B cells in ANCA-associated vasculitides
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Circulating CD24\textsuperscript{hi}CD38\textsuperscript{hi} Regulatory B cells Correlate Inversely with the Th\textsubscript{EM}17 cell Frequency in Granulomatosis with Polyangiitis Patients

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Abstract

Objectives To investigate whether there is a direct relation between expanded proportions of Th17 effector memory (Th$_{EM17}$) cells and regulatory B cells (Bregs) in peripheral blood of granulomatosis with polyangiitis (GPA) patients.

Methods Frequencies of Bregs and Th$_{EM17}$ cells, as well as Th$_{EM1}$ cells, were determined by flow cytometry in blood samples from 42 GPA patients in remission and 18 matched healthy controls (HCs). The Breg frequency was defined as CD24$^{hi}$CD38$^{hi}$CD19$^+$ cells. Th$_{EM17}$ cells were defined as CCR6$^+$CXCR3$^+$CCR4$^+$ cells and Th$_{EM1}$ cells as CCR6$^+$CXCR3$^+$CCR4$^-$ cells within the CD3$^+$CD4$^+$CD45RO$^+$CCR7$^-$ population. In addition, CD3$^+$CD4$^+$ Th cells from 9 GPA patients were co-cultured in vitro with either total B cells or a Breg-depleted B cell fraction. Cultured cells were stimulated with Staphylococcus Enterotoxin B (SEB) and CpG-oligodeoxynucleotides (CpG-ODN). Th17- (IL-17$^+$) and Th1 cell (IFNγ$^+$) frequencies were determined at baseline and day 5 upon restimulation with phorbol myristate acetate (PMA) and Ca-I.

Results A decreased Breg frequency was found in treated GPA patients, whereas an increased Th$_{EM17}$ cell frequency was observed in treated and untreated GPA patients compared to HCs. Additionally, a decreased Th$_{EM1}$ cell frequency was seen in untreated GPA patients compared with HCs. In untreated GPA patients circulating Breg frequencies correlated negatively with Th$_{EM17}$ cells ($r = -0.533; p = 0.007$) and positively with Th$_{EM1}$ cells ($r = -0.473; p = 0.015$). The co-culture experiments revealed a significant increase in the frequency of IL-17$^+$ Th cells in Breg-depleted samples (median: 3%; range: 1-7.5%) compared with Breg-undepleted samples ($p = 0.002$; undepleted samples median: 2.1%; range: 0.9-6.4%), whereas no difference in the frequency of IFNγ$^+$ Th cells in Breg-depleted cultures was observed (undepleted median: 11.8%; range: 2.8-21% vs Breg-depleted median: 12.2%; range: 2.6-17.6%).

Conclusion Bregs modulate Th$_{EM17}$ responses in GPA patients. Future studies should elaborate on clinical and therapeutical implications of the Breg-Th17 interaction in GPA patients.

Key Messages
- CD24$^{hi}$CD38$^{hi}$ Bregs are inversely correlated with Th$_{EM17}$ cells in untreated granulomatosis with polyangiitis patients in remission
- CD24$^{hi}$CD38$^{hi}$ Bregs seem to suppress Th17 cell expansion in vivo
Introduction

Granulomatosis with polyangiitis (GPA) is one of ANCA-associated vasculitides (AAV) affecting small- to medium-sized blood vessels. ANCA are predominantly directed against proteinase 3 (PR3). Approximately 50% of GPA patients presenting with PR3-ANCA experience a relapse. Although the exact GPA pathogenesis is not fully known, evidence points to involvement of B- and T cell responses. The abundance of CD4+ T helper (Th) cells in granulomatous lesions of AAV patients and the IgG1 and -3 isotype predominance of ANCA indicate that Th cells are needed for both autoimmune inflammation and autoantibody formation. Previously, we demonstrated an increased frequency of circulating effector memory Th cells (ThEM; CD45RO+CCR7-) in remission compared with active GPA patients and healthy controls (HCs). Interestingly, these ThEM cell subsets showed an expansion of ThEM 17 cells and a decrease of ThEM 1 cells. Also, elevated serum IL-17A levels (i.e. Th17 signature cytokine) were demonstrated in active and inactive GPA patients. It has been reported that IL-17 enhances CXC-chemokine release and induces adhesion molecule expression responsible for neutrophil recruitment to sites of inflammation. IL-17 also promotes production and release of pro-inflammatory cytokines, which are essential for neutrophil priming. Thus, IL-17 is likely involved in the recruitment of neutrophils and other immune cells to sites of inflammation, which may contribute to tissue injury and granuloma formation. Together, these data provide important evidence for Th17 cell and IL-17 involvement in the GPA pathogenesis.

In addition to Th cells, B cells are considered central players in the GPA pathogenesis, as they are precursors of ANCA-producing plasma cells. However, other B cell properties are likely involved as well. For example, a subpopulation of B cells referred to as regulatory B cells (Bregs), phenotypically identified by CD24hiCD38hi expression, were demonstrated to exert immune-regulating properties, mainly via IL-10 secretion. Interestingly, alterations in Breg numbers and/or function are associated with progression of several autoimmune diseases such as SLE, multiple sclerosis, and RA. Bregs are able to inhibit Th cell proliferation and to suppress Th17 responses, indicating that these cells are important in dampening inflammatory responses and autoimmune diseases.

To date, most studies on Bregs in GPA patients have demonstrated that their function is not compromised as both Th1 cell and monocyte cytokine production can be suppressed by Bregs from GPA patients. However, several studies did report a decreased circulating Breg frequency in GPA patients. We hypothesized that the reduced Breg frequency may contribute to aberrant Th cell responses and may explain enhanced Th17 cell responses in GPA patients. To test this hypothesis, we assessed the circulating Breg and ThEM 17 cell frequencies, as well as the ThEM 1 cell frequencies, in GPA patients, and investigated the functional impact of these Bregs on Th17 cells in vitro.


Materials and Methods

Study Population

We included 42 remission GPA patients and 18 age-matched HCs (38.9% male; mean age: 58.7; range: 37.4-78.3) to compare circulating B- and T cell subsets. The GPA diagnosis was according to definitions of the Chapel Hill Consensus Conference and patients fulfilled the ACR classification criteria. All patients tested at least once positive for PR3-ANCA. Furthermore, patients were at least one year off rituximab treatment. For patient characteristics see Supplementary Table 1.

Nine GPA patients and three HCs were included for the functional experiment. All GPA patients were in remission and received no immunosuppressive therapy (Supplementary Table 1). The study was carried out in compliance with the Helsinki Declaration, was approved by the ethics committee of the UMCG (METc no. UMCG 2012/151) and informed consent was obtained from all participants.

Flow Cytometry Analysis

Blood samples were washed and incubated with anti-human CD3-AlexaFluor700, CD4-eFluor450 (eBioscience, San Diego, USA), CD45RO-FITC, CCR7-PE-Cy7 (BD-Biosciences, Franklin Lakes, USA), CXCR3-APC-Cy7, CCR4-PerCP-Cy5.5 and CCR6-BV605 (BioLegend, San Diego, USA) to determine CD45RO+CCR7-CCR6+CCR4+CXCR3- Th EM17 cells and CD45RO+CCR7-CCR6+CCR4+CXCR3+ Th EM1 cells, or anti-human CD19-eFluor450, CD38-PE-Cy7 (eBioscience) and CD24-FITC (BD-Biosciences) to determine CD24hiCD38hi Bregs. Samples were fixed, washed and acquired on a LSR-II (BD Biosciences). For gating strategies see Supplementary Figure 1.

Cell Sorting and Co-culture Assay

Peripheral blood mononuclear cells (PBMCs) were isolated and stained with anti-human CD19-eFluor450, CD24-FITC and CD38-APC (BD Biosciences) to sort total B cells or CD24hiCD38hi Breg-depleted B cells. The purity of the sorted Breg-depleted fraction was >98%.

Simultaneously, untouched CD4+ Th cells were sorted directly into a polypropylene tube containing either total B cells or Breg-depleted B cells (Supplementary Figure 2). Sorted cells were washed in RPMI + 10% FCS (Lonza, Basel, Switzerland) + 50 μg/ml gentamycin (GIBCO, Life-Technologies, Grand Island, USA) and cultured in the presence of 500 ng/ml CpG-oligodeoxynucleotides (CpG-ODN) 2006 (Hycult Biotech, Uden, the Netherlands) and 5 μg/ml Staphylococcal Enterotoxin B (SEB; Sigma-Aldrich, St Louis, USA). At baseline and day 5, samples were restimulated with 2 mM Ca-I and 50 ng/ml phorbol myristate acetate (PMA) for 4.5 h in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich).
Determination of Intracellular Cytokines in Th cells

After restimulation, cells were washed twice in PBS (GIBCO). To exclude dead cells, Zombie-Dye Aqua (BioLegend) was added. Cells were washed, stained with anti-human CD3-BV786 (BD Biosciences) for 15 min, and incubated with the Fix&Perm kit (Invitrogen, Life Technologies, Grand Island, USA). Next, cells were stained with anti-human IL-17-APC-eFluor780 (eBioscience) and IFNy-BUV395 (BD Biosciences) to determine the frequencies of Th17- (IL-17-producing) and Th1 (IFNy-producing) cells. Samples were acquired on a LSR-II and analyzed in Kaluza v1.7 (Beckman Coulter, Brea, CA, USA). For representative gating examples see Figure 2A.

Statistical Analysis

Data were analyzed with GraphPad Prism (GraphPad Software, San Diego, USA). Correlations were assessed using Spearman’s rank correlation coefficient. The Wilcoxon signed rank test was used to compare paired data. P<0.05 was considered significantly different.

Results

Circulating CD24hiCD38hi Bregs Correlate Inversely with the Th_{EM}17 cell Frequency of Untreated GPA Patients

First, frequencies of circulating Bregs, Th_{EM}17 and Th_{EM}1 cells were compared between treated and untreated GPA patients and HCs. Representative gating plots are given in Figure 1A. A significant decrease in the circulating Breg frequency was observed in treated GPA patients compared with untreated patients and HCs, whereas a significant increase in the Th_{EM}17 cell frequency was observed in treated and untreated GPA patients as compared with HCs (Figure 1B). In addition, the Th_{EM}1 cell frequency was significantly decreased in untreated GPA patients, whereas treated GPA patients showed a decreased trend (not reaching significance), compared with HCs (Figure 1B). No differences in Breg frequencies were found comparing ANCA-positive and ANCA-negative patients (data not shown).

Subsequently, the association between circulating Bregs and both Th_{EM}17 and Th_{EM}1 cells was assessed. Importantly, Bregs correlated negatively with Th_{EM}17 cells (r = -0.53; p = 0.007) and positively with Th_{EM}1 cells (r = -0.47; p = 0.01) in untreated GPA patients (Figure 1C). In treated GPA patients, Breg and Th_{EM}1 cell frequencies were not correlated (r = 0.3; p = 0.09), whereas a trend towards a negative correlation was observed with Th_{EM}17 cells (r = -0.36; p = 0.05). Furthermore, no correlation between Breg and Th_{EM}17 or Th_{EM}1 cells were found in HCs (both r = -0.125; p = 0.311; Figure 1C). These findings support an association between decreased Bregs and expanded Th17 cell responses in untreated GPA patients.
Figure 1. Association between Th EM17 cells and CD24 hiCD38 hi Bregs in peripheral blood of untreated and treated GPA patients. A. Representative flow cytometry plots from a HC and GPA patient for CD24 hiCD38 hi Bregs (left), Th EM17 cells (middle) and Th EM1 cells (right). B. Comparison of the circulating CD24 hiCD38 hi Breg, Th EM17 cell and Th EM1 cell frequencies between HCs and treated and untreated GPA-patients. C. Correlation of circulating CD24 hiCD38 hi Bregs with Th EM17- and Th EM1 cells in HCs (left), with Th EM17 cells (middle) and Th EM1 cells (right) in untreated and treated GPA patients. *p<0.05; **p<0.001; ***p<0.0001. Bregs: regulatory B cells; HCs: healthy controls; GPA: granulomatosis with polyangiitis; Th EM: T helper effector memory
Circulating Bregs and Th$_{EM}$17 cells in GPA Patients

Figure 2. Bregs from GPA patients decrease IL-17$^+$ Th cell proportions at day 5 in B cell:Th cell co-cultures. PBMCs were depleted of monocytes, NK cells, and CD8$^+$ T cells. CD4$^+$ Th cells (CD19 negative) were negatively selected and sorted directly into a polypropylene tube containing either total CD19$^+$ or Breg-depleted B cells in the same ratio as present in peripheral blood of each patient. Min. 0.5*10$^6$ cells/mL were cultured in polypropylene tubes, stimulated with SEB + CpG and restimulated for 4.5 hours with PMA + Ca-I in the presence of BFA at baseline and day 5. A. Representative flow cytometry dot plots from a GPA patient sample showing frequencies of IL-17$^+$ Th cells (top) and IFN$\gamma$+ Th cells (bottom) at baseline (left) and at day 5 (right) of the co-culture. Lymphocytes were gated based on FCS/SSC, dead cells were excluded and CD3$^+$ T cells were gated to determine single IL-17$^+$ or IFN$\gamma$+ T cells. B. IL-17$^+$ and C. IFN$\gamma$+ Th cell frequencies of GPA patient samples co-cultured with either undepleted or Breg-depleted B cell fractions. D. The IL-17$^+$:IFN$\gamma$+ Th cell ratio was determined for both undepleted and Breg-depleted co-cultures. *p<0.05; **p<0.01
Circulating CD24hiCD38hi Bregs Suppress Th17 cell Responses in vitro

To elucidate the impact of Bregs on Th17 cell expansion, we sorted and co-cultured CD4+ Th cells with Breg-depleted B cells or with total B cells in the presence of CpG and SEB. The IL-17-producing Th cell frequencies (i.e. Th17 cells) were determined at baseline and day 5. We also determined the impact of Bregs on the IFNγ+-producing Th cell frequencies (i.e. Th1 cells).

At baseline, no differences were seen in the Th17- or Th1 cell percentages between both cultures (Figure 2B and C). In Breg-depleted and undepleted cultures, the Th1 cell frequency decreased at day 5 compared with baseline, whereas the Th17 cell frequency was not different (Figure 2C). Intriguingly, the Th17 cell frequency was significantly increased in Breg-depleted samples in comparison to Breg-undepleted cultures at day 5, whereas no such difference was seen at baseline (Figure 2B). Importantly, no differences were found in these frequencies in HCs (Supplementary Figure 3). Additionally, the IL-17+:IFNγ+ Th cell ratio was increased in undepleted and Breg-depleted samples over time (Figure 2D).

Discussion

Here we show a significant inverse correlation between Th17 cells and Bregs in untreated GPA patients, which implicates that Bregs allow Th17 cell expansion. We further explored this finding by assessing the impact of circulating Bregs on Th17 cells in vitro, and confirmed that Breg depletion resulted in a Th17 cell expansion. The cross-talk between B- and Th cells was previously revealed in animal studies. B cell depletion in a mouse model of atherosclerosis switched the immune response towards diminished IFNγ and enhanced IL-17 production154. In a mouse model of arthritis, mice lacking IL-10-producing Bregs developed exacerbated arthritis and presented with increased Th17 cells, whereas adoptive transfer of Bregs to those mice reduced Th17 cells and ameliorated disease154. Additionally, Bregs not only dampened the Th17 cell response, differentiation of naïve T cells into Th17 cells was also inhibited by IL-10-producing Bregs154.

Human studies indicate a similar relationship between B and Th cells. It was reported by Blair et al.131 that CD24hiCD38hi Bregs from HCs could decrease IFNγ and TNFα production by Th cells and suppress their differentiation, but are functionally impaired in SLE patients. In GPA patients, an inhibitory effect of Bregs on IFNγ-producing Th1 cells was demonstrated by Todd et al.45, which appears inconsistent with our results. However, it is important to note that the set-up of our co-culture experiments was significantly different from those performed by Blair et al.131 and Todd et al.45, in which Bregs were kept unstimulated, whereas in our co-culture B cells were stimulated with CpG. Recently, Mielle et al.155 have shown that CpG-induced Bregs failed to suppress IFNγ- and TNFα-producing Th cells in vitro. This is consistent with our finding and clearly
explains the discrepancies in results relating the suppressive effect of stimulated and unstimulated Bregs on cytokine production by Th cell subsets. To date, little data is available on the effect of Bregs on the Th17 cell response. Interestingly, Zhang et al. demonstrated that human Bregs negatively regulate Th17 cell responses in patients with tuberculosis. Another study demonstrated that healthy CD24hiCD38hi Bregs were able to limit Th1- and Th17 cell differentiation, whereas Bregs from RA patients failed to do so. Furthermore, data from rituximab-treated patients support the putative link between Bregs and the Th17 cell response. Following B cell depletion by rituximab, CD24hiCD38hi Bregs are the first to emerge from the bone marrow, and become the dominant circulating B cell subset. Enrichment in CD24hiCD38hi Bregs upon rituximab may affect Th cell balances with a major effect on the human Th17 cell population. It has been postulated that the effectiveness of rituximab is mediated by inhibition of the Th17 cells. Together, these studies provide new insight into regulation of Th17 cells by Bregs.

In contrast to previous reports showing altered Breg function in autoimmune diseases, our study suggests that Bregs from GPA patients retain the ability to control the Th17 cell response. This is in line with previous reports that have also shown that Breg function, in terms of IL-10 production and suppression of monocyte and Th1 cell activation, is not compromised in GPA patients. However, a decrease in circulating CD24hiCD38hi Bregs in GPA patients was repeatedly reported. These findings suggest that the numerical decrease of Bregs in GPA patients results in expansion of potentially pathogenic Th17 cells. In GPA patients, rituximab treatment is effective in remission induction and increased frequencies of circulating Bregs have been demonstrated in these patients following B cell repopulation. Importantly, patients who repopulated with a normalized CD5+ Breg frequency had more sustained remission than patients with low CD5+ Breg frequency repopulation. It is conceivable that the efficacy of rituximab in GPA patients is achieved in part by expansion of rare regulatory B cells, which in turn inhibit expansion of Th17 cells. This could depend on the ratio Bregs and pro-inflammatory T cells (including Th17 cells) after rituximab treatment, as a decreased ratio was related to the occurrence of future disease relapses in GPA patients. Further investigations are clearly warranted to dissect the impact of rituximab in GPA patients on the distribution of Th cells with a major focus on Th17 cells.

Further research should also assess the impact of other proposed Breg subsets, such as memory Bregs (CD24hiCD27+) or CD5+ Bregs, on Th cell responses, as the relation between these Breg subsets and ThEM17-/ThEM1 cells is currently not known. Functionally, it is not known whether Bregs need direct cell contact for their suppressive actions or exert these effects via IL-10 production and/or other anti-inflammatory cytokines. This could be investigated by using a trans-well system and addition of blocking monoclonal antibodies to the cell culture.
In conclusion, both $\text{Th}_{\text{EM}}^{17}$ and $\text{Th}_{\text{EM}}^{1}$ cells are correlated with the Breg population in untreated GPA patients. Mechanistically, we showed that Bregs diminish Th17 cell expansion in GPA patients \textit{in vitro}. Future research should focus on the Breg and Th17 cell interaction to elucidate underlying mechanisms responsible for inhibition of Th17 cells. Better understanding of signals that induce Breg expansion could provide a new strategy to control Th17 cell responses in GPA patients.

\textbf{Acknowledgments}

A research grant from the Dutch Kidney Foundation (grant no. 13OKJ39) was given to J.S.S. W.H.A. and P.H. are supported by the European Union's Horizon 2020 research and innovation program project RELENT (grant no. 668036).

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\textbf{Disclosure Statement}

The authors have declared no conflicts of interest.
Supplementary Files

Supplementary Table 1. Clinical characteristics GPA patients.

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<th>GPA patients included in phenotyping study</th>
<th>GPA patients included in functional study</th>
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AZA: Azathioprine; ANCA: anti-neutrophil cytoplasmic autoantibodies; CRP: c-reactive protein; eGFR: estimated glomerular filtration rate; IS: immunosuppressive; MMF: mycophenolate mofetil; MTX: methotrexate; Pred: prednisone
Supplementary Figure 1. Gating strategy used to identify CD24<sup>hi</sup>CD38<sup>hi</sup> Breg and Th<sub>EM</sub> cells.

**A.** Gating strategy used to identify CD24<sup>hi</sup>CD38<sup>hi</sup> Bregs. First lymphocytes were gated using the SSC/FSC plot. Using the total lymphocyte population, we determined high expression of both CD38 and CD24 using the CD38/CD19 and CD24/CD19 plot, respectively. Within the lymphocytes, B cells were gated using the SSC/CD19 plot. Within the B cell population Bregs were defined as CD24<sup>hi</sup>CD38<sup>hi</sup> cells in the CD24/CD38 plots, applying the same gates as determined for high expression of both markers.

**B.** Gating strategy used to identify Th<sub>EM17</sub>- and Th<sub>EM1</sub> cells. CD4<sup>+</sup> T cell subsets were gated using the CCR7/CD45RO plot. Within the CCR7<sup>-</sup>CD45RO<sup>+</sup>CD4<sup>+</sup> Th<sub>EM</sub> cells, CCR6<sup>-</sup> and CCR6<sup>+</sup> cells were identified. Within the CCR6<sup>+</sup>CD4<sup>+</sup> Th<sub>EM</sub> cells, the CXCR3/CCR4 plot was used to identify CXCR3<sup>-</sup>CCR4<sup>+</sup> Th<sub>EM1</sub> cells and within the CCR6<sup>-</sup>CD4<sup>+</sup> Th<sub>EM</sub> cells the same plot was used to identify CXCR3<sup>-</sup>CCR4<sup>+</sup> Th<sub>EM17</sub> cells.
Supplementary Figure 2. Gating strategy used for sorting. Lymphocytes were gated using the FCS/SSC and CD4 Th cells were negatively gated by depleting CD8^+ T cells, NK cells and monocytes using anti-CD8, anti-CD16, anti-CD56 and anti-CD14, respectively. Untouched CD4^+ Th-cells were directly sorted into a polypropylene tube containing either Breg-undepleted B cells (total CD19^+) or Breg-depleted fraction (by depleting CD24^hiCD38^hi B cells). The sort purity was almost 99%. At least 0.5*10^6 cells/mL were cultured and stimulated with SEB and CpG and restimulated for 4.5 hours with PMA and Ca-I in the presence of BFA at baseline and at day 5.
Supplementary Figure 3. IFNγ⁺ and IL-17⁺ T cell proportions over time in co-cultures of CD4⁺ Th cells with either undepleted or CD24⁺CD38⁺ Breg-depleted B cells from HCs. The frequency of IL-17⁺ and IFNγ⁺ Th cells of GPA patient samples co-cultured with either undepleted or Breg-depleted B cell fractions in the presence of CpG and SEB. IL-17⁺ and IFNγ⁺ Th cell frequencies were determined at day 0 and day 5 upon restimulation with Ca-I and PMA.