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Macrophage diversity in the pathogenesis of vasculitides

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CHAPTER 5

CD8+ T cells infiltration is associated with lesional GM-CSF expression in giant cell arteritis

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WORK IN PROGRESS

ABSTRACT

Background

Giant cell arteritis (GCA) is a large-vessel vasculitis characterized by granulomatous T cell and macrophage infiltration. Granulocyte-macrophage colony stimulating factor (GM-CSF) is present in GCA-affected vessels where it may be involved in skewing of tissue destructive macrophages. Furthermore, a recent phase 2 clinical trial demonstrated that GM-CSF receptor blockade was superior to placebo at 26 weeks in GCA. However, the cellular source of GM-CSF in GCA is still unclear.

Objectives

As T cells have been identified as a major source of GM-CSF in other immune-mediated diseases such as multiple sclerosis, we investigated whether T cells are GM-CSF producers in GCA patients, both systemically and locally.

Methods

GM-CSF production capacity by PMA-stimulated PBMCs from newly diagnosed, untreated GCA patients and HCs (n=10 each) was assessed using flow cytometry. Temporal artery biopsies (TABs, n=5) were immunohistochemically stained for CD3, CD8 and GM-CSF. Colocalization of these markers was assessed with immunofluorescence.

Results

Proportions of CD4+ (median GCA=3.1%; HCs=2.76%) and CD8+ (median GCA=5.8%; HCs=5.8%) T cells produced GM-CSF after *in vitro* stimulation, but no significant differences were found between the groups. Immunofluorescence staining confirmed that CD3+ T cells (both CD8+ and CD8-) were positive for GM-CSF in TABs. Interestingly, GM-CSF positivity in TABs correlated strongly with the extent of CD8+ T-cell infiltration (r=0.74, p<0.01), but not with CD3+ T-cell infiltration (r=0.38, p=0.16).

Conclusion

Our data imply that T cells are an important source of GM-CSF in GCA lesions. The correlation of the extent of CD8+ T-cell infiltration with GM-CSF positivity suggests that CD8+ T cells are the major source of local GM-CSF overexpression in tissue.

INTRODUCTION

Giant cell arteritis (GCA) is an inflammatory disease affecting medium- to large-sized arteries in patients above 50 years of age. Depending on the arteries involved, GCA patients may present with various signs and symptoms. Vasculitis of the cranial arteries often presents as headache and symptoms such as jaw claudication and vision loss, while the involvement of larger arteries (such as the aorta) may lead to limb claudication, aortic aneurysm development and dissection (1). To date, high-dose and long-term glucocorticoid (GC) treatment still remains the standard treatment for GCA. However, long-term usage of GC may lead to the development of severe adverse effects including diabetes, hypertension, glaucoma, osteoporosis and malignancy (2). More recently, interleukin-6 (IL-6) receptor blockage (tocilizumab), was shown to be effective as a GC-sparing treatment for GCA (3). However, even with tocilizumab treatment, up to 44% of patients do not achieve longstanding remission emphasizing that there is still a clinical need for more specific and remission inducing treatment strategies for GCA.

Histologically, GCA is characterized by granulomatous inflammation of the vessel wall comprising mainly of T cells and macrophages. The infiltrating lymphocytes produce pro-inflammatory mediators that further prime and activate infiltrating macrophages, leading to the production of tissue destructive and remodeling factors such as matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) (4). One such mediator that is currently gaining attention for its role in the pathogenesis of GCA is the granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF is a hematopoietic growth factor that promotes the survival and maturation of myeloid cells and that can be expressed by a wide range of both hematopoietic and non-hematopoietic cells (5). In recent years, studies have pointed to a critical role of GM-CSF in the pathogenesis of various autoimmune and inflammatory disorders such as multiple sclerosis (MS), rheumatoid arthritis and spondyloarthritis (6–9). In these diseases, both CD4 and CD8+ T cells have been associated with increased GM-CSF expression (7,9–11).

In GCA, the first report on GM-CSF mRNA expression in GCA lesions was published more than a decade ago (12). Subsequently, it was shown that activated peripheral blood mononuclear cells (PBMCs) from GCA patients produce higher levels of GM-CSF compared to PBMCs from healthy controls (HCs) *in vitro* (13). Its importance in the pathology of GCA however, has only just recently become appreciated due to several important findings. Our prior work showed the expression of GM-CSF in GCA-affected vessels and we postulated that GM-CSF may play an important role in the local skewing of tissue destructive macrophages (14). Ongoing preliminary studies confirm the high expression levels of GM-CSF in inflamed GCA vessels compared to noninflamed control vessels (15). Pre-clinical studies of GM-CSF receptor blocking in an engrafted artery mouse model showed a reduction in leukocyte infiltration, neo-vessel formation and intimal hyperplasia (16). Recently, a phase II clinical trial of GM-CSF receptor blocking

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(NCT03827018) in GCA showed an encouraging 62% reduction in risk of flare compared to placebo (17). Collectively, these studies emphasize the importance of GM-CSF in the vasculopathy of GCA. However, to date, the cellular source of GM-CSF production in GCA remains to be identified. In multiple sclerosis, another immune mediated disease, both CD4+ and CD8+ T cells have been implicated as the main producers of GM-CSF (7,9–11).

These observations prompted us to investigate whether T cells play a major role in GM-CSF production in the vasculopathy of GCA, both systematically and locally. To this end, we first assessed the frequencies of GM-CSF producing T-cell subsets in a cohort of baseline GCA patients and patients in glucocorticoid-free remission (GC-FR) compared to healthy controls (HCs). We next evaluated the expression of GM-CSF by lesional T cells in temporal artery biopsies obtained from treatment naïve GCA patients using immunohistochemistry and immunofluorescence analysis.

MATERIALS AND METHODS

Patient characteristics

For flow cytometry experiments, newly diagnosed GCA (baseline) patients, GCA patients in glucocorticoid free remission (GC-FR) and healthy controls (HCs) (n=10 each) were included (Table 1). GCA baseline patients did not receive any glucocorticoids or other disease modifying anti-rheumatic drugs prior to blood withdrawal. GCA GC-FR patients were symptom-free and did not receive glucocorticoids in the three months prior and 6 months after blood withdrawal. Health status of HCs was determined by a clinician through questionnaires, physical examination and lab tests. Only healthy HCs without any inflammatory conditions were included.

For immunohistochemistry, five inflamed temporal artery biopsy (TAB) tissue samples from treatment naïve histologically proven GCA patients were included. The diagnosis of GCA was based on a pathologist's assessment of a positive panarteritic TAB. Three non-inflamed TABs from PET proven GCA patients (n=2) and polymyalgia rheumatica (PMR) patient (n=1) were included as controls. The study was approved by the institutional review board of the University Medical Center Groningen (METc2010/222). Written informed consent was obtained from all study participants. All procedures were in compliance with the Declaration of Helsinki.

Table 1. Patient characteristics

	HC	GCA baseline	GCA GC-FR
n	10	10	10
Age (yr); median	71	68	74
Sex (M/F)	4/6	3/7	2/8
CRP (mg/L); median (range)	5 (0.5-5)	52.5 (4.7-138)	4 (0.4-13)
ESR (mm/h); median (range)	7 (2-28)	77 (7-106)	13.5 (5-43)

PBMC stimulation and flow cytometric analysis

To assess the capacity of circulating/peripheral blood T cells to produce cytokines after *in vitro* stimulation, we used cryopreserved PBMCs that were thawed with Roswell Park Memorial Institute 1640 (RPMI, Lonza, Basel, Switzerland) +10% fetal calf serum (FCS). After thawing, $1x10^6$ cells/100µL in RPMI+5% FCS were stimulated with phorbol 12-myristate 13-acetate (PMA, 5ng/mL), calcium ionophore A23187 (0.16µg/mL) and incubated with brefeldin A (10µg/mL) for 16 hours at 37°C + 5% CO_2 in polypropylene tubes. From each patient, one unstimulated sample of $1x10^6$ cells/100µL incubated with brefeldin A (10µg/mL) was used to facilitate gate setting.

After stimulation, viability was assessed by incubating the cells for 15 minutes at room temperature (RT) with a Zombie NIR fixable viability kit (1:1000, Biolegend, San Diego, California, USA). Next, cells were incubated with fluorochrome-conjugated monoclonal antibodies for 15 minutes at RT to assess the surface expression of CD3, CD4 and CD8 (Supplementary table 1). After fixation and permeabilization using FIX & PERM Cell Fixation & Cell permeabilization Kit reagents (Life Technologies, Carlsbad, California, USA), cells were stained intracellularly for 20 minutes at RT for IL-4, GM-CSF, IFN-γ and IL-17A (Supplementary table 1). GM-CSF expression was determined within type 1 CD4+ (Th1; IFN-γ+CD4+) and CD8+ T cells (Tc1; IFN-γ+CD8+), Th2 and Tc2 (IL-4+CD4+ and IL-4+CD8+) and Th17 and Tc17 cells (IL-17+CD4+ and IL-17+CD8+). Samples were measured on a BD-LSR-II flow cytometer. Gate settings for cytokine measurements were based on unstimulated samples. As stimulated cells downregulate CD4 expression, cytokine production is presented as the percentage of positive cells within CD8+ and CD8-CD3+ T cells (Supplementary figure 1).

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissues (3 µm) were deparaffinized and rehydrated, followed by antigen retrieval for 15 minutes in a microwave (for buffers, see Supplementary table 1). Tissues were incubated with primary antibodies (Supplementary table 2), followed by endogenous peroxidase blocking. The tissues were subsequently incubated with peroxidase labeled secondary antibodies, 3,3'Diaminobenzidine (DAB) (DAKO, Glostrup, Denmark) for peroxidase activity detection, and finally haematoxylin (MERCK, Kenilworth, NJ, USA) as a counterstain. Matching isotype controls were also included. Reactive appendix or tonsil tissues were used as positive controls. Stained slides were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U 10074-01; Hamamatsu Photonics K.K., Shizuoka, Japan).

Histo-score (H-score) analysis

For each tissue, a region of interest (ROI) was selected in representative areas that contained infiltrating cells. Three layers of the vessel wall (including intima, media and adventitia) were scored. The percentage of positive cells per layer was assessed with QuPath image analysis software (V0.2.2) (18). The relative intensity of each staining was

defined as absent=0, weak but detectable above control=1, moderate=2 and strong=3 (see Supplementary figure 2), as assessed by two independent investigators. The final H-score was calculated as the sum of the relative intensity of specific staining multiplied by the percentage of positive cells. As both CD8 and CD3 staining intensity were always strong, the intensities were always scored as 3.

Triple fluorescence multispectral imaging

Immunofluorescence stainings were carried out on paraffin TAB sections that were first deparaffinized and rehydrated (for the antibody panel used, see Supplementary table 3). Antigen retrieval was performed using Tris-EDTA buffer (pH 9) in a microwave for 15 minutes. Tissues were then incubated with anti-CD3 antibody overnight at 4oC. Subsequently, the tissues were incubated with anti-GM-CSF and anti-CD8 antibody cocktails for an hour followed by incubation with secondary antibody cocktail for another hour at room temperature. Finally, a cocktail of fluorescently tagged tertiary antibodies was applied and incubated for one hour. Afterward, autofluorescence was blocked with TrueView autofluorescence quenching kit (Vector Labs) for 5 minutes followed by counterstaining with DAPI for 10 minutes and sealed. Image cubes were captured at a magnification of 20X or 40X using Nuance Multispectral Imaging System 3.0.1 (PerkinElmer) using NuanceFX 3.0.1 software (PerkinElmer). To acquire the image cube, multiple wavelengths were used; 440:460 for DAPI, 490:530 for Alexa 488, 570:600 for Alexa 568, 710:720 for Alexa 647. Spectral unmixing was performed with spectral libraries of each fluorophore assigned different colors (DAPI = blue, AF488 = green, AF568 = red, AF647 = yellow), subtracting the background autofluorescence. Colocalization of CD3 and GM-CSF was assigned the color magenta, while the colocalization of CD8 and GM-CSF was assigned the color cyan.

Statistical analysis

Flow cytometry data was analyzed with Kaluza Analysis Software (Beckman Coulter). Graphs were created with GraphPad Prism version 8 (GraphPad Software, San Diego, USA). Two-tailed Kruskal-Wallis tests were used to compare multiple groups. If the Kruskal-Wallis test was significant, the Mann-Whitney U-test was then used to compare two groups. Correlation analyses were performed using Spearman's correlation. P-value < 0.05 was considered statistically significant.

RESULTS

No differences in frequencies of GM-CSF producing T cells and intensity of GM-CSF expression between HCs and GCA patients

To determine the capacity of T cells to produce GM-CSF in HCs and GCA patients, we assessed the frequencies, absolute numbers and the mean fluorescence intensity (MFI) of total GM-CSF-producing CD4+ and CD8+ T cells (Figure 1A-B). In all groups, variable proportions of CD4+ (range 0.10%-8.21% in HCs, 1.14%-5.29% in GCA baseline, 0.08%-

9.12% in GCA GC-FR) and CD8+ T cells (range 0.55%-15.64% in HCs, 0.79%-14.65% in GCA baseline, 0.61%-11.61% in GCA GC-FR) were capable of producing GM-CSF. There was also great variation in absolute counts of CD4+ (range 0.08-6.65 x 10^7 cells/L in HCs, 0.87-6.51 x 10^7 cells/L in GCA baseline, 0.18-10.31 x 10^7 cells/L in GCA GC-FR) and CD8+ T cells (range 0.09-4.38 x 10^7 cells/L in HCs, 0.08-6.60 x 10^7 cells/L in GCA baseline, 0.26-7.86 x 10^7 cells/L in GCA GC-FR) that produced GM-CSF. No statistically significant differences were found between HCs and GCA baseline and GCA GC-FR patients.

Next, we determined the frequencies of CD4+ and CD8+ T cell Th1/Tc1, Th2/Tc2 and Th17/Tc17 subsets that also expressed GM-CSF (Figure 1C-D). No significant differences were found regarding GM-CSF-expressing CD4+ and CD8+ T cell subsets between the three groups. Importantly, Th1 and Tc1 cells were the highest producers of GM-CSF in the circulation of patients (median Th1: 1.37% GCA baseline, 2.15% GCA GC-FR; Tc1: 2.89% GCA baseline, 3.11% GCA GC-FR) and controls (Median Th1: 1.37%; Tc1: 3.54%).

Infiltrating T cells in inflamed GCA vessel express GM-CSF

Immunohistochemical detection of CD3 and CD8 revealed that T-cell-infiltrates were abundant in inflamed TABs of GCA patients, primarily in the adventitia (n=5, Figure 2A, for isotype control, see Supplementary figure 3). The expression of GM-CSF was detected in all three layers of inflamed vessel walls including areas that are commonly rich in infiltrating T cells, macrophages (n=5, Figure 2A, for isotype control, see Supplementary figure 3). The expression of GM-CSF was also detected in resident vessel wall cells such as vascular smooth muscle cells, fibroblasts and vasa vasorum endothelial cells (Figure 2A). In non-inflamed TABs (skip lesions), GM-CSF positivity was also detected in endothelial cells of the vasa vasorum, medial smooth muscle cells and occasionally in intimal fibroblasts (Supplementary figure 4).
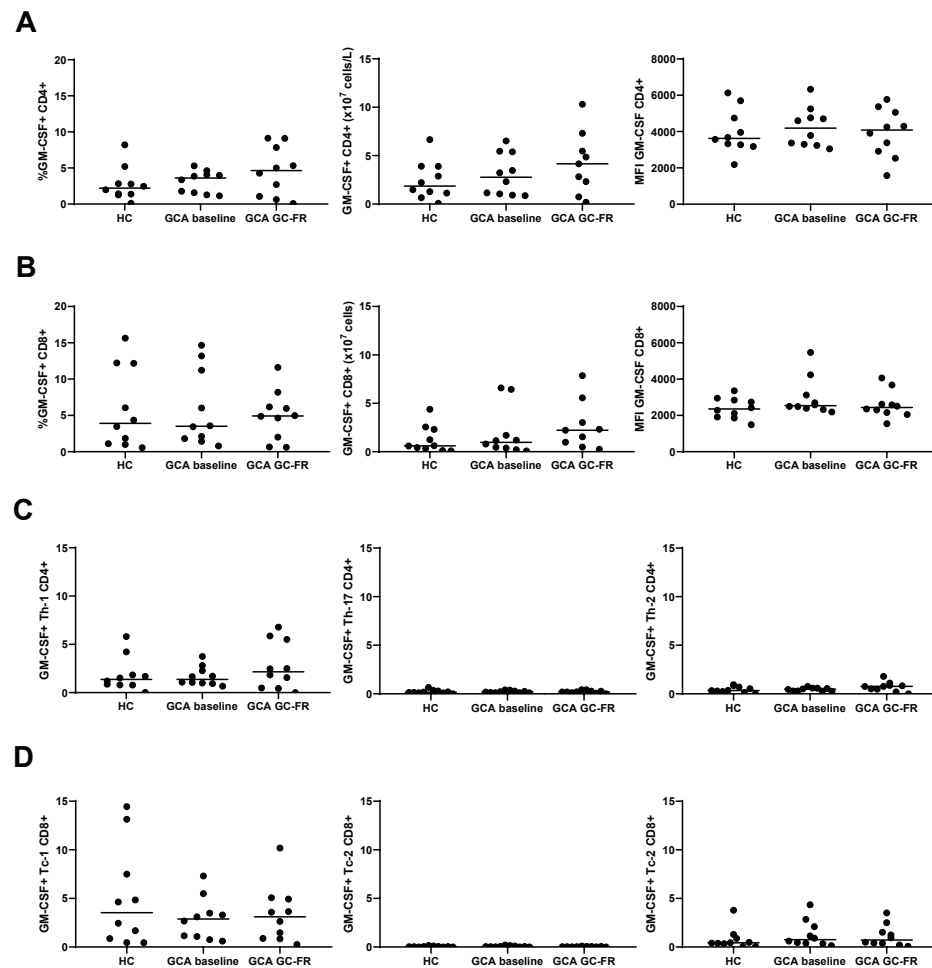
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Figure 1. GM-CSF-producing capacity of circulating T cells. The frequencies, absolute numbers and MFI of total GM-CSF-producing CD4+ (A) and CD8+ (B) T cells were determined after *in vitro* stimulation in HC, GCA at baseline and in GCA in GC-FR (n=10 each group). Then, GM-CSF-producing Th1, Th17 and Th2 (C) and GM-CSF-producing Tc1, Tc17 and Tc2 (D) cells were determined. Horizontal lines represent median values. No significant differences were found between the groups by Kruskal-Wallis tests.

Figure 2. Distribution of T cells and GM-CSF in GCA lesions. (A) GM-CSF, CD3 and CD8 expression was detected throughout the 3 layers of the vessel wall in TABs most prominently in the adventitia. The expression of GM-CSF was detected in T-cells and macrophage rich areas (red arrows), medial smooth muscle cells (green arrow), vasa vasorum endothelial cells (yellow arrows) and fibroblasts (blue arrows). (B) H-scores of the TABs (n =5) further corroborated our observation.

Consecutive staining showed that GM-CSF expression was detected in all layers of the vessel wall, but especially in areas of T-cell infiltration (Figure 2A) suggesting that infiltrating T cells, both CD3+ and CD8+ cells, are an important cellular source of GM-CSF in GCA lesions. Triple fluorescence staining for GM-CSF, CD3 and CD8 confirmed expression of GM-CSF by CD8 T cells and implicated GM-CSF expression by CD4 T cells as well, as can be seen in the regions positive for CD3+GM-CSF+ but negative for CD8 (Figure 3).
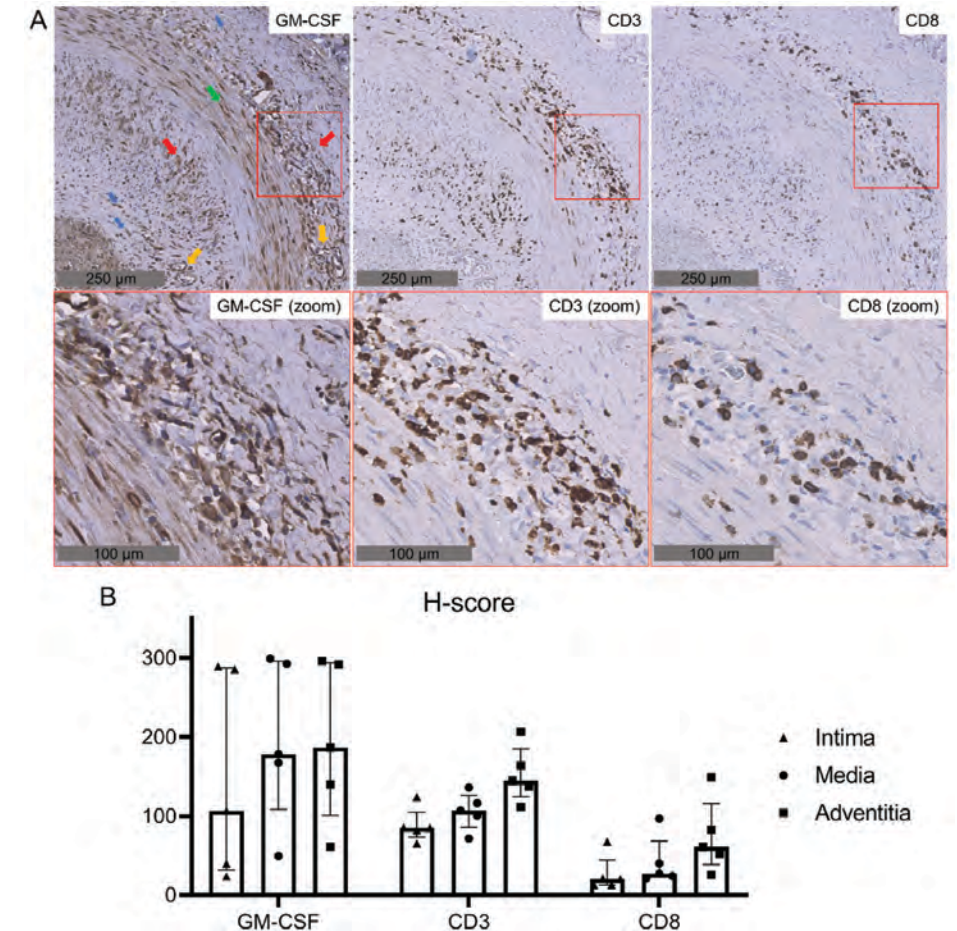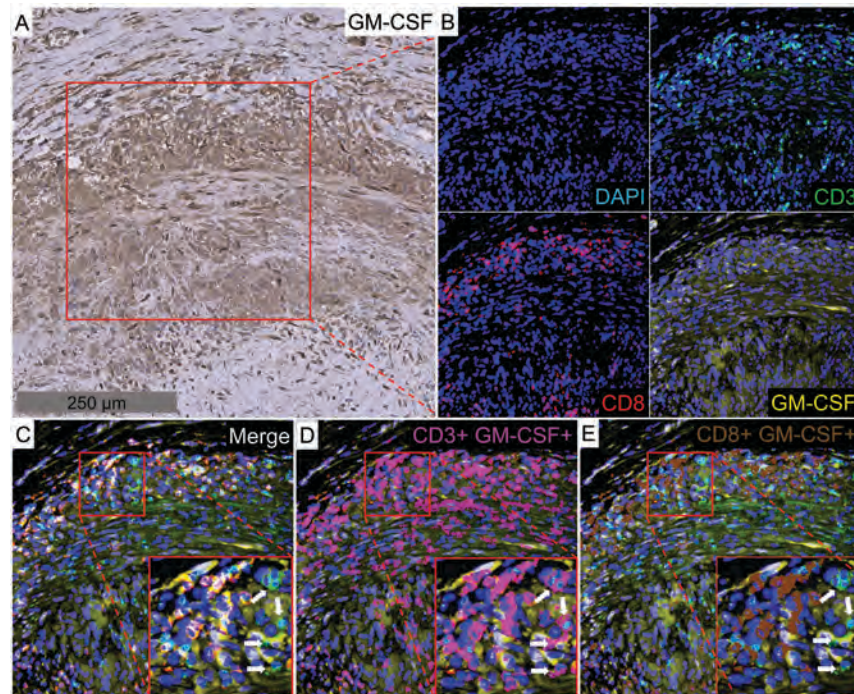
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Figure 3. T cells express GM-CSF in GCA lesion. (A) single staining IHC of GM-CSF. (B) Representative image showing triple fluorescent staining for CD3 (green), CD8 (red), GM-CSF (yellow) and nuclear marker DAPI (blue). (C) Merge image of the four immunofluorescence colours. (D) Overlapping pixels indicating the colocalization of CD3 and GM-CSF are represented in magenta. (E) Overlapping pixels indicating the colocalization of CD8 and GM-CSF, are represented in brown. GM-CSF expressing CD4+ T-cells (defined by CD3+CD8-, magenta positive brown negative cells) are indicated by the white arrows.

CD8+ T cells may be the major producer and orchestrator of GM-CSF overexpression in GCA lesions

As CD8+GM-CSF+ T cells were readily detected at the site of inflammation in GCA, we aimed to further investigate their relation with GM-CSF production. Further analysis of consecutive stainings (Figure 4A) revealed that the degree of GM-CSF positivity increased with the degree of CD8+ T-cell infiltration. Interestingly, GM-CSF positivity also increases in macrophage rich areas and fibroblast rich areas with the degree of CD8+ T-cell infiltration. To confirm our observations, we performed correlation analyses of GM-CSF positivity with the extent of T-cell infiltration. Indeed, GM-CSF positivity strongly correlated with the ratio of CD8/CD3 (r=0.83, p=0.0003, Figure 4B) and the degree of CD8 infiltration (r=0.74, p=0.0023, Figure 4C), but not with the degree of CD3 infiltration (r=0.38, p=0.1649, Figure 4D). Thus, although both CD4+ and CD8+ T cells express GM-CSF our data suggest that CD8+ T cells, in particular, are major producers and drivers of GM-CSF expression by other cells in GCA lesions.
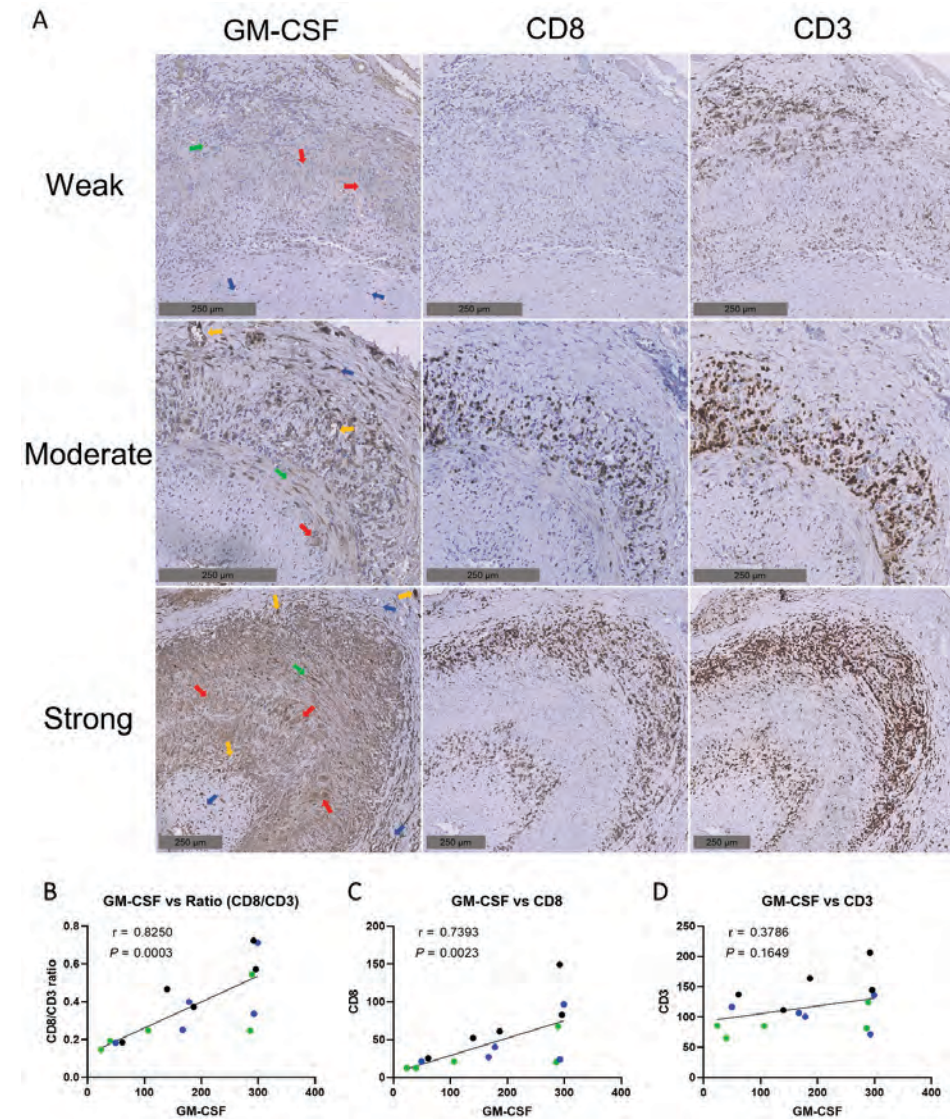

Figure 4. GM-CSF positivity increases with CD8+ T-cell infiltration. (A) Consecutive staining of GM-CSF, CD8 and CD3 demonstrated that the degree of GM-CSF positivity correlates with the abundancy of CD8+ cell infiltrates in the inflamed TAB tissue, not only in the T-cell rich areas but also in the macrophage/giant cell rich areas (red arrows), medial smooth muscle layers (green arrows) and fibroblasts rich areas (blue arrows). For correlation analysis, each data point represents the H-score from the 3 vessel wall layers (intima, media and adventitia) from 5 GCA affected vessels (n=15). (B) Correlation between GM-CSF positivity and the ratio between CD8+/CD3+ T cells (Spearman's r=0.83, P=0.0003). (C) Correlation between GM-CSF positivity and CD8+ T cell infiltration (Spearman's r=0.74, P=0.0023). (D) Correlation between GM-CSF positivity and CD3+ T cell infiltration (Spearman's r=0.38, P=0.1649). Green dots represent the H-score of the intima. Blue dots represent the H-score of the media. Black dots represent the H-score of the adventitia.

DISCUSSION

In the present study we investigated whether T cells play a major role in GM-CSF production in GCA, both systemically and in the inflamed vessel. Our data revealed that both CD4+ and CD8+ T cells express GM-CSF in GCA temporal artery tissue. Our data further implicate CD8+ as the major producer and enhancer of GM-CSF expression in inflamed GCA vessels. Analysis of peripheral blood samples did not reveal differences between patients and controls in the capacities of CD4 and CD8 T cells to produce GM-CSF.

The term granulocyte macrophage colony stimulating factor (GM-CSF) was originally defined based on its capacity to induce granulocyte and macrophage proliferation, differentiation and colony formation from bone marrow progenitor cells (19). More recently, GM-CSF has also been implicated as an important factor in the skewing of macrophages towards a proinflammatory and tissue destructive phenotype (14,20).

Our results revealed no differences in the frequencies of GM-CSF producing CD4+ and CD8+ T-cells in peripheral blood of GCA patients (active and remission) compared to those of HCs. However, both CD4+ and CD8+ T cells in inflamed TABs expressed GM-CSF. Although serum levels of GM-CSF are generally low, high levels of GM-CSF can be induced at sites of inflammation where it may act as a communication conduit between lymphocytes and myeloid cells that drives and amplifies the inflammatory processes (21,22). This characteristic of GM-CSF has also been implicated in the pathogenesis of multiple sclerosis (MS). In MS patients, and in line with our observations, frequencies of circulating GM-CSF-producing CD4+ T-cells are similar to those found in HCs. In contrast, high frequencies of GM-CSF+CD4+ T cells have been detected in the cerebrospinal fluid of MS patients (23). Similarly, in GCA, only low serum levels of GM-CSF have been reported that did not differ from those in HCs (24,25). Moreover, preliminary data from a preclinical artery graft mouse model and an *ex vivo* artery explant model confirmed that GM-CSF mRNA is indeed expressed in the inflamed vessel wall (15,16). Collectively, these studies further support the contention that T cell-derived GM-CSF may actively contribute to the vascular inflammatory process in GCA.

Our data also suggest that CD8+ T cells in particular may be an important source as well as a driver of GM-CSF expression in GCA. Although both CD4+ and CD8+ T cells expressed GM-CSF in inflamed TABs, the expression of GM-CSF increased with the degree of CD8+ T-cell infiltration. Interestingly, this increase in GM-CSF expression was observed not only in the T-cell dominated areas but also in areas dominated by macrophages/giant cells infiltration and in the fibroblast rich areas. All of these cells are known to be able to produce GM-CSF upon priming with cytokines such as IL-1β and TNF-α, both highly expressed in GCA lesions (14,26). Whether lesional CD8+ T cells in GCA TABs indeed release such stimuli and exacerbate local GM-CSF expression remains to be elucidated.

Similar to GCA, Takayasu Arteritis (TA) is a form of vasculitis affecting medium- to large-sized vessels. However, TA manifests itself at a younger age than GCA. Interestingly, in comparison to GCA, TA has been associated with a higher number of CD8+ T cells (27). Serum levels of GM-CSF have also been reported to be higher in TA patients compared to GCA patients (28), implicating that CD8+ T cells could be one of the contributing factors to these differences in circulating GM-CSF.

The strengths of our study are the comprehensive analysis of both CD4+ and CD8+ T cells in the peripheral blood and in the inflamed GCA tissue lesions. The blood and tissue samples used here were obtained from treatment naïve patients to exclude the potential effects of GCs on the expression of GM-CSF. Additionally, we included blood samples obtained from GC free remission patients and age-matched HCs to explore the possible alteration between disease and remission and to minimize age-related changes in circulating GM-CSF expressing cells. We detected a strong correlation between GM-CSF positivity and CD8+ T cell infiltration in spite of the small sample size (n =5). Future studies in different cohorts with bigger sample sizes are warranted to confirm these findings. *In vitro* studies should be conducted to confirm the ability of CD8+ T cells to induce GM-CSF expression in myeloid and non-myeloid cells, including smooth muscle cells and fibroblasts, as well as to identify the factors that may drive this process. Additionally, future studies should explore the effects of GC treatment on the local production of GM-CSF.

Our findings aid in the identification of the cellular sources of GM-CSF and expand our understanding of the involvement of CD8+ T cells in the vasculopathy of GCA. Although CD8+ T cells have previously been linked with the vascular pathology of GCA (29), their contribution has generally been considered to be minor (30). Here, we provide evidence that (massive) CD8+ T-cell infiltration may amplify vascular inflammation by directly and indirectly enhancing local GM-CSF expression.

CONCLUSIONS

T cells (both CD4+ and CD8+) are an important cellular source of GM-CSF production in the inflamed vessels in GCA. Although both CD4+ and CD8+ T cells are capable of expressing GM-CSF, its expression in GCA lesions may be enhanced by the degree of CD8+ T-cells infiltration. Our data, therefore, suggest that CD8+ T cells are active contributors to the exacerbation of vessel wall inflammation in GCA and could constitute a novel therapeutic target to halt disease progression.

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SUPPLEMENTARY MATERIALS
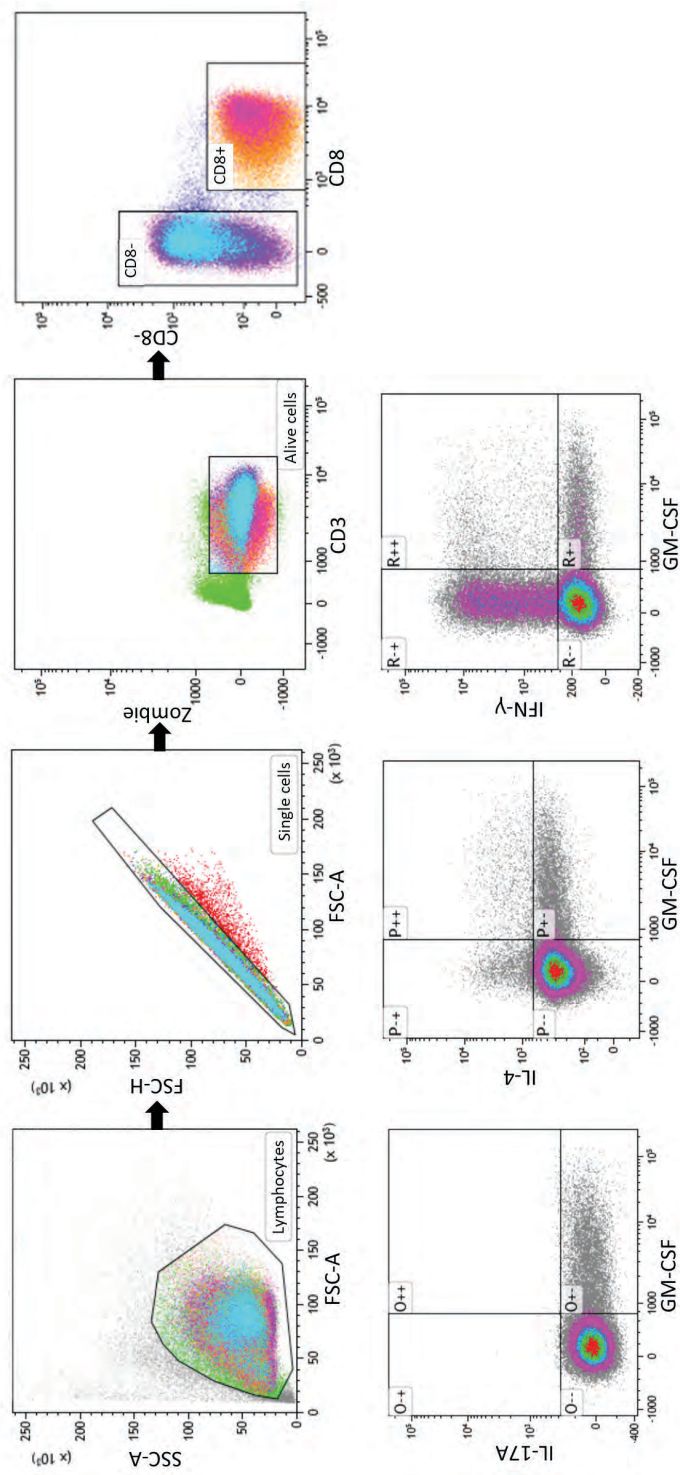
Supplementary figure 1. Flow cytometric strategy for determining cytokine-expressing T cells. Lymphocytes were determined based on their forward and side scatter after which single cells were selected. Living cells were selected by gating for Zombie negative and CD3 positive cells. Cytokine expression was assessed as a percentage of positive cells within CD8+ and CD8- T cells. Cytokine expression was determined by gating on unstimulated cells. The example shows one stimulated sample.
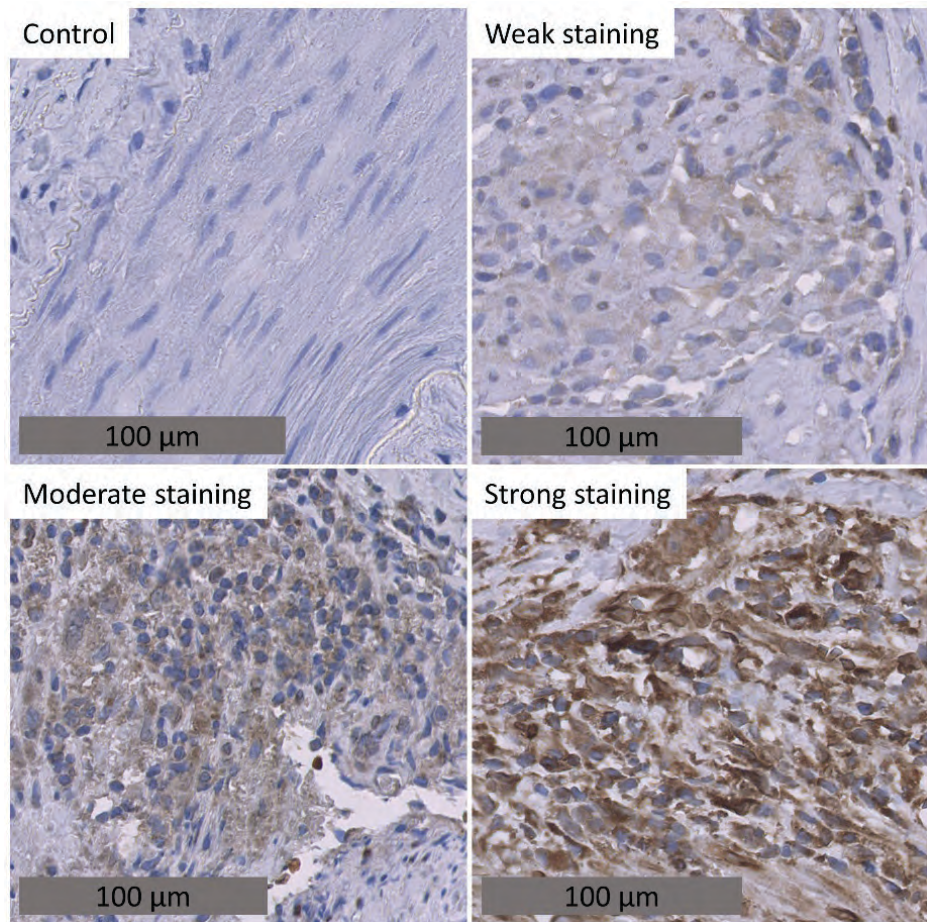
Supplementary figure 2. The intensity of GM-CSF staining compared to the isotype control staining. The relative intensity of each staining was defined as not present, weak but detectable above control, moderate and strong.
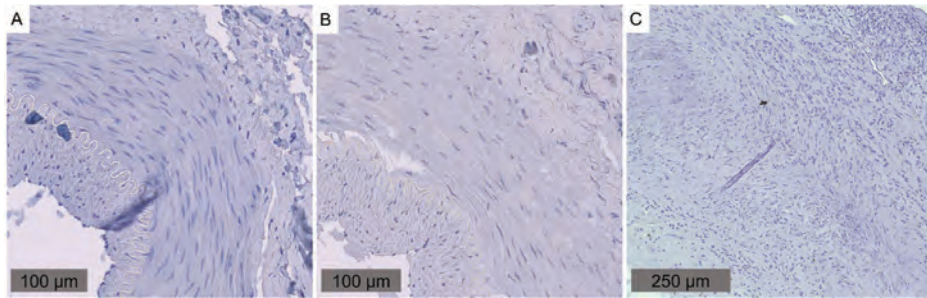
Supplementary figure 3. Isotype control for (A) GM-CSF, (B) CD3 and (C) CD8.

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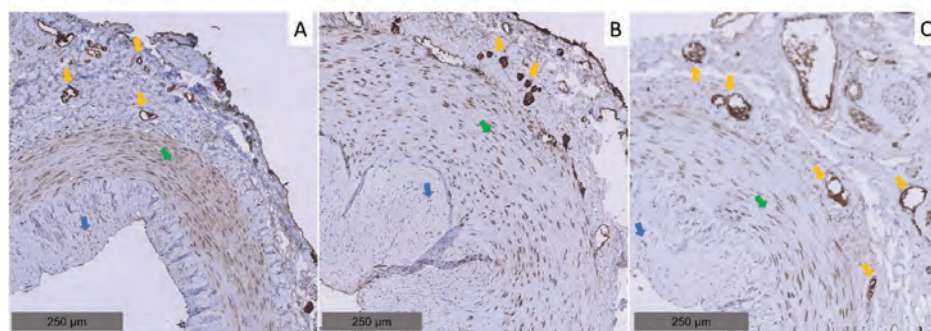
Supplementary figure 4. Distribution of GM-CSF in non-inflamed TABs. Shown are non-inflamed TABs from GCA patients (A&B) and a PMR patient (C). GM-CSF positivity was detected in endo-thelial cells of the vasa vasorum (yellow arrows), medial smooth muscle cells (green arrows) and occasional intimal fibroblasts (blue arrows).

Supplementary table 1. Fluorescent-conjugated monoclonal antibodies.

Antibody	Clone	Company	Catalogue number
IL-4 BV605	MP4-25D2	Biolegend	500828
GM-CSF APC	BVD2-21C11	Biolegend	502310
IFN-γ FITC	4s.B3	Biolegend	502506
IL-17A PE-Cy7	eBio64DEC17	ThermoFisher	25-7179-42
CD3 PerCPcy5.5	SK7	Biolegend	344808
CD4 BUV395	SK3	BD	612748
CD8 BUV737	SK-1	BD	612754

Supplementary table 2. List of immunohistochemistry antibodies.

Single Staining

Target	Antigen retrieval	Manufacturer & code	Host	Isotype	Dilution	Secondary antibody	Manufacturer & Code	Dilution
GM-CSF	pH 9	Abcam, ab9741	Rabbit	Polyclonal IgG	1:200	Envision α-rabbit polymer-HRP	DAKO, K4003	Prediluted
CD3	pH 9	Abcam, ab699	Mouse	IgG2a	1:20	Envision α-mouse polymer-HRP	DAKO, K4006	Prediluted
CD8	pH9	Abcam, ab75129	Mouse	IgG1	Prediluted	Envision α-mouse polymer-HRP	DAKO, K4006	Prediluted

Supplementary table 3. Triple fluorescence staining panel.

Targets	CD3 (Abcam, ab699)	CD8 (Abcam, ab75129)	GM-CSF (Abcam, ab9741)	Nucleus
Isotype (primary ab)	Mouse IgG2a 1:20	Mouse IgG1 Prediluted	Rabbit polyclonal IgG 1:150	
Secondary ab	Rat α-mouse IgG2a (Biolegend, RMG2a-62) 1:40	Goat α-mouse IgG1 (Southern Biotech, 1071-01) 1:50		
Tertiary ab	Donkey α-rat IgG (Abcam, ab150153) 1:40	Donkey α-goat IgG (Abcam, ab175704) 1:50	Donkey α-rabbit IgG (Abcam, ab150075) 1:40	
Conjugate/dye	AF488	AF568	AF647	DAPI
Longpass filter channel	515LP	590LP	610LP	475LP

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