 patients. At the time of diagnosis, 8 patients had vision loss, and PMR was associated in 17 patients. After GCA diagnosis, in untreated patients prednisone was commenced at a starting dosage of 50 mg/day and then tapered in all patients according to the same fixed schedule after 1 month of therapy if symptoms had resolved. Biopsy specimens were evaluated by two experienced pathologists with an expertise in vasculitides (A.R. and A.C.) who had no access to clinical data. The length of the temporal artery biopsy specimens was ≥ 0.5 cm in all cases. After histological review, biopsy specimens were classified into one of the following three categories according to the criteria defined in a recent article by our group [2]: biopsy specimens with classic GCA (with histological evidence of transmural infiltration, predominantly of lymphomononuclear cells, with or without giant cells); biopsy specimens with SVV surrounding an uninfamed temporal artery; and biopsy specimens with VVV in the adventitia as the only lesion observed in the temporal artery. Four parameters (score range 0–4), as described by Hernández-Rodríguez J *et al.* [13], were used to evaluate the baseline inflammatory response at the time of diagnosis: fever, weight loss, an ESR of ≥ 85 mm/h and a haemoglobin level < 11.0 mg/dl. Patients were considered to have a weak inflammatory response if they had two or fewer inflammatory parameters, and a strong inflammatory response if three or four parameters were present. The controls comprised 15 histologically normal temporal artery samples from 15 consecutive patients (10 women, 5 men; median 74 years, range: 60–84 years) with suspected GCA but with negative biopsy results. The ultimate diagnoses in these patients were: fever of unknown origin (five patients), isolated PMR (five patients), non-specific headache in the presence of OA (five patients). This study was approved by the Ethics Committee of the University of Palermo. Signed informed consent for the collection and storage of biological material was also obtained from all the patients enrolled in this study. All patients gave their informed consent before enrolment into the study.

TABLE 1 List of antibodies and primers used

Antibodies	Source	Primers
Rabbit polyclonal anti-human IL-8	Novus Biological, Littleton CO; 1:100	IL-9 (Hs00914237_m1)
Monoclonal mouse anti-human IL-9	Novus Biological, Littleton CO; 1:200	IL-9R (Hs01108522_m1)
Rabbit polyclonal anti-human IL-17	Sigma Aldrich, St Louis, MO; 1:250	IL-4 (Hs00174122_m1)
Rabbit polyclonal anti-human IL-9R	Novus Biological, Littleton CO; 1:100	IL-17A (Hs00174383_m1)
Rabbit polyclonal anti-human IL-4	AbCam, Cambridge, UK; 1:250	TSLP (Hs00263639_m1)
Rabbit polyclonal anti-human TGF- β	AbCam, Cambridge, UK; 1:250	TGF- β (Hs00998133_m1)
Rabbit polyclonal anti-human TSLP	AbCam, Cambridge, UK; 1:250	GAPDH (Hs99999905_m1)
Mouse IgG1 monoclonal antibody	AbCam, Cambridge, UK	
Rabbit IgG polyclonal antibody	AbCam, Cambridge, UK	
Rabbit polyclonal anti-human PU.1	AbCam, Cambridge, UK	
Mouse monoclonal anti-human CD15	Dako, Glostrup, Denmark	

Immunohistochemical analysis of GCA biopsy samples

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5- μ m-thick paraffin-embedded sections from arteries and from tonsils used as positive controls, as previously described [9]. A list of primary antibodies is provided in Table 1. The number of immunoreactive cells was determined by counting positively stained cells on photomicrographs obtained from three random high-power microscopic fields ($\times 400$ magnification) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera (Leica, Rijswijk, The Netherlands). Staining was semi-quantitatively scored on a four-point scale (range: 0–3) at $\times 200$ magnification as previously described [14]: score of 0 = no or minimal staining, score of 1 = up to 40% positive cells, score of 2 = 40–60% positive cells and score of 3 = staining of >60% of the cells. Double staining was performed on paraffin-embedded sections of arteries in order to characterize Th9 cells and IL-9R-expressing cells. Sections were incubated with primary anti-human IL-9 and PU.1 antibodies (Th9 cells), IL-9 and IL-17 (Th17 cells), and IL-9R and CD15 (neutrophils), then treated with FITC- or rhodamine red-conjugated anti-rabbit or anti-mouse antibodies plus RNasi (200 mg/ml) and counterstained using Toto-3 iodide (642/660; Invitrogen). Confocal analysis was used to acquire fluorescence staining.

RNA extraction from artery biopsies and quantitative TaqMan RT-PCR

Temporal artery biopsies prepared soon after removal were also stored in RNAlater solution (Applied Biosystems, Foster City, CA, USA). For quantitative TaqMan real-time PCR, sets of primers and probes were obtained from Applied Biosystems and are listed in Table 1. Samples were run in triplicate using the Step-One Real-Time PCR System (Applied Biosystems). Relative changes in gene expression between controls and patients were determined using the $\Delta\Delta C_t$ method, using GAPDH to normalize target gene levels in each

sample as previously described [9]. Final values were expressed as relative expression between patients and controls.

Statistical analysis

Statistical analysis of quantitative variables was performed using the Mann-Whitney rank-sum test. Pearson's correlation analysis was utilized to quantify the expression associations between the genes of interest. $P < 0.05$ was considered significant.

Results

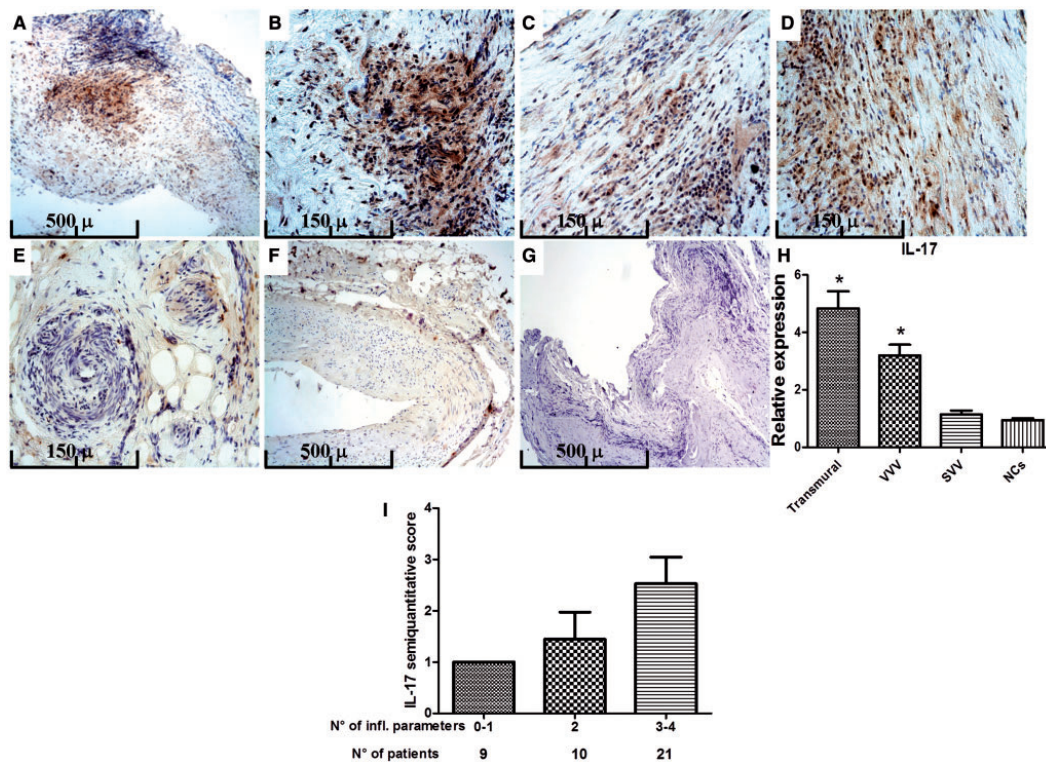
Histological characterization

Classic transmural inflammation was observed in 23 GCA patients (12 with giant cells and granulomatous reaction). SVV was observed in 10 patients and VVV in 7. None of the GCA patients, regardless of the histologic phenotype, had clinical manifestations suggesting a systemic vasculitis other than GCA when temporal artery biopsy was performed or during the follow-up period (median 18 months, range 8–33 months). All but the 9 patients with transmural inflammation, 7 of the 10 patients with SVV, and 3 of the VVV patients had a weak inflammatory response (two or fewer inflammatory parameters). No differences between the three histological groups were observed with respect to the time from onset of symptoms to diagnosis.

IL-17 overexpression predominates in arteries with transmural inflammation and VVV

IL-17 has previously been demonstrated to be involved in the pathogenesis of GCA [5]. In our series, a significant IL-17 overexpression was present in the arteries with transmural inflammation (Fig. 1A and B) and was more pronounced in those displaying granulomatous reaction (Fig. 1A and B) compared with those without granuloma (Fig. 1C). Significant IL-17 immunoreactivity was also observed in the arteries of GCA patients with VVV (Fig. 1D), was virtually absent in SVV (Fig. 1E), and was never observed in normal controls (Fig. 1F; Fig. 1G shows isotype control staining). Expression of IL-17 in arteries

Fig. 1 IL-17 expression in GCA



IL-17 expression in arteries with transmural inflammation and granulomatous reaction (**A** and **B**), with transmural inflammation without granulomatous reaction (**C**), with VVV (**D**) and with SVV (**E**). (**F**) IL-17 expression in controls. (**H**) m-RNA expression of IL-17 was assessed by quantitative RT-PCR in artery samples obtained from 17 GCA patients and 10 control subjects. (**I**) Correlation between IL-17 semi-quantitative score systemic inflammatory response. (**B-E**) Original magnification $\times 250$, (**A, F** and **G**) $\times 100$. (**H**) Data are expressed as mean (s.e.m.); * $P < 0.05$ compared with SVV and NCS.

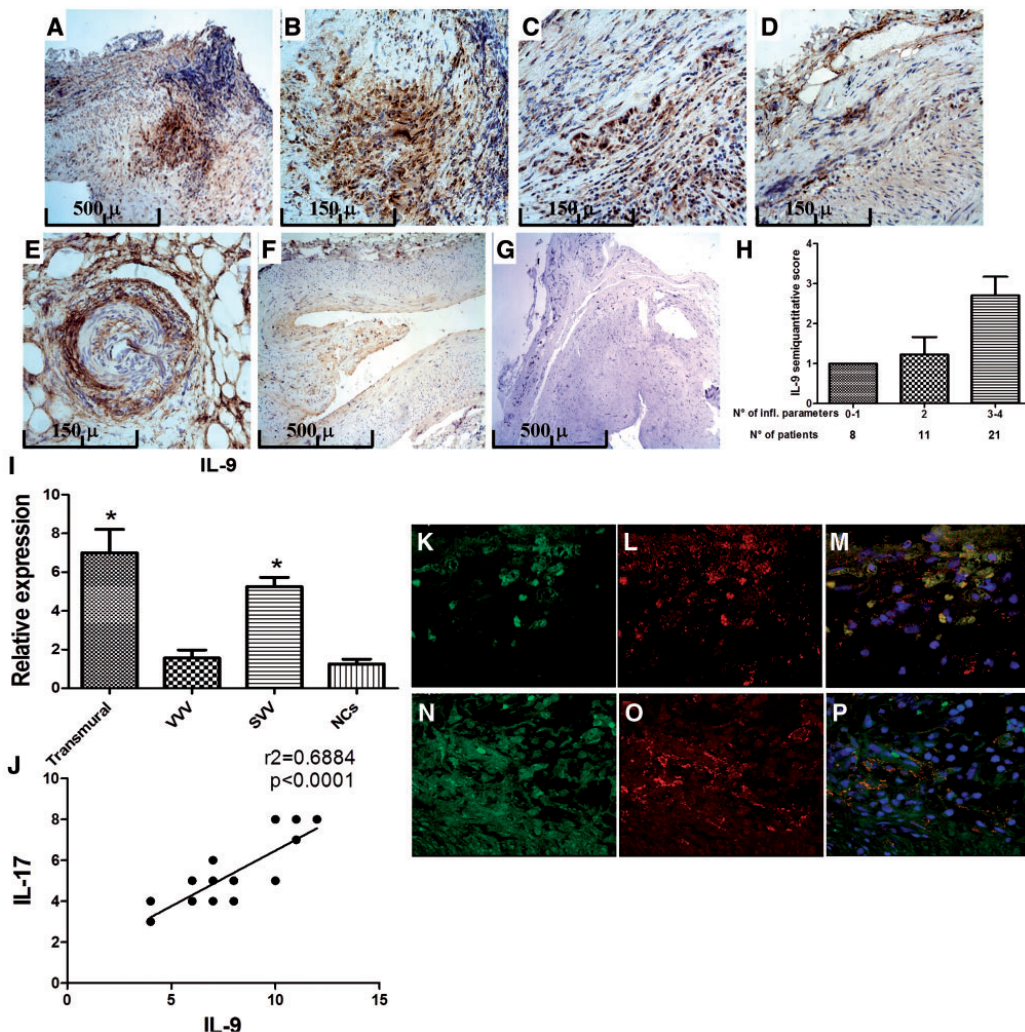
with transmural inflammation and VVV was confirmed by RT-PCR (Fig. 1H). In patients with transmural inflammation and VVV, the highest scores for IL-17 expression were associated with the highest numbers of inflammation parameters as described in the Patients and methods section (Fig. 1I).

Th9 polarization predominates in arteries with transmural inflammation and SVV

Various patterns of IL-9 expression were observed in GCA patients. Significantly higher IL-9 expression characterized temporal arteries with classic transmural inflammation, independent of the presence of granuloma (Fig. 2A-C) or SVV (Fig. 2E). Conversely, in VVV IL-9 was only weakly expressed (Fig. 2D), and it was virtually absent in normal controls (Fig. 2F; Fig. 2G shows isotype control staining). Inflammatory cells infiltrating the artery wall and giant cells, where present, were positive for IL-9 immunostaining (Fig. 2A-E). IL-9 positivity was also observed in endothelial cells of vessels scattered throughout the inflammatory infiltrates and among vascular smooth muscle cells (VSMCs) (Fig. 2A-E). IL-9 tissue

expression was correlated with the intensity of the systemic inflammatory response (Fig. 2H). Significantly increased IL-9 expression was, in fact, observed in patients with a strong versus those with a weak systemic inflammatory response. IL-9 expression, in particular, correlated with acute-phase reactants, including ESR ($r = 0.4322$, $P = 0.002$) and CRP concentration ($r = 0.3435$, $P = 0.003$) (data not shown). IL-9 expression was also evaluated by quantitative RT-PCR. As shown in Fig. 2I, IL-9 was predominantly expressed in arteries with transmural inflammation and SVV compared with VVV and normal controls. In patients with transmural inflammation, IL-9 expression was strongly and directly correlated with IL-17 expression (Fig. 2J). Analysis of the tissue distribution of IL-17- and IL-9-expressing cells in arteries with transmural inflammation suggested a different cellular source for these cytokines in the context of mononuclear cells and an apparent co-localization with giant cells. To better clarify the cellular source of IL-9-producing cells, we also performed confocal microscopy analyses. As shown in Fig. 2K-M, significant co-localization was observed between IL-9 and PU.1, but not

Fig. 2 IL-9 expression in GCA



IL-9 in arteries with transmural inflammation and granuloma (A and B), without granuloma (C), with VVV (D), with SVV (E) and in controls (F). (G) Isotype control antibody staining. (H) IL-9 semi-quantitative score in arteries with transmural inflammation ($n = 23$), SVV ($n = 10$) and VVV ($n = 7$) was correlated with the systemic inflammatory response. (I) m-RNA expression of IL-9 in arteries from 17 GCA patients and 10 controls. (J) Correlation between IL-9 and IL-17 expression in arteries with transmural inflammation. (K-M) IL-9 (K) and PU.1 (L). (M) Merged double staining for IL-9 and PU.1. (N and O) IL-9 (N) and IL-17 (O). (P) Merged double staining for IL-9 and IL-17. (A, F and G) original magnification $\times 100$; (B-E) $\times 250$; (K-P) $\times 400$. (I) Data are expressed as mean (s.e.m.); * $P < 0.05$ compared with SVV and NCs.

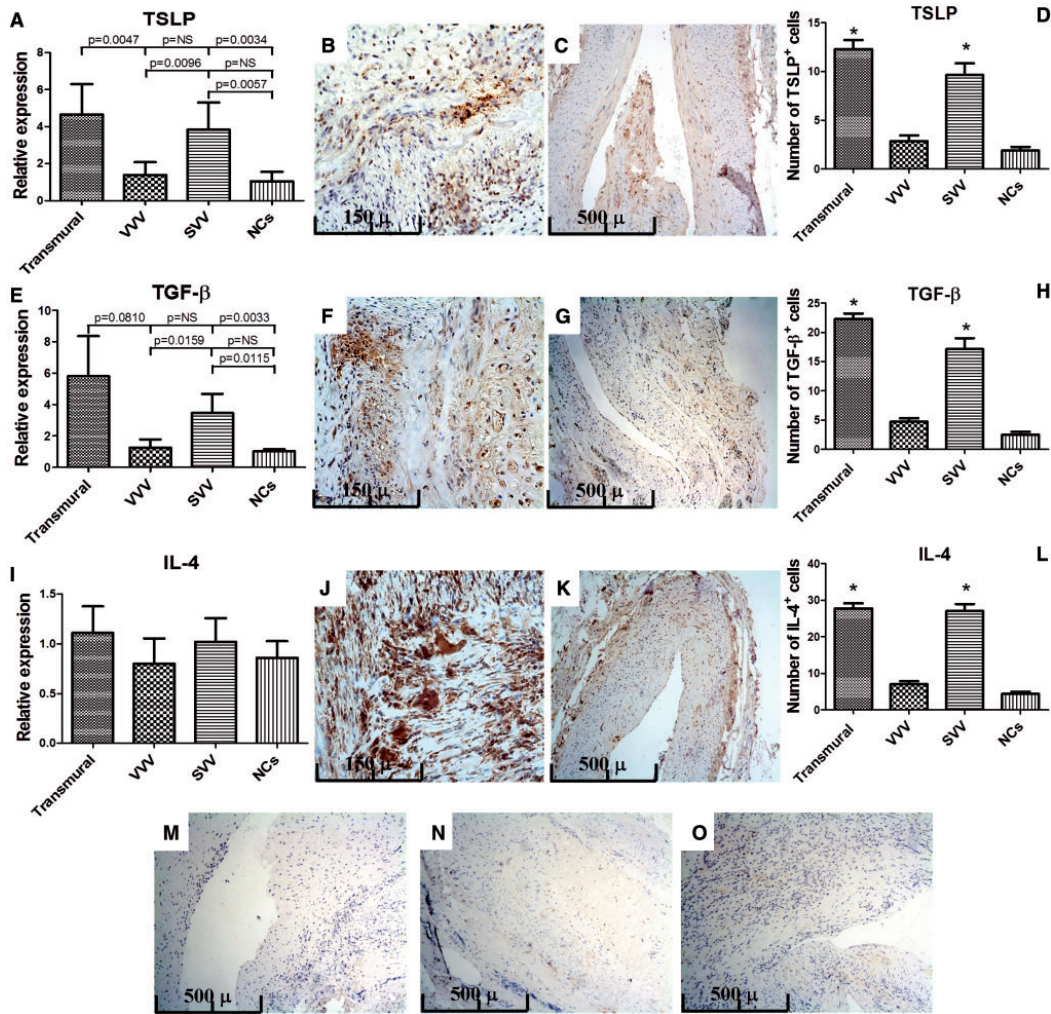
between IL-9 and IL-17 (Fig. 2N-P), suggesting the prevalent Th9 phenotype of mononuclear IL-9-producing cells. Interestingly, giant cells, occasionally scattered throughout the inflamed arteries, showed an intense co-expression of IL-9 and IL-17 (Fig. 2P).

IL-4, TGF- β and TSLP are overexpressed in GCA arteries

Because of the intense tissue expression of IL-9, we also evaluated the behaviour of IL-4, TGF- β and TSLP, which

are known to be involved in the modulation of IL-9 production [11, 12]. RT-PCR analysis demonstrated a significant upregulation of TSLP (Fig. 3A) and TGF- β (Fig. 3E) but not of IL-4 (Fig. 3I) in GCA arteries with transmural inflammation and SVV compared with VVV and control arteries. Immunohistochemical analysis, however, demonstrated a significant overexpression of TSLP (Fig. 3B-D), TGF- β (Fig. 3F-H) and IL-4 (Fig. 3J-L) in transmural inflamed arteries and SVV compared with VVV and controls (Fig. 3M-O shows isotype control staining for TSLP, TGF- β and IL-4, respectively).

Fig. 3 IL-4, TGF- β and TSLP are overexpressed in GCA arteries



(A, E and H) m-RNA expression of TSLP (A), TGF- β (E) and IL-4 (I) in arteries obtained from GCA patients (7 with transmural inflammation, 5 with VVV and 5 with SVV) and 10 controls. (B, C, F, G, J and K) Representative microphotographs showing TSLP (B and C), TGF- β (F and G) and IL-4 (J and K) immunostainings. (D, H and L) Quantification of TSLP- (D), TGF- β - (H) and IL-4- (L) positive cells. (M and N) Isotype control staining for TSLP (M), TGF- β (N) and IL-4 (O). (B, F and J) Original magnification $\times 250$, (C, G, K, M-O) $\times 100$. Data are expressed as mean (S.E.M.); * $P < 0.05$.

IL-9R is expressed mainly on the surface of neutrophils and is accompanied by IL-8 expression

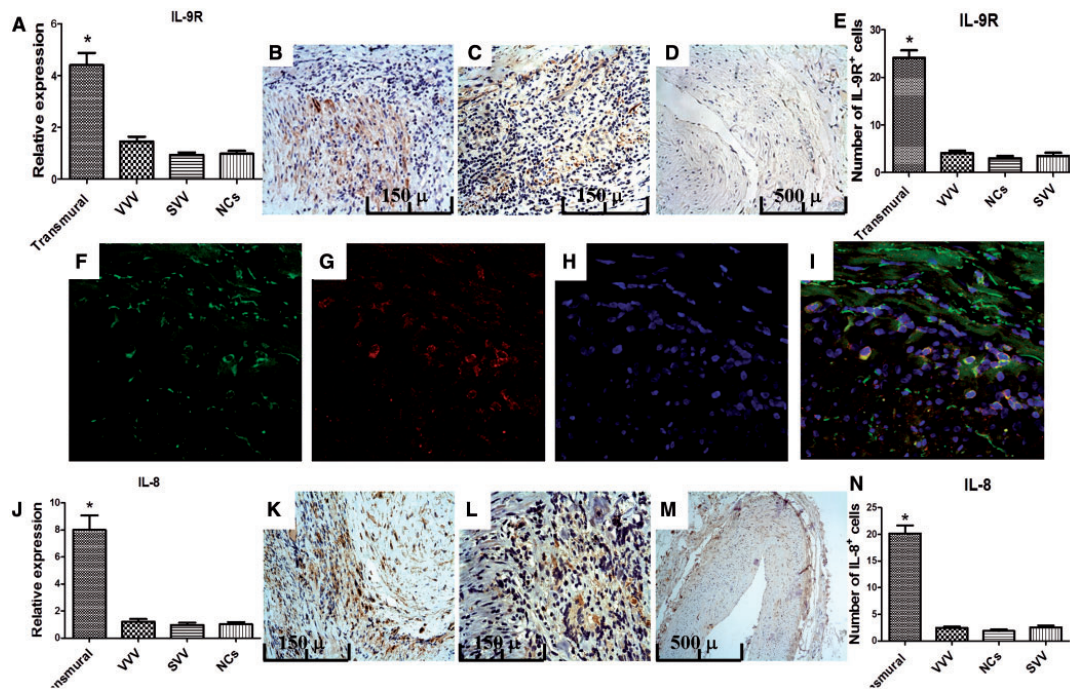
We also evaluated the expression of IL-9R. GCA arteries with transmural inflammation displayed the more intense expression for IL-9R (Fig. 4A-C and E). By immunohistochemistry, IL-9R positivity was observed among VSMCs (Fig. 4B), endothelial cells of vessels distributed in the inflammatory infiltrates and cells with multinucleated nuclei strongly resembling neutrophils (Fig. 4C). Significant infiltration by neutrophils was confirmed by specific staining in patients with transmural inflammation and by confocal microscopy analysis (Fig. 4F-I), which showed that neutrophils are the main IL-9R-expressing cell type in GCA arteries (Fig. 4I).

Since it is known that IL-9R activation in human neutrophils results in IL-8 release [15], we next evaluated the expression of IL-8 in arteries from GCA patients. IL-8 expression was significantly modulated only in arteries with transmural inflammation (Fig. 4J-L and N). Expression of IL-8 was observed mainly among infiltrating mononuclear cells, VSMCs and neovessels (Fig. 4K-L; D and M show isotype control staining for IL-8 and IL-8R, respectively).

IL-9 expression is not modified by glucocorticoid treatment

To analyse the effect of glucocorticoid therapy on IL-9 and IL-17 expression, we compared their protein levels in

Fig. 4 IL-9R is expressed on neutrophils in GCA arteries and accompanied by IL-8 overexpression



(A) m-RNA expression of IL-9R in arteries of GCA patients (7 with transmural inflammation, 5 with VVV and 5 with SVV) and 10 controls. (B–D) Representative microphotographs showing IL-9R immunostainings in GCA (B and C) and controls (D), with quantification of positive cells (E). (F–I) Single staining for IL-9R (F), CD15 (G) and toto-3 (H); (I) merged double staining for IL-9R (green) and CD15 (red). (J) m-RNA expression of IL-8. (K–M) IL-8 immunostainings in GCA (K and L) and controls (M), with quantification of IL-8-positive cells (N). (B, C, F–I, K and L) Original magnification $\times 250$; (D and M) $\times 100$. Data are expressed as mean (S.E.M.); $*P < 0.05$.

arteries from five glucocorticoid-treated GCA patients. As shown in Fig. 5, in glucocorticoid-treated arteries the expression of IL-17 and the numbers of IL-17-immunoreactive cells was significantly decreased (Fig. 5A–C). Conversely, there was no statistically significant reduction in the numbers of IL-9-immunoreactive cells in specimens obtained from glucocorticoid-treated patients (Fig. 5D–F).

Discussion

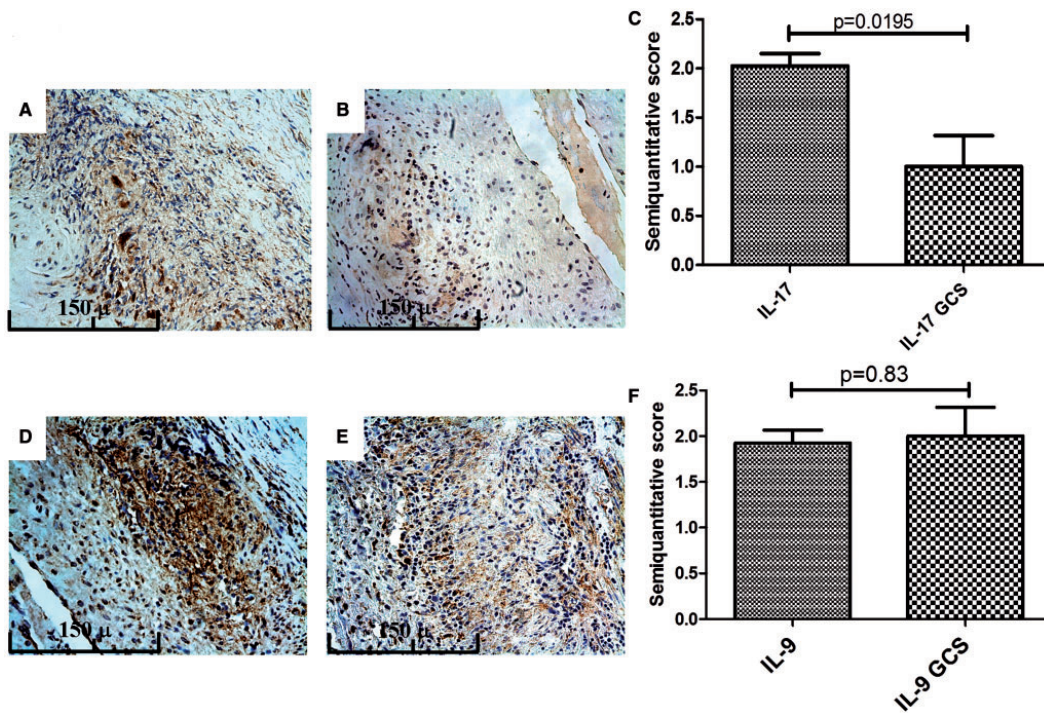
In GCA, two main cytokine pathways—the IL-6/IL-17 axis and the IL-12/IFN- γ axis—have been demonstrated to play a prominent role in the pathogenesis of the vasculitic process [16]. Yet the potential involvement of other cytokine pathways and/or their expression correlated with particular histological patterns has not been adequately studied. In this study, we evaluated the expression of two cytokine pathways, IL-9/Th9 and IL-17/Th17, in various histological subsets of GCA patients.

Distinct immunological signatures were observed in the various subsets of GCA. IL-17 expression was observed mainly in those arteries displaying granulomatous transmural inflammation and VVV, and was virtually absent in those with SVV. Conversely, intense IL-9 immunoreactivity

was observed in arteries with granulomatous transmural inflammation and SVV, compared with arteries with VVV. Interestingly, a strong co-expression of IL-9 and IL-17 was observed in giant cells, highlighting the pro-inflammatory role of these cells in GCA. The expression of both IL-9 and IL-17 was strongly and directly correlated with the intensity of the systemic inflammatory response, supporting a prominent pro-inflammatory role for these cytokines. We also evaluated the role of glucocorticoid treatment in the expression of IL-9 and IL-17. Studies conducted on small series have, in fact, previously shown that IL-17A-expressing cells are dramatically reduced in specimens obtained from glucocorticoid-treated patients [5, 17]. In our study, IL-17 expression was significantly reduced by glucocorticoid treatment, with IL-9 being only marginally modified, indicating a potential role for Th9 cells in glucocorticoid-resistant GCA.

In GCA arteries with transmural inflammation, IL-9-expressing cells were to a large extent Th9 cells, since they co-expressed the specific Th9 transcription factor PU.1. Th9 cells are a distinct subpopulation of CD4⁺ effector T cells that preferentially secrete high levels of IL-9, requiring TGF- β and IL-4 for their differentiation. These cytokines induce expression of the transcription factors

Fig. 5 Artery specimens from five GCA patients treated with glucocorticoids at the time of biopsy were evaluated for the expression of IL-17 and IL-9



(**A** and **B**) Representative staining of IL-17 in arteries obtained from GC-treated patients. (**C**) Semi-quantitative analysis of IL-17 expression in GC-treated arteries compared with untreated GCA arteries. (**D–F**) Representative stainings of IL-9 in arteries obtained from GC-treated patients. (**F**) Semi-quantitative analysis of IL-9 expression in GC-treated arteries compared with untreated arteries. (**A**, **B**, **D** and **E**) Original magnification $\times 250$. (**C** and **F**) Data are expressed as mean (s.e.m.). GC: glucocorticoids.

PU.1/Spi-1 and IFN regulatory factor 4, which subsequently regulate expression of the IL-9 gene [11]. More recently, it has been demonstrated that Th9 cells also express greater amounts of the TSLP receptor than Th2 cells, and addition of TSLP to Th9 cultures increases IL-9 production *in vitro* [12]. In our study, arteries with higher expression of IL-9 (transmural inflammation with granulomatous reaction and SVV) displayed intense overexpression of IL-4, TGF- β and TSLP, suggesting that the upstream Th9 cytokine network is also upregulated in GCA. Interestingly, basal TSLP expression was also observed in VSMCs of normal arteries, suggesting constitutive expression of this cytokine in physiological conditions. Despite the absence of IL-4 upregulation at m-RNA level, IL-4 evaluation by immunohistochemistry demonstrated its strong upregulation in inflamed arteries.

IL-9 expression was also accompanied by significant overexpression of the specific IL-9 receptor on neutrophils. A potential role for neutrophils in GCA disease progression has recently been hypothesized, providing evidence of a role for neutrophil phenotypic changes in GCA pathology [18]. Functional expression of IL-9R by

human neutrophils has been demonstrated in asthmatic patients to have an important role in IL-8 release [15]. In this regard, high levels of IL-8 were observed in GCA, especially in patients with transmural inflammation, providing a functional immunological link between IL-9R expression and neutrophil activity in GCA arteries. Taken together, these findings seem to suggest the relevance and functionality of the IL-9 axis and highlight the importance of neutrophils in the pathogenesis of GCA.

In conclusion, in this study we confirmed a role for Th17 in the pathogenesis of GCA and for the first time demonstrated a putative pro-inflammatory role for IL-9 and Th9 cells in the pathogenesis of GCA. We have also provided the first evidence that distinct populations of potentially autoreactive T cells expressing particular cytokines (Th17 vs Th9) characterize patients with particular histological subsets of GCA, and may thus contribute to the heterogeneity of tissue lesions observed in these patients. The concomitant presence of a Th9 response may be responsible for the chronicity of tissue damage with the emergence of more severe tissue inflammation.

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