Granzyme B producing regulatory B-cells in patients with giant cell arteritis

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Abstract

Objective

Giant cell arteritis (GCA) is a serious inflammatory rheumatic disease driven by T-cells which are regulated by B-cells with anti-inflammatory activity. However, the role of these regulatory B-cells has not been studied in detail. The aim of this study is to investigate the anti-inflammatory B-cell compartment in patients with GCA.

Methods

Peripheral blood mononuclear cells (PBMC) of GCA Patients (n=47) and healthy controls (HC) (n=49) were isolated to assess the granzyme B (GrB) and Interleukin- 10 (IL-10) production of regulatory B-cells (Breg) after in vitro stimulation.

Results

The fraction of GrB producing Breg in GCA patients was diminished as compared to HC, and this was independent of current disease activity. In contrast, there were no significant differences between patients and HC with regard to IL-10 producing Breg. In GCA patients with active disease, $CD4^+$ T-cells produced less IFNγ than HC. Regarding other T-cell derived pro-inflammatory cytokines, a trend towards a lower expression as compared to HC was seen.

Conclusion

Regulatory B-cells were differentially altered in patients with GCA. While GrB producing Breg were persistently diminished, IL-10 producing Breg showed no differences between patients and controls. This may indicate a lack of B-cell based suppressive capacity which has influence on the T-cell compartment.

Key word

giant cell arteritis, immune system diseases, B-lymphocytes, T-lymphocyte subsets

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Introduction

Giant cell arteritis (GCA) is a large-vessel vasculitis that affects aortic branches, especially the carotid and temporal arteries, and the thoracic aorta itself (1, 2). Due to inflammatory thickening of the vessel walls, GCA may lead to arterial occlusion resulting in ischaemia causing stroke (2, 3). The insufficient blood supply may cause anterior ischaemic optic neuropathy which is associated with loss of vision or also loss of a hemifield.

The pathophysiology of GCA is incompletely understood. However, most research published to date suggests a major role of T cells with pro-inflammatory Th1 und Th17 cells as dominant drivers of disease (4). As a result of an unknown trigger, a toll-like-receptor (TLR) mediated activation of vascular dendritic cells is believed to stimulate chemotaxis of pro-inflammatory $CD4^+$ T-effector cells (T_{eff}) into the vessel wall with subsequent differentiation into Th1 and Th17 cells (5-10). Subsequently, the vessel wall is inflamed and can even be destroyed resulting in a much thicker neo-intima, potentially leading to occlusion of the arterial lumen (7, 11, 12).

The role of B-cells has not been studied in detail but recent data have suggested that B-cells may well play a role in the pathogenesis of GCA. Indeed, a variety of B-cell specific mediators such as B-cell activating factor (BAFF) and Lymphotoxin-α1-β2 (LT) as well as B-cell and plasma-cell containing artery-tertiary-lymphoid-organs (ATLO) were identified in temporal biopsies of GCA patients (13-15). In addition, B-cells are known to act as a regulator of T-cells restraining pro-inflammatory responses. These so-called regulatory B-cells (Breg) are characterised by secretion of anti-inflammatory interleukin-10 (IL-10) or granzyme B (GrB) (16, 17). IL-10 affects Th1 and Th17 cells by reducing the expression of IFN-γ and IL-17 (18). In addition, the quantity of co-stimulatory surface molecules of antigen presenting cells is decreased by IL-10 (19). The serin protease GrB degrades the T-cell receptor and thereby inhibits T-cell activation (16, 20). A dysfunction of these suppressive features may promote and drive the inflammatory cascade in GCA. In other autoimmune diseases, such as lupus nephritis, ANCA-associated vasculitis and rheumatoid arthritis, diminished Breg have been shown to correlate with the inflammatory burden (21-23).

Therefore, the primary aim of this study was to investigate the role of regulatory B-cells in GCA patients compared to healthy controls.

Material and methods

Study population

GCA patients were identified from the hospital information system of the Rheumazentrum Ruhrgebiet, Ruhr-University Bochum, Germany. All patients were included prospectively after giving informed consent. HC were defined as patients without any inflammatory disease. Demographics as well as clinical characteristics were documented and blood samples were taken at one timepoint. Disease activity was defined as clinical evidence of GCA-related symptoms leading to a change in therapy according to the treating rheumatologist. This included either unequivocal evidence of symptoms of GCA, and/or an increased concentration of C-reactive protein (CRP) due to GCA, and/or presence of a halo-sign in duplex-sonography. Remission was defined as absence of clinical symptoms as assessed by the treating rheumatologist. Relapse was defined as a re-increase of CRP and cranial or extracranial symptoms attributed to GCA. The lab studies were performed in the experimental laboratory of the Department of Nephrology, University Hospital Essen. This study was approved by the ethical committee of the Ruhr-University Bochum, Germany, register nr. 19-6767.

Peripheral blood mononuclear cell isolation

About 20-30 ml of heparinised whole blood from each individual was used to isolate peripheral blood mononuclear cells (PBMC). Using Lymphoprep (Stemcell Technologies, Cologne, Germany), the density gradient centrifugation was performed by carefully layering the blood on top of Lymphoprep.

Table I. Demographics of study population.

Variables	GCA patients, pooled (n=47)	GCA patients, active disease (n=14)	GCA patients, remission (n=33)	Healthy controls (n=49)
Age, years	71 *(± 7)*	72 *(± 8)*	70 *(± 7)*	65 *(± 9)*
Sex, n (%)	31 female (65.9)	8 female (57)	23 female (70)	40 female (81.6)
Disease activity	14 with active disease (9 first diagnosis, 5 relapse) 33 in remission	All	None	NA
Therapy	Prednisolone: n= 29 Methotrexate: n=23 Tocilizumab: n=13	Prednisolone mono: n= 8 Methotrexate mono: n= 1 Tocilizumab mono: n= 0 MTX+Prednisolone: n= 2 Toci+Prednisolone: n= 3 MTX+Toci: n= 0	None: n= 4 Prednisolone mono: n= 1 Methotrexate mono: n= 8 Tocilizumab mono: n= 4 MTX+Prednisolone: n= 10 Toci+Prednisolone: n= 4 MTX+Toci: n= 1 MTX+Toci+Pred: n=1	none
Dosage of prednisolone	None: n=18 1-7.5 mg/d: n=15 > 7.5 mg/d: n=14	Mean dosage: 65 mg per day (± 62.5 mg)	Mean dosage: 2,6 mg per day (± 3.7 mg)	NA
Disease duration (mean, in months)		29 ±22 (relapsing patients)	40 ±34	

Centrifugation was done with 800 g for 20 minutes followed by the lowest deceleration.

Cell culture

PBMC at a concentration of $2x10^6$ cells per millilitre (ml) were cultured in RPMI 1640 + Glutamax (Thermo Scientific, Burladingen, Germany), supplemented by Penicillin at 100 U/ml (Sigma Aldrich), Streptomycin at 100 µg/ml (Sigma Aldrich), non-essential amino acids (Thermo Scientific, Burladingen, Germany) and sodium-pyruvate (Thermo Scientific, Burladingen, Germany), as well as 10% fetal calf serum (Biowest, Nuaillé, France). To assess the expression of pro-inflammatory T-cell cytokines (IFN-γ, IL-2, GMCSF, IL-17a), cell stimulation cocktail (eBioscience, Thermo Scientific, Burladingen, Germany) was added to the PBMC followed by incubation for 4 hours at 37°C under 5% CO_2 atmosphere. To investigate the granzyme B (GrB) production of Breg, four different stimulation setups were performed. PBMC were stimulated with (I) anti-human immunoglobulin G/M (IgG/IgM, 6 µg/ml and 6,5 µg/ml, Jackson Immuno Research, Europe Ltd., Cambridge, UK), (II) anti-human IgG/IgM and interleukin-21 (IL-21, 50 ng/ml, Miltenyi Biotec), (III) anti-human IgG/IgM and IL-21 and trimeric CD40 ligand (CD40L, 100 µg/ml, Enzo Life Sciences, Lörrach, Germany), or (IV) IL-21 and anti-human CD40L, in a 24-well plate (BD Biosciences), respectively. After 16 hours of incubation at 37°C under 5% CO_2, 1 µg brefeldin A (BFA, Sigma Aldrich) was added to the culture. To determine the IL-10 production of Breg, PBMC were stimulated with CpG (ODN2006, 500μM stock solution, Invivogen) and incubated for 72 hours at 37°C under 5% CO_2, followed by restimulation with phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich), Ionomycin (Sigma-Aldrich) and BFA. After 6 hours of incubation at 37°C under 5% CO_2, the cells were harvested.

Flow cytometry

To detect T-cell cytokines, PBMC were harvested after stimulation and stained with anti-CD3 Pacific Blue (clone UCHT1, Beckman Coulter, Krefeld, Germany) and anti-CD8 APC-H7 (clone SK1, BD Biosciences). Thereafter, cells were fixed and permeabilised for intracellular staining, using the Cytofix/Cytoperm kit (BD Biosciences). The intracellular staining process contained IL-2 (clone JES6-5H4, PE, Biolegend) or GMCSF (clone BVD2-21C11, PE, Biolegend), IL-17a (clone BL168, PerCP, Biolegend) and IFN-γ (clone B27, FITC, BD Biosciences).
Breg were stained with anti-CD3 Pacific Blue and anti-CD19 (clone J3-119, FITC, Beckman Coulter, Krefeld, Germany) followed by fixation and permeabilisation. Thereafter, cells were intracellularly stained with either anti-GrB (clone GB11, PE, Thermo Scientific, Burladingen, Germany) or anti-IL-10 (clone JES3-9D7, APC, Biolegend). 7AAD was used as a dead cell marker prior to intracellular staining. To ensure the specificity of stainings, appropriate isotype controls were used. Flow cytometric measurements were performed by using the NAVIOS™ fluorescence activated cell scanner (FACS) from Beckman Coulter. The data analysis was based on the Kaluza™ Software v. 2.1 by Beckman Coulter.

Statistical analyses

All values are expressed as mean ± standard deviation (SD). Data are displayed as scatter-plots. Statistical significance between the groups was determined by using the Mann-Whitney U-test. Correlation coefficients were calculated with Spearman rank correlation coefficient. A p-value ≤0.05 was considered significant.

Results

Study population

A total of 47 GCA patients and 49 HC were included (Table I). At the time of enrolment, 14 of 47 patients (29.8 %) had signs of active disease. All GCA patients received treatment prior to enrolment, *i.e.* no treatment-naive pa-

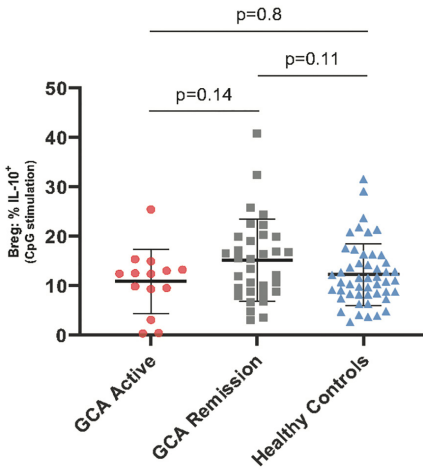

Fig. 1. IL-10 producing regulatory B-cells are detectable in patients. PBMC were stimulated with CpG (Cytosin-Guanin oligopeptides) followed by flow cytometric determination of IL-10 producing B-cells.
The fraction of Il-10 producing B-cells was comparable between patients and healthy controls. No significant differences were obtained. Statistical significance between the groups was determined by using the Mann-Whitney U-test.

tients had been included. The different therapeutic regimes are included in Table I. Patients with active disease had a mean glucocorticoid dosage of 65 mg per day at time of enrolment and quiescent patients had a mean glucocorticoid dosage of 2.6 mg per day. The high mean dose in the group of active patients results from the majority of first diagnosis, who partially received high-dose i.v. prednisolone at the time of enrolment. The mean age of the GCA Patients and HC was 70.6 ±7.1 years *versus* 64.5 ±9.1 years (Table I).

IL-10 producing regulatory B-cells are detectable in HC and GCA patients

Two different types of Breg were assessed: IL-10 producing Breg and GrB expressing Breg. In patients with GCA, IL-10 producing Breg were detectable independent of disease activity. The fraction of IL-10 expressing Breg was similar between patients with active disease and HC (CD19⁺ B-cells: %IL-10⁺, 10.85±6.5 % *vs*. 12.24±6.3 % I=0.8) (Fig. 1). There was no difference between GCA patients in remission and HC (CD19⁺ B-cells: %IL-10⁺, 15.13 ±8.3% *vs*. 12.24 6.3% *p*=0.11) (Fig. 1). Likewise, patients with active *versus* quiescent disease showed comparable expression of L-10 within CD19⁺ B-cells (CD19⁺ B-cells: %IL-10⁺, 10.85% ±6.5% *vs*. 15.13±8.3 % *p*=0.14).

GrB expressing regulatory B-cells are diminished in GCA patients independent of disease activity

Next, GrB producing Breg were studied. After *in vitro* stimulation with anti-human IgG/IgM, there was a significantly lower fraction of GrB expressing Breg in GCA patients versus HC. GCA patients with active disease as well as quiescent GCA patients showed a diminished fraction of GrB producing Breg as compared to HC (Active GCA *vs*. HC, CD19⁺ B-cells: %GrB⁺: 1.13±0.75% *vs*. 3.35±4.22%, *p*=0.04; GCA in remission *vs*. HC, CD19⁺ B-cells: %GrB⁺: 1.83±2.67% *vs*. 3.35±4.22%, *p*=0.021) (Fig. 2A). However, when IL-21 was added to the culture system, the fraction of GrB expressing Breg increased in patients and in HC. After *in vitro* stimulation with IgG/IgM and IL-21, GrB expression in active GCA patients was still lower than in HC but statistical significance was missed (CD19⁺ B-cells: %GrB⁺, 25.94±16.48% *vs*. 39.12±21.84% *p*=0.07) (Fig. 2B). GrB expression was comparable between quiescent patients and HC after *in vitro* stimulation with IgG/IgM and IL-21 (CD19⁺ B-cells: %GrB⁺, 31.46±18.41% *vs*. 39.12±21.84% *p*=0.12) (Fig. 2B).
No significant difference in GrB expression was found between active GCA patients versus HC after combined *in vitro* stimulation with IgG/IgM, IL-21 and anti-human CD40L (CD19⁺ B-cells: GrB⁺, 34.82%±27.45% *vs*. 48.69%±27.47% *p*=0.11) (Fig. 2C), or GCA patients in remission *versus* HC (CD19⁺ B-cells: GrB⁺, 34.78%±20.91% *vs*. 48.69%±27.47% *p*=0.09) (Fig. 2C).
In contrast, quiescent GCA patients showed a reduced fraction of GrB producing Breg after stimulation with IL-21 and anti-human CD40L when compared to HC (CD19⁺ B-cells:

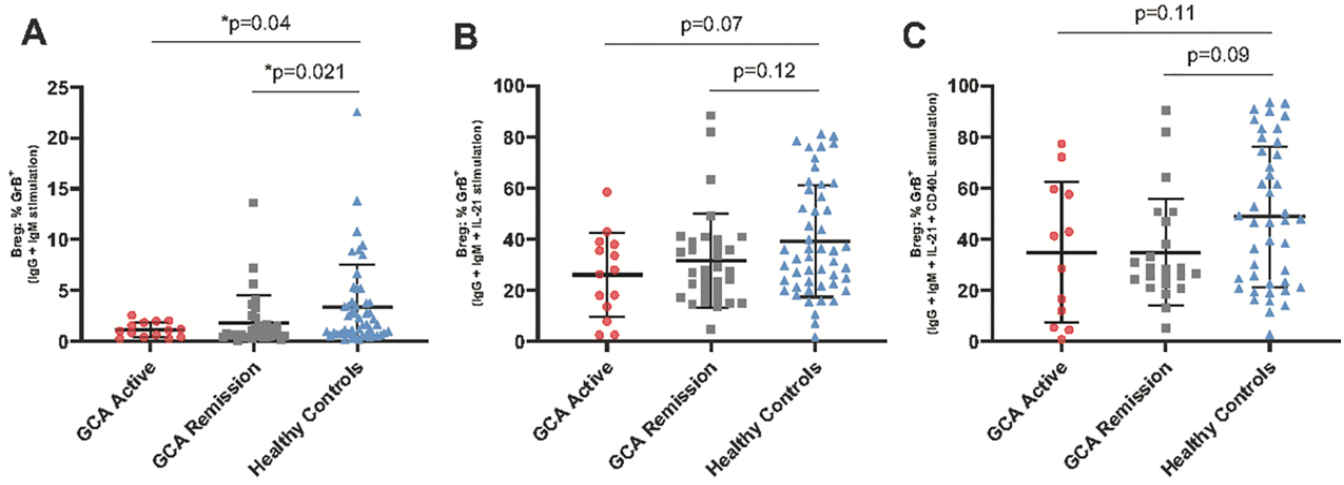

Fig. 2. Granzyme B expression of B-Cells is diminished in patients with GCA. PBMC were stimulated with anti-human IgG and IgM (**A**), anti-human IgG and IgM and IL-21 (**B**) or IgG and IgM, IL-21 and anti-CD40L (**C**). GrB expression was determined by flow cytometry after culture. GrB producing B-cells induced by anti-human IgG and IgM were diminished in GCA patients as compared to HC independent of disease activity. Combination of stimuli led to an increased fraction of GrB producing B-cells in patients and HC. Statistical significance between the groups was determined by using the Mann-Whitney U-test.

GrB$^+$, 7.56±13.11% *vs*. 12.76±13.96, p=0.02). No significant difference was observed between HC and patients with active disease (CD19$^+$ B-cells: GrB$^+$, 12.76±13.96% *vs*. 6.9±7.44%, p=0.07). GCA patients in remission and patients with active disease were not significantly different regarding GrB B-cells (CD19$^+$ B-cells: GrB$^+$, 7.56±13.11% *vs*. 6.9±7.44% p=0.8).

A correlation analysis revealed a significant association between the relative number of circulating Th1 cells (IFN-γ producing T-helper-cells) and the fraction of GrB producing B-cells after stimulation with IgG/IgM/IL-21 in HC (r=-0.45, p=0.001). Interestingly, this association was neither found in GCA patients in remission (r=-0.3, p=0.09) nor in GCA patients with active disease (r=-0.34, p=0.23). Th17 cells did not correlate GrB producing Breg after stimulation with IgG/IgM/IL-21 as assessed in HC and GCA patients. Details on the frequency of Th1, Th17, Th10 and GMCSF producing T-cells are provided in the supplemental data (Supplementary Fig. S1). Moreover, the impact of steroids on GrB producing Breg was analysed. Steroid treatment had no impact on the fraction of GrB$^+$ Breg (Supplementary Fig. S2 and S3).

Discussion

This is one of the first studies investigating the role of B-cells in GCA. The most striking finding of our study is that Bregs are differentially altered in GCA. Even though the pathophysiology of GCA has not been completely understood, the T-cell compartment has been much in the focus of recent research showing that effector Th1 and Th17 cells are likely to be the dominant drivers of disease (10). In line with that, T-cell directed therapy was shown to be efficacious in patients with GCA (24). However, the dysregulation of pro-inflammatory T-cell responses may have several underlying causes. One of these may be a failed regulation of activated T-cells. Indeed, it has already been demonstrated that a defective regulation of effector T-cells may lead to breakdown of immune tolerance facilitating autoimmunity and B-cells have been identified as potent regulators of effector T-cells (16, 18, 24, 25). These Bregs have anti-inflammatory capacity and IL-10 as well as GrB were identified as key molecules mediating suppressive function (16, 18, 24, 25). That was the basis to hypothesise that T-cell driven inflammation in GCA is the result of a failed suppression mediated by Bregs.

Our study shows that Breg are differentially altered in GCA. While IL-10 producing Bregs were detectable to a similar extent in GCA and HC, this was clearly not the case for GrB producing Bregs. IL-10 producing B-cells were assessed by van der Geest *et al*. previously (14). The authors found, in line with our results, no difference between HC and GCA patients. However, the methodology to detect IL-10 producing B-cells was different from our experimental approach as B-cells did not undergo TLR-stimulation prior IL-10 detection. This may limit comparability of the results. In other types of vasculitis, *e.g*. ANCA-vasculitis, a persistent reduction of TLR-induced IL-10 producing Bregs has been reported (21).

GrB producing Breg were detected in our study using different approached for stimulation. Thus, IgG/IgM stimulation was used to activate B-cells via the B-cell receptor (BCR) utilising a T-cell independent pathway. The combination of CD40L and IL-21 was used to stimulate B-cells in addition via CD40 and IL-21R. Both molecules are important when B-cells are activated by T-cells. This strategy made it possible to differentiate whether the T-cell dependent or independent mechanisms of action to induce GrB producing B-cells were functional. GrB producing Breg have been studied under healthy conditions in the past and a potent immunosuppressive capacity mediating immune tolerance was demonstrated by several different groups (16, 22, 24-27). Thus, GrB Breg were assessed in multiple patient cohorts and numerical or functional deficiencies were found in a number of autoimmune diseases (23, 27-29).

In our study, sole IgG/IgM stimulation via BCR revealed a decreased fraction of GrB producing Breg in GCA patients as compared to HC. This effect was reversed when IL-21 or IL-21 CD40L were used as stimulators in addition to BCR activation. Our data indicate that regulatory B-cells of patients with GCA show less GrB production upon T-cell independent stimulation. However, adding T-cell derived factors such as CD40L and IL-21 led to an increase of GrB production by B-cells in these patients and the observed difference to controls was not present anymore. Therefore, regulatory B-cell function may in principle be more dependent on proper function of T-cells in patients than in controls.

In HC, there was a negative statistical correlation between GrB$^+$ Breg and IFNγ producing T-cells, *i.e*. a higher fraction of GrB producing B-cells was associated with a lower fraction of circulating Th1 cells. This is in line with the reported suppressive capacity of GrB$^+$ Bregs in co-culture experiments showing a strong inhibitory effect of GrB$^+$ Bregs on IFNγ producing T-cells (26). Surprisingly, the statistical association of GrB$^+$ Bregs and IFNγ producing T-cells was not found in GCA patients suggesting either resistance of T-cells against regulation or decreased Breg function with insufficient inhibition.

A limitation of our study is that no treatment-naïve patients were included – which is apparently not easy in patients with GCA. Higher doses of glucocorticoids may alter B-cell activation and down-regulate BCR-mediated signalling (30). Thus, the current treatment may have influenced the results. However, neither steroid dosage nor type of immunosuppressive treatment was found to have a significant impact on GrB producing Breg of GCA patients in our study. Nevertheless, this was a cross-sectional study lacking longitudinal data. Likewise, data of patients who switched or tapered immunosuppressive treatment would have provided interesting insights on the impact of therapy on GrB Breg. The recruitment of patients and healthy controls was challenging due to the Covid pandemic, so that another limitation of our study is the matching of patients to healthy controls, which could not be exactly 1:1 considering age and gender (Table I).

In summary, our results indicate a reduced suppressive function of B-cells

in GCA. This could be of importance in the pathogenesis of GCA. These findings should be confirmed and investigated further in treatment naive GCA patients assessing additional functional properties of Breg populations.

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