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Cytokine producing B-cells and their capability to polarize macrophages in giant cell arteritis

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A B S T R A C T

Objective: The lack of disease-specific autoantibodies in giant cell arteritis (GCA) suggests an alternative role for B-cells readily detected in the inflamed arteries. Here we study the cytokine profile of tissue infiltrated and peripheral blood B-cells of patients with GCA. Moreover, we investigate the macrophage skewing capability of B-cell-derived cytokines.

Methods: The presence of various cytokines in B-cell areas in temporal artery (n = 11) and aorta (n = 10) was identified by immunohistochemistry. PBMCs of patients with GCA (n = 11) and polymyalgia rheumatica (n = 10), and 14 age- and sex-matched healthy controls (HC) were stimulated, followed by flow cytometry for cytokine expression in B-cells. The skewing potential of B-cell-derived cytokines (n = 6 for GCA and HC) on macrophages was studied *in vitro*.

Results: The presence of IL-6, GM-CSF, TNFα, IFNγ, LTβ and IL-10 was documented in B-cells and B-cell rich areas of GCA arteries. *In vitro*, B-cell-derived cytokines (from both GCA and HC) skewed macrophages towards a pro-inflammatory phenotype with enhanced expression of IL-6, IL-1β, TNFα, IL-23, YKL-40 and MMP-9. *In vitro* stimulated peripheral blood B-cells from treatment-naïve GCA patients showed an enhanced frequency of IL-6+ and TNFα+IL-6+ B-cells compared to HCs. This difference was no longer detected in treatment-induced remission. Erythrocyte sedimentation rate positively correlated with IL-6+TNFα+ B-cells.

Conclusion: B-cells are capable of producing cytokines and steering macrophages towards a pro-inflammatory phenotype. Although the capacity of B-cells in skewing macrophages is not GCA specific, these data support a cytokine-mediated role for B-cells in GCA and provide grounds for B-cell targeted therapy in GCA.

1. Introduction

Giant cell arteritis (GCA) is the most frequent form of vasculitis of the medium- and large-arteries in people older than 50 years [1] and it frequently overlaps with polymyalgia rheumatica (PMR) [2,3]. Both diseases are still largely treated with glucocorticoids (GC), with only tocilizumab (interleukin 6 (IL-6) receptor blocker) being approved as a GC-sparing drug for patients with GCA [4]. Improved insight into the pathogenesis of GCA may aid the discovery of new targets for intervention in this disease.

GCA is a granulomatous vasculitis in which dendritic cells, macrophages and T-cells are well known players [5]. More recently, studies on both temporal artery and aortic GCA tissue revealed a variable presence and degree of organisation of B-cells ranging from scattered cells to tertiary lymphoid organs (TLOs) [6–8]. A potential pathogenic role for B-cells in GCA is underlined by two case-reports describing B-cell depletion therapy with rituximab in GCA [9,10]. The lack of disease-specific autoantibodies in GCA [11–13] suggests that non-humoral B-cell functions may be important. Reduced B-cell numbers in the peripheral blood during active disease in GCA and PMR patients have been described [14,15]. In treatment-induced remission, peripheral B-cell numbers normalized, likely due to B-cell redistribution from the tissues to the peripheral blood. These redistributed effector B-cells showed an enhanced potency to produce IL-6 [14], which is a central cytokine in the inflammatory cascade in GCA [4]. B-cells may thus contribute to GCA pathogenesis by cytokine production.

In addition to IL-6, effector B-cells can produce several pro-inflammatory cytokines such as tumor necrosis factor (TNF)α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)γ, while regulatory B-cells are able to produce the anti-inflammatory cytokine IL-10 [16,17]. Furthermore, B-cells can secrete lymphotoxin (LT) which is involved in the organization of TLOs [16,17]. Through cytokine production, B-cells may contribute to the local cytokine milieu thereby polarizing other cellular players in GCA and being

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involved in the perpetuation of inflammation rather than initiating the vasculitic cascade. In aorta tissue from patients with GCA, approximately half of the TLOs were located adjacent to granulomas, which are dominated by macrophages [8], suggesting communication between these two organized inflammatory structures. In GCA, activated macrophages play a central role by secreting cytokines, chemokines and proteases that amplify the inflammatory process and contribute to chronic vascular damage and remodelling [18,19]. Recent reports documented the presence of CD206+MMP-9+YKL-40+ macrophages likely skewed by GM-CSF signalling in GCA [20,21]. These macrophages are associated with tissue destructive functions and the production of pro-inflammatory cytokines.

To gain insight into the role of B-cells in GCA pathogenesis and their interaction with macrophages, we studied the cytokine profile of tissue infiltrated B-cells in temporal artery and aorta of patients with GCA and of the *in vitro* stimulated peripheral blood B-cells. In addition, we investigated the macrophage skewing capability of B-cell derived cytokines *in vitro*.

2. Materials & methods

2.1. Population characteristics for the in vitro studies

Newly diagnosed, treatment naïve consecutive patients with GCA (n = 11) and PMR (n = 10) participated in the flow cytometry study (Table 1). Nine of the GCA patients fulfilled the 2022 ACR/EULAR classification criteria [22]. Two of the patients who did not fulfil these criteria had positive [^{18}F]fluorodeoxyglucose-positron emission tomography-computed tomography ([^{18}F]FDG-PET-CT) but not higher than liver uptake in both axillary arteries or in the whole aorta. All PMR patients (age- and sex-matched) fulfilled ACR/EULAR 2012 criteria for PMR. The HCs were age- and sex-matched, had no relevant past or present morbidities including cardiovascular and auto-immune diseases, and did not take any immunosuppressive drugs. Informed consent was signed by all subjects. Samples were collected in compliance with the Declaration of Helsinki and the study was approved by the institutional review board of the University Medical Centre Groningen (GCA/PMR: METc2010, HC: METc2012/375).

2.2. Detection of intracellular cytokines in stimulated peripheral blood B-cells by flowcytometry

Cryopreserved peripheral blood mononuclear cells (PBMCs) collected from patients before starting GC treatment, and HC were thawed and cultured at a concentration of 10^6 cells/mL in 1 mL Roswell Park Memorial Institute medium (RPMI 1640, Gibco) containing 10% heat inactivated fetal calf serum (FCS) and 0.3% gentamycin in polypropylene tubes. Cells were either left untreated or treated with 500 ng/mL CpG-oligodeoxynucleotides (ODN) 2006 (Hycult Biotech). The cells were incubated for 72 h at 37 °C with 5% CO_2. During the last 5 h, cells were treated with 10 μg/mL Brefeldin A (BFA; Sigma-Aldrich) to inhibit cytokine secretion, and were either left untreated or stimulated with 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich), and 2 mM calcium ionophore (CaI; Sigma Aldrich). After incubation, cells were harvested and stained with viability dye and surface makers CD19, CD22, CD40, CD80/86 and HLA-DR. Subsequently, cells were fixed and permeabilised using Fix&Perm (Invitrogen) and stained for the following intracellular cytokines: TNFα, IFNγ, LTα, IL-6, GM-CSF and IL-10 (see Supplementary Table 1). Samples were measured using an LSR-II flow cytometer (BD) and data were analysed using Kaluza software (Beckman Coulter). The unstimulated cells (without CpG and PMA) from each individual donor were used to set the gates for the stimulated cells as their own internal control for cytokine production (see Supplementary Fig. 1). Follow-up samples of 10 GCA and 9 PMR patients (1 GCA and 1 PMR patient were lost to follow up), were collected at 3 months after GC-induced remission and were similarly processed, stimulated and measured. In the PMR patient group, there was one patient who also received methotrexate in a weekly dosage of 20 mg and one patient who also received leflunomide 20 mg daily at the 3-month timepoint. Data are presented as the frequencies of cytokine positive B-cells within the

Table 1
Pre-treatment characteristics of newly diagnosed patients with giant cell arteritis (GCA). Age- and sex-matched polymyalgia rheumatica (PMR) patients and healthy controls (HC) are included. TA: temporal artery, FDG-PET-CT: 18F-fluorodeoxyglucose-positron emission tomography-computed tomography, US: ultrasound; ACR: American College of Rheumatology, EULAR: European League Against Rheumatism, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, Hb: hemoglobin, NS: not significant.

	HC	GCA	PMR	p-value HC vs GCA	p-value HC vs PMR	p-value GCA vs PMR
n	14	11	10	–	–	–
Age in years; Median (range)	73 (59–91)	74 (59–84)	72 (56–82)	NS	NS	NS
Females (%)	11 (79)	9 (82)	7 (70)	NS	NS	NS
TA Positive/performed	NA	6/7	NA	–	–	–
FDG-PET/CT positive for GCA/PMR/GCA + PMR	NA	3/0/3	0/10/0	–	–	–
US positive for GCA (%)	–	9 (82)	–	–	–	–
Fulfilled ACR/EULAR 2022 criteria (GCA); Yes/No	NA	9/2	NA	–	–	–
Fulfilled ACR/EULAR 2012 criteria (PMR); Yes/No	NA	NA	10/0	–	–	–
Cumulative glucocorticoid dose at 3 months (mg) Median (range)	NA	3478.5 (1660–4540)	1000.12 (240–1850)	–	–	<0.0001
CRP mg/L; Median (range)	5 (0–7)	54 (5–105)	33 (12–93)	<0.0001	<0.0001	NS
ESR mm/h; Median (range)	10 (3–20)	63 (7–116)	55 (24–109)	<0.0001	<0.0001	<0.05
Hb mmol/L; Median (range)	8.7 (7.3–9.4)	7.2 (5.6–8.3)	7.5 (6.2–8.8)	<0.0001	<0.0001	<0.05
Lymphocytes 10^9/L; Median (range)	1.90 (1.19–1.48)	1.71 (0.92–2.24)	1.89 (1.31–2.69)	NS	NS	NS
CD19$^+$ B-cells 10^9/L; Median (range)	0.20 (0.07–0.37)	0.15 (0.08–0.21)	0.25 (0.07–0.41)	NS	NS	NS

total CD19+CD22$^+$ B-cell population.

2.3. Generation of B-cell-conditioned medium

Cryopreserved PBMCs of 6 GCA and 6 HC from the study population used for the detection of intracellular cytokines (see Supplementary Table 2 for additional clinical information) were thawed and B-cells were isolated with the EasySep™ Human B-cell isolation kit (Stemcell Technologies) according to the manufacturer's instructions. After isolation, B-cell enrichment led to a purity of 91.5% as measured by flow cytometry (Supplementary Fig. 2). B-cells in a concentration of 4×10^5 cells/mL in RPMI+10%FCS+0.3% Gentamycin were stimulated with CpG ODN 2006 for 72 h, followed by stimulation with PMA for 5 h. The cells were then pelleted and washed to remove the PMA and CpG. For unstimulated controls, B-cells were left untreated during the whole process. The cells were subsequently incubated in fresh 200 μl RPMI+10%FCS+0.3% Gentamycin at 37 °C with 5% CO_2 for 24 h to allow cytokine secretion into the medium. Supernatants of stimulated cultures (B-cell-conditioned medium, BCCM) and unstimulated cultures were collected and stored at −20 °C. Selected cytokines (IL-6, TNFα, GM-CSF, IFNγ and IL-10) were quantified in the BCCM with a human premix magnetic Luminex screening assay kit (R&D systems). The assay was read on a Luminex Magpix instrument (Luminex) and with xPONENT 4.2 software (Luminex).

2.4. Macrophage activation with B-cell-conditioned medium

THP-1 human monocytic cells (ATCC) were cultured and maintained in RPMI+10%FCS+0.3% Gentamycin. To differentiate THP-1 cells into macrophages, the cells were cultured for 48 h in the presence of 10 ng/mL PMA followed by 48 h culture in RPMI+5%FCS+0.3% Gentamycin. For macrophage activation, the differentiated THP-1 macrophages were cultured in the presence of 50% BCCM for 24 h. THP-1 cultured in the presence of 50% medium derived from unstimulated B-cell cultures and a medium only control group were included. Cells were then lysed for RNA extraction.

2.5. RNA extraction and qPCR

Total RNA was extracted from THP-1 derived macrophages treated with BCCM using the RNeasy® micro Kit (Qiagen, Hilden, Germany). Total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamers (Promega). Real-time qPCR was conducted with a ViiA™ 7 Real-Time PCR System with TaqMan™ probes (Thermo Fisher IL-23P19 (Hs00372324_m1), IL-6 (Hs00174131_m1), MMP-9 (Hs00234579_m1), YKL-40 (Hs01072228_m1), TNF-alpha (Hs00174128_m1) and IL-1β (Hs01555410_m1). Amplification plots were analysed with QuantStudio™ Real-Time PCR software v1.3 (Applied Biosystems). Relative gene expression was normalized to β-actin (ACTB, Hs99999903_m1) as an internal control.

2.6. Immunohistochemistry for detection of B-cells and cytokines in arterial tissues

Temporal artery (TA, n = 11, see Supplementary Table 3 for additional patient characteristics) and aorta tissue (n = 10) of GCA patients who underwent aortic aneurysm surgery (average age of 70; 4 male/6 female patients; all without steroid treatment within 3 months before surgery) was formalin fixed, paraffin embedded and sectioned at 3 μm thickness. For comparison, aorta tissue of age-matched patients with an atherosclerotic aortic aneurysm (n = 14) (average age of 69; 5 male/9 female patients; without evidence of GCA) were included, as previously described [8]. Tonsil tissue was used as positive control for optimization of the immunostainings. Consecutive sections were deparaffinized and rehydrated. Heat-induced antigen retrieval and endogenous peroxidase block were performed. Sections were incubated with anti-human antibodies for the detection of the following markers: CD20, TNFα, IFNy, LTβ, IL-6, IL-10 (Supplementary Table 4). Tissues were then incubated with secondary antibodies, 3,3′-Diaminobenzidine (DAB, Dako) and counterstained with hematoxylin (Merck). All stained tissue sections were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U 10074-01, Hamamatsu Photonics K.K.). B-cell clusters, defined here as >10 B-cells in close proximity, were identified in all tissues. Cytokine expression by B-cell clusters was scored in a semi-quantitative fashion by two independent reviewers on a four-point scale with none = no cytokine expression, low = >1–25% of B-cells in cluster are positive, mid = 25–50% of B-cells in cluster are positive and high = >50% of B-cells in cluster are positive (see Supplementary Fig. 3 for examples of none and high expression).

Dual staining for transcription factor PAX5 in combination with IL-6 was performed with the MultiVision Polymer Detection System (ThermoScientific) in agreement with the manufacturers protocol. Ultra-V-block was applied after deparaffinization, heat-induced antigen retrieval and endogenous peroxidase block and before primary antibody incubation. Sections were then incubated with MultiVision Polymer Cocktail and visualized by LVBlue and LVRed.

2.7. Statistical analysis

GraphPad Prism 8.0 software was used for statistical analysis. Non-parametric tests were used for data analysis as data did not follow a Gaussian distribution. Multiple group comparisons were analysed with the Kruskal-Wallis test followed by Mann-Whitney *U* test or the Dunn's post hoc test. Paired samples (pre-treatment and after 3 months of treatment) were analysed with the Wilcoxon signed rank test. Correlations were assessed with the Spearman's rank correlation coefficients. A p-value of less than 0.05 (2-tailed) was considered statistically significant.

3. Results

3.1. Cytokines are present in B-cells and in B-cell rich areas of GCA arteries

All GCA TA tissues displayed transmural inflammation and all GCA aorta tissues showed adventitial and medial leukocyte infiltration typical for GCA. B-cell clusters were observed in 9/11 of GCA TA, 9/10 of GCA aortas (mostly in close proximity to granulomas, as previously reported [8], Supplementary Fig. 4) and 10/14 of atherosclerosis aortas. In all tissues most of the B-cells were observed in the adventitia, but also in the media (mostly in aorta) and intima (TA). More extensive B-cell infiltration was observed in the aorta compared to the TA. Moreover, B-cells in the aorta showed a higher level of organization in clusters. Cytokine staining revealed expression of IL-6, GM-CSF, TNFα, IFNγ, LTβ and IL-10 in the area of B-cell clusters in both TA and aorta (Fig. 1). Semi-quantitative scoring of all tissues revealed that IL-6, GM-CSF, TNFα, IFNγ and LTβ were expressed in the vast majority of B-cell clusters (Supplementary Fig. 5). The regulatory B-cell cytokine IL-10 was also detected within the B-cell clusters but expression varied from none to marked (Fig. 1 and Supplementary Fig. 5). Double staining of IL-6 and nuclear B-cell marker PAX5, and GM-CSF and PAX5 (Fig. 1) confirmed that B-cells express IL-6 and GM-CSF, respectively. Overall, semi-quantitative scoring revealed that the percentage of cytokine expressing B-cell clusters is comparable between GCA TA, GCA aorta and atherosclerosis aorta (Supplementary Fig. 5). Of note, the expression of these cytokines was also found outside of the B-cell clusters (Supplementary Fig. 6).

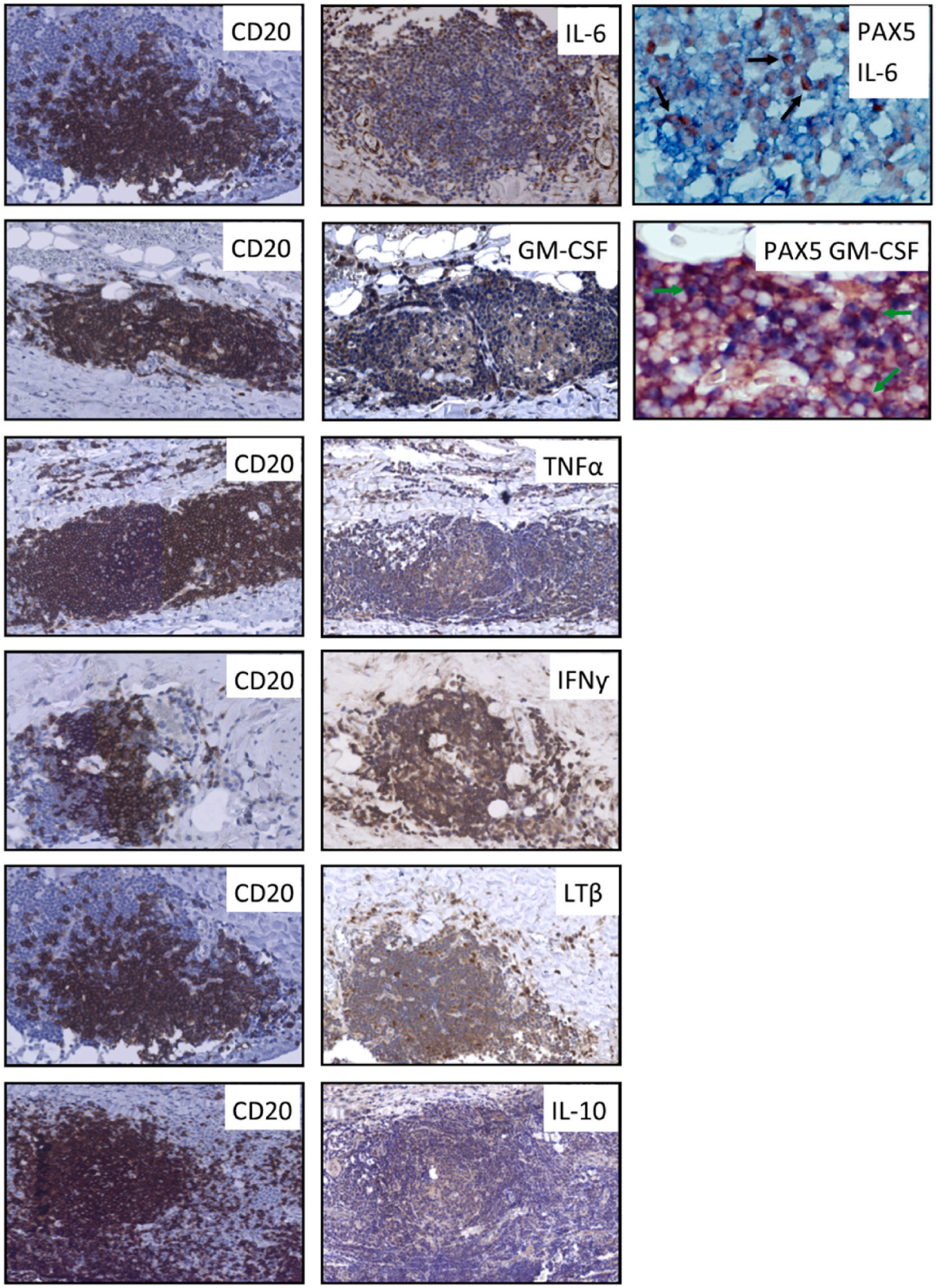

Fig. 1. Local expression of cytokines by B-cells in GCA inflamed arteries. The left panel shows $CD20^{+}$ B-cells in brown while the middle panel shows cytokine expression within B-cell clusters for respectively IL-6, GM-CSF, TNFα, IFNγ, LTβ, and IL-10. The right panel displays double-staining of nuclear marker PAX5 (B-cells, red) with IL-6 (blue) in GCA artery. The black arrows point at double positive (PAX5+IL-6+) B-cells. It also displays a double-staining of nuclear marker PAX5 (B-cells, blue) with GM-CSF (red) in GCA artery. The green arrows point at double positive (PAX5+GM-CSF+) B-cells.

3.2. B-cell-derived cytokines skew macrophages towards a pro-inflammatory, MMP-9+YKL-40+ phenotype

Given our observation that approximately half of the TLOs were located adjacent to a granuloma [8], we further sought to gain insight into the interplay between B-cells (which are the dominating cell type in TLOs) and macrophages (which are the dominating cell type in granulomas) in GCA. To this end we studied the effect of B-cell-derived cytokines on the phenotype of THP-1 derived macrophages *in vitro*. The cytokines IL-6, TNFα, GM-CSF, IFNγ and IL-10 were detected in the

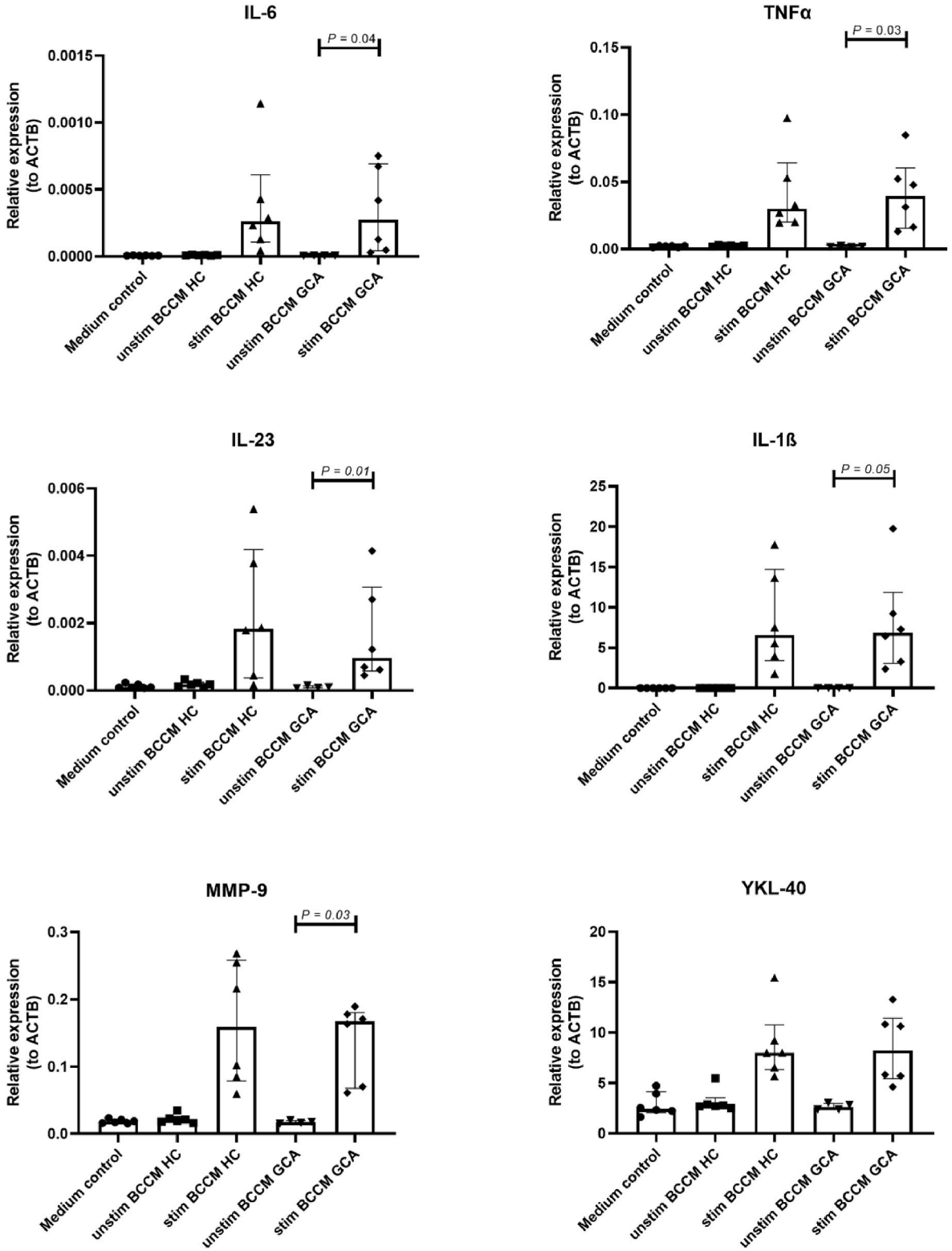

Fig. 2. Cytokine-mediated effect of B-cells on macrophages in giant cell arteritis (GCA) and healthy controls (HC). Depicted are mRNA expression levels of pro-inflammatory cytokines (IL-6, TNFα, IL-23, IL-1β), tissue destructive marker (MMP-9) and neovascularization marker (YKL-40) by THP-1-derived macrophages after incubation with B-cell-conditioned medium (BCCM) obtained from either HC (n = 6) or patients with GCA (n = 6). ACTB = beta actin (housekeeping gene).

stimulated B-cell-conditioned medium (BCCM) of both GCA patients and HC at similar levels (see Supplementary Fig. 7). Upon incubation with the BCCM, THP-1 derived macrophages expressed significantly more transcripts for the pro-inflammatory cytokines IL-6, TNFα, IL-1β and IL-23 compared to untreated THP-1 cells and THP-1 cells cultured in the presence of medium from unstimulated B-cells (Fig. 2). In addition, the tissue remodelling factor MMP-9 and the neovascularization stimulating factor YKL-40, which are both known to play a role in the vasculopathy of GCA, were also strongly upregulated at the mRNA level in macrophages that had been stimulated with BCCM. Although there were no significant differences between GCA and HC groups, these data suggest that presence of B-cells in aorta and TA *in vivo* may promote local macrophage polarization.

3.3. Effector B-cell frequencies are increased in stimulated peripheral blood of GCA and PMR patients at diagnosis

We next investigated circulating B-cells from treatment-naïve patients with GCA and PMR and from HC for their potency to produce cytokines. There were no differences in total B-cell frequencies between the groups after stimulation with CpG and PMA (Fig. 3). GCA patients had significantly increased frequencies of IL-6+ B-cells (median (IQR); 44 (41–52)) and IL-6+TNFα+ B-cells (12 [8–24]) compared to HC (IL-6+: 28 [23–39], IL-6+TNFα+: 6 [4–24]) (p-value 0.009 and 0.01, respectively, see Fig. 3). We did not observe differences in frequencies of TNFα, IFNγ, LTα, GM-CSF and IL-10 producing B-cells between GCA and HC. Since the ratio of pro-to anti-inflammatory cytokines, e.g., TNFα/IL-10 and IL-6/IL-10, can be informative on the immune function of B-cells, we also investigated these ratios but did not observe differences between patients and HC (data not shown). There were no differences in cytokine producing B-cell frequencies between the groups of patients with GCA and PMR.

We next assessed the relation between the capacity of peripheral B-cells to produce cytokine and markers of GCA disease activity. To this end, frequencies of cytokine producing B-cells were correlated with ESR and CRP in treatment-naïve GCA patients (Fig. 3). There was no correlation between sole IL-6 and TNFα producing B-cells with ESR and CRP but the frequency of TNFα+IL-6+ double positive B-cells correlated significantly with ESR (r value 0.680, p value 0.025, Fig. 3). This finding was specific for GCA, as it was not found in PMR (r value −0.127, p value 0.733).

3.4. GC treatment suppresses cytokine producing capacity of peripheral B-cells

Previous work showed that after three months of GC-treatment, peripheral B-cell numbers increase, likely as a result of B-cell redistribution to the circulation [14,23]. To assess possible changes in cytokine production over time and upon GC-treatment, we analysed the cytokine producing capacity of peripheral B-cells after three months of GC treatment, when patients were in remission. As previously reported, the total frequency of B-cells in the circulation of patients with GCA was significantly increased compared to the pre-treatment samples (Fig. 4A). Frequencies of IL-6+, GM-CSF+, IFNγ+ B-cells and double positive TNFα+IL6+, GM-CSF + IL-6+ and GM-CSF + TNFα B-cells were significantly reduced after three months of GC treatment in patients with GCA. The frequencies of IL-10+, TNFα+, and LTα+ B-cells did not change after treatment in both GCA and PMR (Fig. 4A). Furthermore, correlation analyses revealed significant strong and moderate negative correlations between cumulative GC dose (from baseline to 3 months) and the percentage of IL-6+ B-cells and GM-CSF + IL-6+ B-cells in GCA patients, respectively (Fig. 4B). No significant correlations were found in PMR patients (Fig. 4B). The cumulative GC dose at three months for GCA patients was significantly higher than for the PMR patients (Table 1), which may play a role in the lack of significant correlation in PMR patients. These findings indicate that the peripheral B-cell compartment in active GCA is more easily skewed towards production of pro-inflammatory cytokines and that GC treatment reduces this skewing potential.

4. Discussion

Here we document that B-cells at the site of arterial inflammation in GCA express pro-inflammatory cytokines such as IL-6 and GM-CSF. Moreover, we demonstrated that B-cell derived cytokines skewed macrophages towards a pro-inflammatory, tissue destructive phenotype *in vitro*. Furthermore, upon *in vitro* stimulation, frequencies of IL-6+ and TNFα+IL-6+ B-cells derived from PBMCs of treatment-naïve patients with GCA were increased compared to HC. The frequencies of these effector B-cells correlated with ESR at diagnosis and were down-modulated upon GC-induced remission at three months. Overall, these data support a cytokine-mediated role for B-cells in the immunopathology of GCA, as schematically depicted in Fig. 5, and suggest B-cells as cellular players involved in the perpetuation/chronicity of inflammation in GCA. As such, B-cell targeted therapy may constitute a new modality to halt the disease process in GCA.

First, we provided evidence for the presence of cytokine-expressing effector B-cells in the inflamed arteries of GCA patients. We found high expression of IL-6, GM-CSF, IFNγ, LTβ and TNFα in B-cell clusters, while expression of IL-10 varied. B-cells were generally positioned in the adventitia, close to the adventitia-media border and often adjacent to a granuloma. We and others have previously reported that B-cells in GCA arteries are organized into TLOs with high endothelial venules allowing the influx of circulating immune cells [6–8]. The local production of cytokines by B-cells may not only aid the organization and perpetuation of the TLOs but may also skew infiltrating and vascular resident cells by shaping the local inflammatory cytokine milieu especially in the adventitia. TLOs in GCA presented different levels of organization, with or without a germinal center [8]. IL-6, a critical regulator of germinal centers, enhances BCL6 expression and IL-21 production, and is involved in the generation of T follicular helper cells [24]. Also, IFNγ produced by B-cells can fuel the accumulation of T follicular helper cells and the formation of germinal centers [25]. In addition, IFNγ can induce the chemokine CXCL9 [26], which attracts CXCR3+ T-cells, B-cells and macrophages. We previously reported on the expression of CXCL9 and CXCR3 in the inflamed vessel wall and their role in vascular B-cell migration [27]. Thus, persistent expression of IFNγ derived from local B-cells could be a factor sustaining the influx of CXCR3+ cells thereby perpetuating inflammation [28]. In addition to IL-6 and IFNγ, the observed high expression of LTβ in B-cell rich areas adds to the formation of TLOs, as LTβ was found to be a driving force behind TLO formation [29]. LTβ is especially involved in follicular dendritic cell (FDC) maturation and CXCL13 secretion [30]. Both, FDCs and CXCL13 were observed in GCA artery TLOs [6,8]. Notably, these cytokines were not B-cell exclusive as their expression was previously reported in other cellular infiltrates such as macrophages and T-cells [20,28,31,32].

We further hypothesized that the cytokines secreted by B-cells influence the phenotype of other infiltrating inflammatory and resident vascular cells in GCA. As monocytes/macrophages play a crucial role in the perpetuation of inflammation and destruction of the vessel wall in GCA and given our observation that B-cell clusters in GCA arteries are often adjacent to granulomas, we focused here on the interplay between B-cells and macrophages. Monocytes enter the inflamed artery through the vasa vasorum or high endothelial venules in the B-cell rich adventitia and subsequently migrate towards the media. Several cytokines produced by arterial B-cells are indeed capable of polarizing macrophages. Especially the combination of IL-6, GM-CSF, IFNγ, and TNFα was shown to prime macrophages towards a pro-inflammatory M1-like macrophage phenotype by activation of the NF-kB and MAPKs pathways [21,33,34]. In an *in vitro* model, we showed that B-cell conditioned medium contains these cytokines and that it skews macrophages towards a pro-inflammatory, destructive phenotype expressing IL-6,

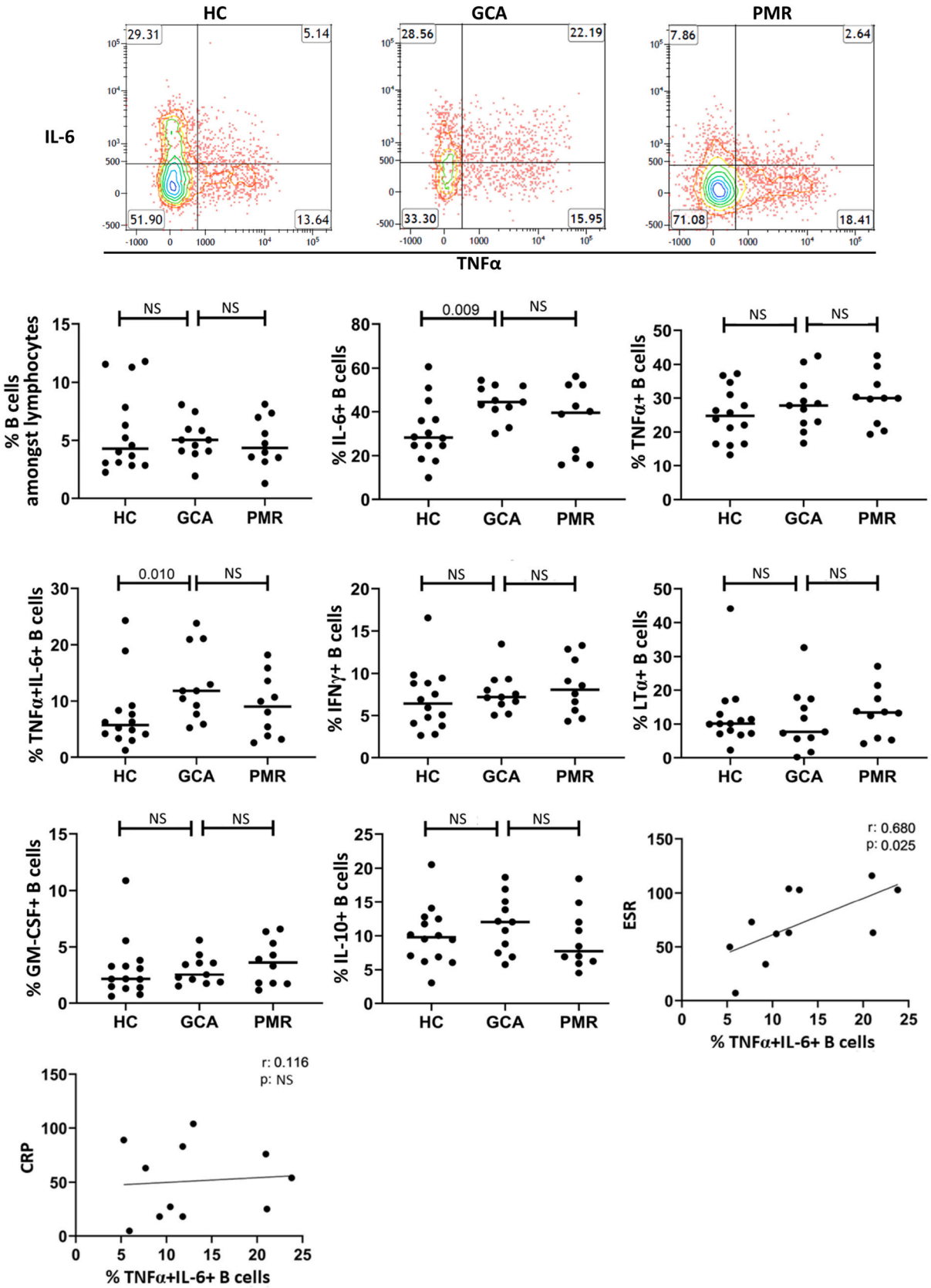

Fig. 3. Results of *in vitro* stimulation with CpG and PMA of peripheral blood B-cells from GCA patients (n = 11), PMR patients (n = 10) and HC (n = 14). Depicted are representative dotplots of TNFα+IL-6+ B-cells (upper panel), frequencies of total B-cells and IL-6, TNFα, TNFα+IL-6+, IFNγ, LTα, GM-CSF and IL-10 expressing B-cells. The last two figures show correlations of disease activity markers ESR and CRP with the frequencies of TNFα+IL-6+ B-cells in GCA patients. GCA: Giant Cell Arteritis, PMR: Polymyalgia Rheumatica, HC: healthy controls, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, NS: not significant.

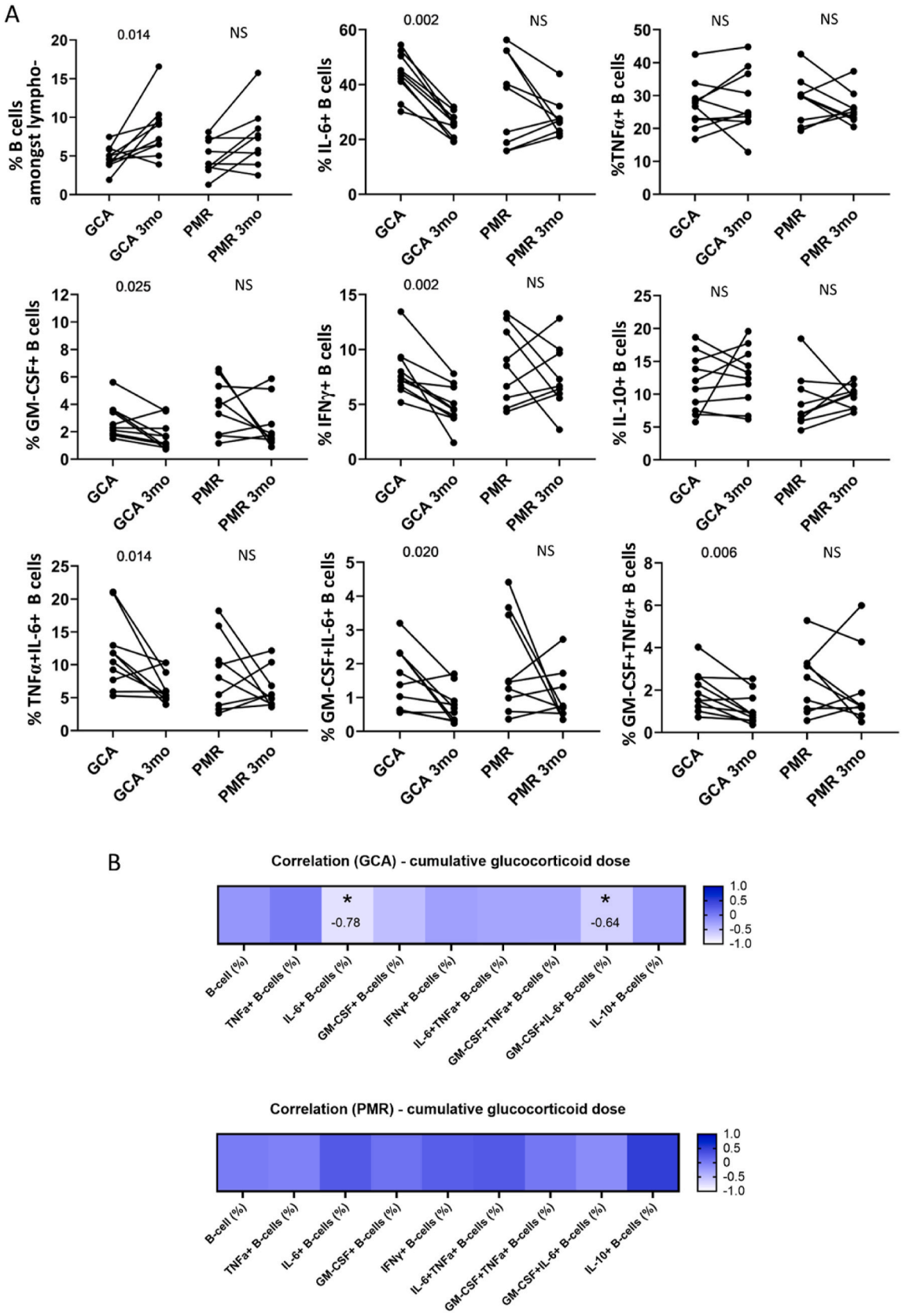

Fig. 4. Frequency of cytokine producing B-cells significantly reduced after treatment in GCA patients. (A) Graphs depicting the frequencies of cytokine producing B-cells after 3 months of glucocorticoid treatment compared to pre-treatment for both GCA (n = 10) and PMR (n = 9) patients. (B) Correlation analysis of cumulative glucocorticoid dose with the frequency of cytokine producing B cells at 3 months. NS: not significant. *P $\leq$ 0.05.

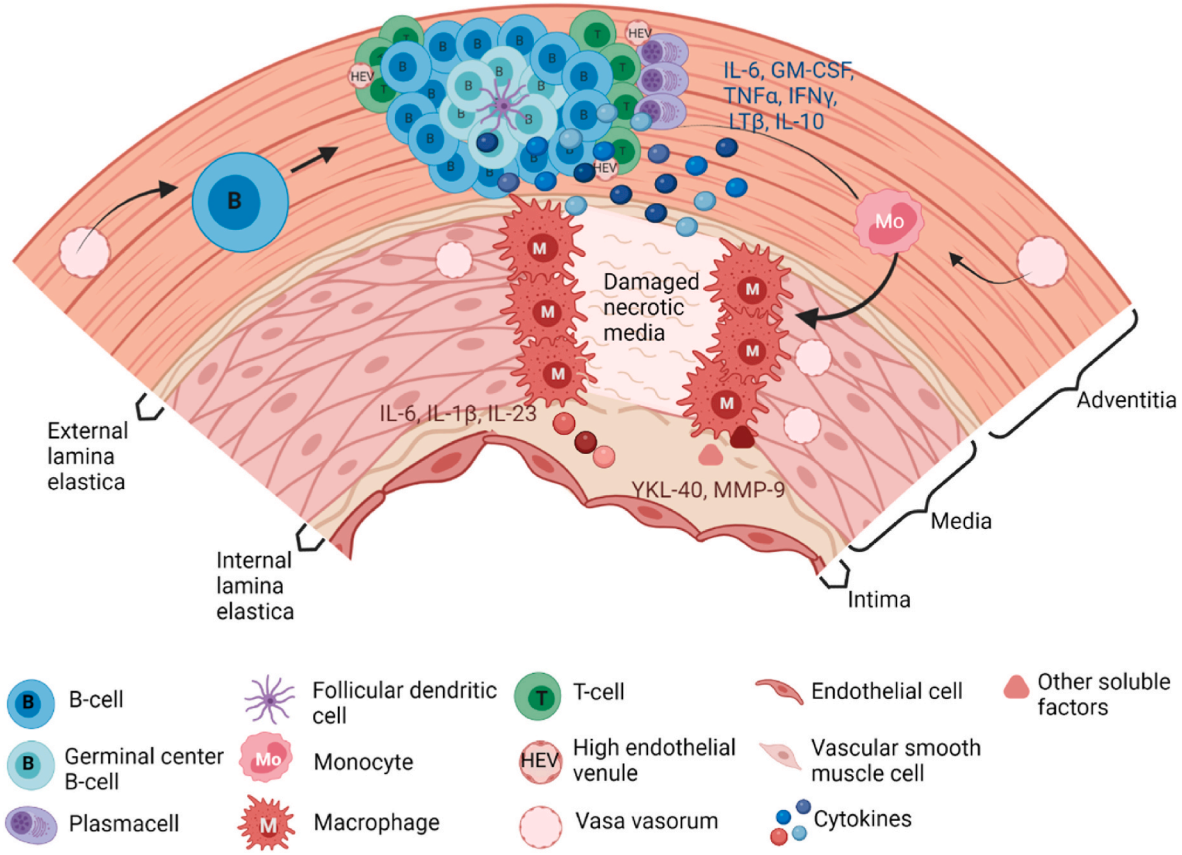

Fig. 5. Graphical representation of the inflamed vessel wall in giant cell arteritis (GCA) integrating the main study findings. B-cells enter the blood vessel wall via the vasa vasorum in the adventitia. Here, B-cells organize into active TLOs with a germinal center. B-cells from the TLO secrete multiple pro-inflammatory cytokines (IL-6, GM-CSF, TNFα, LTβ) and the anti-inflammatory cytokine IL-10 to a varying degree. These B-cell-derived cytokines may activate monocyte/macrophages that enter via the vasa vasorum and migrate towards the media. The skewed macrophages have a pro-inflammatory phenotype producing IL-6, IL-1β and IL-23. In addition, macrophages secrete MMP-9 and YKL-40 which are involved in breakdown of the vessel wall and neovascularization, respectively. Created with BioRender.com.

TNFα, IL-1β, IL-23, MMP-9 and YKL-40. The phenotype of these macrophages resembles that of a tissue destructive CD206+MMP-9+YKL-40+ subset of macrophages identified in the inflamed GCA artery, likely induced by local GM-CSF [20,21]. The cytokine-mediated effect of B-cells onto other immune cells may link the presence of infiltrated B-cells to the perpetuation of GCA rather than its initiation. This was also shown for other diseases such as experimental autoimmune encephalomyelitis (EAE), in which IL-6 producing B-cells aggravated disease severity but were not necessary in the early phase of disease development [35]. Elimination of these IL-6 producing B-cells in EAE reduced IL-17 indicating a role for B-cell-derived IL-6 in skewing T-cells towards Th17 cells [35]. The observed B-cell cytokine secretion pattern (other than IL-6 and IL-6+TNFα) and the macrophage skewing potential was similar between GCA and HC and thus not GCA-specific. This fits with our hypothesis that B cells in GCA are involved in the perpetuation of the inflammatory response rather than being involved in GCA initiation. Although our study focused on the influence of B-cell--derived cytokines on macrophages, it would be interesting to study the effects of B-cell-derived cytokines on Th1 and Th17 cells of GCA patients as well.

As the source of vascular infiltrated B-cells in GCA resides largely in the peripheral B-cell pool, we next investigated the capacity of PBMC-derived B-cells to produce the cytokines found in vascular B-cell rich areas. Patients with active GCA had increased frequencies of IL-6+ B-cells and IL-6+TNFα+ B-cells compared to HC upon *in vitro* stimulation with CpG and PMA. The frequency of TNFα+IL-6+ B-cells correlated with the disease activity marker ESR. Thus, for these cytokines, there is an increased skewing potential of the peripheral blood B-cells towards production of pro-inflammatory cytokines as assessed after 72 h *ex vivo* stimulation with CpG and PMA in active GCA. This feature was lost after 3 months of treatment. Previously, the percentage of *ex vivo* IL-6 producing B-cells was increased during remission as compared to active GCA [14]. This apparent discrepancy is explained by differences in the experimental set-up in these two studies. The prior study showed that an increased number of B-cells (likely redistributed from the site of vascular inflammation) that can immediately produce IL-6 (i.e., upon short, 4 h stimulation with PMA) is present in the circulation of GCA/PMR patients in remission. Here we show that the enhanced skewing potential of B-cells towards production of pro-inflammatory cytokines in active GCA is lost upon GC-treatment thus preventing more expansion of the circulating pool of B-cells that can immediately produce IL-6. Both studies point towards the impact of GC therapy on circulating effector B-cells. In line with previous findings [14], no differences were observed in the frequencies of circulating IL-10+ B-cells (Breg) in GCA patients.

Despite the impact of disease on the quality of life of patients with GCA and PMR, the therapeutic arsenal presently available is very limited. Both diseases are still largely treated with GC, with their known side effects [36]. Therefore, there is a high unmet need for GC-sparing drugs in both GCA and PMR, with only tocilizumab being approved for patients with GCA [4]. As we here show expression of IL-6 by lesional B-cells, the effect of tocilizumab could partly be explained by blockade of the effects of B-cell derived IL-6, in addition to other cellular sources of IL-6, such as macrophages and endothelial cells [20]. GM-CSF is another cytokine recently shown to be an important target for intervention in both *in vitro* [32] studies and in a phase 2 randomized controlled trial with mavrilimumab (GM-CSF receptor blocker) [37].

Inhibition of B-cell-derived GM-CSF effects may partly underlie these promising results, in addition to blockade of GM-CSF from other inflammatory cells and endothelial cells [37]. Several other targeted therapies are currently being explored in GCA [38] and PMR [39].

As B-cells are capable of producing several pro-inflammatory cytokines, which may influence other major cellular players in GCA and PMR, it is appealing to study the effects of B-cell targeted therapy in these diseases. Recently, a phase 2 clinical trial in PMR patients showed a significant effect of B-cell depletion therapy with a single 1000 mg rituximab infusion compared to placebo on top of a GC taper [40]. The GC-sparing effect of rituximab was reflected by both the primary outcome being GC-free remission at week 21 (rituximab 48% vs placebo 21%), and a smaller proportion of patients on a prednisolone maintenance dose (5 mg per day or less).

A limitation of our study is that we have not identified which cytokine or combination of cytokines expressed by the B-cells is responsible for the polarization of macrophages. Based on prior data, GM-CSF would be a prime candidate. Second, we used THP-1 derived macrophages for our *in vitro* study. It would be interesting to investigate the effect of the B-cell derived cytokines on GCA-derived monocytes. Ideally, future investigations into the role of B cells in GCA should consider incorporating B cells isolated from affected arteries although the feasibility of such an approach could be hampered by the limited availability of arterial tissue.

B-cells have long been neglected in GCA mainly because of the lack of disease specific autoantibodies. Our data implicate a possible involvement of cytokine-producing B-cells in the vasculopathy of GCA and provide a rationale for a clinical trial with B-cell targeted therapy in this disease.

Declaration of competing interest

KSMvdG reports personal fees from Roche, outside the submitted work. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2023.103111.

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