Regulatory B cells are numerically but not functionally deficient in anti-neutrophil cytoplasm antibody-associated vasculitis

Sarah Katrina Todd1, Ruth J. Pepper1, Juliana Draibe1, Anisha Tanna2, Charles D. Pusey2, Claudia Mauri3 and Alan D. Salama1

Abstract

Objectives. B cells are central to the pathology of ANCA-associated vasculitis (AAV), a disease characterized by autoantibodies and effectively treated by rituximab. In addition to promoting inflammation, a subset of B cells act to suppress harmful autoimmune responses (Breg). The balance of effector and regulatory B cell subsets in AAV is not known. This study was conducted to assess the relative frequency of these subsets during different states of disease activity.

Methods. B memory (Bmem), naive (Bnaive) and regulatory (Breg) subsets were defined by their relative expression of CD24 and CD38. Function was assessed by cytokine production and suppressive action on CD4+ Th1 activation evaluated in a co-culture system.

Results. Compared with healthy controls, the frequency of Breg (CD24hiCD38hi) was significantly reduced during disease remission in both proteinase 3 (PR3)- and MPO-ANCA patients and during acute disease in PR3-ANCA patients, while the frequency of memory cells (CD24hiCD38lo) was reduced during active disease and restored during remission. Breg cell frequency showed a positive correlation, while Bmem had an inverse correlation with IL-10 production in vitro. B and T cell co-cultures revealed that memory and naive B cell subsets augmented Th1 activation in vitro, which was prevented by Breg, and this pattern did not differ between remission AAV patients and controls.

Conclusion. In remission there is a numerical, but not functional, deficiency in Breg and preservation of Bmem associated with reduced IL-10 production and increased Th1 activation in vitro. This imbalance may contribute to the high rate of relapse observed in AAV, especially in PR3-ANCA patients.

Key words: ANCA-associated vasculitis, B lymphocyte subsets, B regulatory cells.

Introduction

ANCA-associated vasculitides (AAVs) are characterized by autoantibodies against MPO (MPO-ANCA) or proteinase 3 (PR3-ANCA). ANCAAs are thought to contribute directly to pathogenesis by activation of neutrophils and monocytes, which promotes adherence to the endothelium and release of proteolytic granule proteins, cytokines and chemokines [1]. The importance of B cells is further demonstrated by the use of B cell depleting agents such as rituximab, which has been shown to induce clinical remission with similar efficacy to CYC [2, 3].

B cells can modulate immunity independently of antibody production. They are effective antigen-presenting cells [4] and a potent source of cytokines [5]. Some B cell subsets may act to limit inflammation, and this function has been attributed to the CD19+ CD24hiCD38hi population of transitional B regulatory cells (Bregs) [6–8]. Bregs suppress T cell proliferation and production of proinflammatory cytokines in vitro, partly mediated by IL-10 and dependent on direct T cell contact [8]. Other inhibitory mechanisms may include the production of TGF-β and inhibitory antibodies [9]. In this study we set out to assess the balance of the different B cell
subsets—Breg (CD24hiCD38hi), B memory (Bmem, CD24hiCD38hi) and naive B cells (Bnaive, CD24intCD38int)—in an AAV cohort during different disease states [7–8].

Materials and methods

Subjects

Samples were obtained in accordance with the 1975 Declaration of Helsinki, after informed patient consent with local ethical approval from the National Research Ethics Service, London, Southeast (reference 10/H1102/77). All patients had a history of ANCA positivity and fulfilled the Chapel Hill definitions for granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA) [10]. The study group comprised 19 healthy controls and 53 patients categorized by disease activity. Active disease was defined as having clinical symptoms or signs due to systemic vasculitis with consistent immunological and pathological findings. Acute samples were obtained at initial presentation (see supplementary Table S1, available at Rheumatology Online). Remission was defined as the complete absence of clinical disease attributable to vasculitis for a minimum of 1 month. Tolerant patients were classified as those with a history of active AAV who subsequently became negative for ANCA by ELISA, remaining free from pathology after withdrawal of treatment for a minimum of 2 years.

Cell isolation and enrichment

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation on lymphoprep (Alere, Stockport, UK). B cell subsets were isolated from PBMCs by cell sorting on the basis of 4,6-diamidino-2-phenylindole (DAPI) exclusion (Sigma-Aldrich, Dorset, UK) and relative expression of CD19, CD24 and CD38. CD4+CD25− T cells were isolated by serial magnetic bead isolation (Miltenyi Biotec, Surrey, UK).

B cell immunophenotyping

PBMCs were stained with CD19 (HIB19), CD24 (eBioSN3) and CD38 (HIT2) antibodies (eBioscience, Hatfield, UK). Data analysis was conducted using FlowJo version 7.6.3 (TreeStar, Ashland, OR, USA). B cell frequencies were expressed as corrected percentages, with the sum equal to 100%, excluding the contribution of CD19+CD24− cells [11, 12]. Relative B cell numbers were calculated from full blood count (lymphocytes per litre) and flow cytometry data (raw percentages). Full blood counts were not conducted on healthy controls, so comparison was only possible between patient groups.

B cell IL-10 and TNF-α production

Cytokine production was assessed in consecutive samples from the main cohort: 16 remission patients (see supplementary Table S2, available at Rheumatology Online) and 8 controls (4 males). PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 2 mM L-glutamine (Life Technologies, Paisley, UK) and 10% fetal calf serum (FCS; Sigma-Aldrich) for 48 h at 37°C in 5% CO₂. Untreated cells were compared with CpG-stimulated cells [40 ng/ml ODN 2006-05 (InvivoGen, San Diego, CA, USA), with or without CD154 [4 μg/ml CD154 and 10 μg/ml cross-linking antibody (R&D Systems, Abingdon, UK)]. For the last 5 h, 50 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin (Sigma-Aldrich) were added to stimulated PBMCs; brefeldin A, a Golgi-transport inhibitor, was added to all cells (Golgi-Plug, BD Biosciences, San Jose, CA, USA) [13]. Viability was assessed with BD Horizon™ Fixable Viability Stain (BD Biosciences). Cell surface staining was performed and intracellular staining conducted (eBioscience fixation and permeabilization kit) with IL-10 (JES3-9D7; Biolegend, London, UK) and TNF-α (MAb11; eBioscience) antibodies.

B cell co-cultures

Effects on T cell activation were assessed in consecutive samples from the main cohort in five patients (see supplementary Table S2, available at Rheumatology Online) and five controls (four males). CD4+CD25+ T cells were cultured alone or with B cell subsets at a fixed ratio of 1 B:4 T cells in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FCS, non-essential amino acid (NEAA) solution (Fisher, Loughborough, UK), 1 mM sodium pyruvate (Sigma-Aldrich) and penicillin/streptomycin (Life Technologies). T cells were stimulated with soluble anti-CD28 (CD28.8) at 2 μg/ml (eBioscience) and anti-CD3 (HI3a) at 10 μg/ml (BD Biosciences). Unstimulated T cells were included as a control. Cells were cultured for 5 days at 37°C in 5% CO₂. For the last 4 h, 50 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) were added to CD3/28-stimulated cells and Golgi-transport inhibitors were added to all wells (Golgi-Plug and Stop, BD Biosciences) [13]. Viability was assessed and stained co-cultured for CD4 (SK3) (Biolegend). Cells were fixed in 4% paraformaldehyde (PFA) and permeabilized in 0.5% saponin (Sigma-Aldrich). Staining was conducted for IFN-γ (4 S.B3) (Biolegend) and TNF-α (MAb11) (eBioscience) in 0.1% saponin (Sigma-Aldrich). The results were expressed as the percentage change relative to T cells cultured alone (normalized to zero).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Chi-square tests were performed for discrete variables, one-way analysis of variance (ANOVA) was used to compare single parameters in multiple test groups and two-way ANOVA was used to compare multiple parameters in patients and controls. The Mann-Whitney U-test was used when comparing two groups for a single parameter. Regression analyses were performed as indicated, with correlation determined by Spearman’s rank correlation.

Sarah Katrina Todd et al.
**Results**

**AAV and control subjects**

The control group comprised 10 males and 9 females; sex distribution did not differ from the AAV cohort. The median age of the controls was 50 years [interquartile range (IQR) 40–60], which differed only from the remission group at 66 years (49–77) ($P = 0.0130$). The characteristics of 46 patients are summarized in Table 1, separated according to disease activity. Rituximab-treated patients were in clinical remission ($n = 7$), but their demographics and results are provided in Table 2. Analysis of this group was performed separately because of the profound effects of therapy on B cell homeostasis.

The results in Table 1 show that the proportions of patients with an MPA or GPA diagnosis, MPO- or PR3-ANCA specificity and lymphocyte counts did not differ between the patient groups. The remission group contained a higher proportion of women and had lower serum creatinine levels than the active or tolerant groups. Samples were taken from acute patients at the time of initial presentation (see supplementary Table S1, available at Rheumatology Online) and there was no difference between remission and tolerant patients with regards to the time from initial diagnosis. Standard induction therapy was high-dose prednisolone and CYC. Maintenance therapy comprised AZA or MMF in combination with low-dose prednisolone.

**Reduction in Breg in remission and acute PR3-ANCA**

Breg frequency was reduced in remission patients compared with controls (Fig. 1A). Breg frequency was also significantly lower in PR3-ANCA acute patients relative to acute MPO-ANCA patients ($P = 0.0101$) or controls ($P = 0.0499$). Breg frequency in tolerant patients (IQR 6.22–8.01) and MPO-ANCA acute patients (IQR 4.17–9.56) was comparable to controls (IQR 4.09–8.10).

Changes in the relative Breg numbers showed a similar trend (see supplementary Fig. S1, available at Rheumatology Online), with fewer Breg per litre in acute disease (IQR 0.36–4.71 × 10^6) than in acute disease (IQR 0.36–4.71 × 10^6) and MPO-ANCA (2.37–5.68 × 10^6).

**Reduction in Bmem in acute AAV and tolerant patients**

Bmem frequency was reduced in acute patients relative to controls ($P = 0.0121$) and showed a trend towards a reduction in tolerant patients ($P = 0.0624$). When separated according to ANCA specificity, acute MPO-ANCA patients (IQR 10.03–18.51) did not differ significantly from acute PR3-ANCA patients (IQR 15.10–21.58). However, while overall Bmem frequency in remission was comparable to that of controls (Fig. 1B), the frequency was higher in PR3-ANCA (IQR 27.69–52.75) than MPO-ANCA (IQR 9.70–40.93, $P = 0.0382$).

There was no significant difference in relative Bmem numbers when patients were compared; however, the highest Bmem numbers were still observed in remission (see supplementary Fig. S1, available at Rheumatology Online). The distribution pattern was normal in acute disease.

**Table 1** Comparison of patients according to disease activity

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Remission</th>
<th>Acute</th>
<th>Tolerant</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>12</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>Median</td>
<td>66</td>
<td>64</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>IQR</td>
<td>49–77</td>
<td>43–70</td>
<td>52–83</td>
</tr>
<tr>
<td>Sex, n</td>
<td>Male:female</td>
<td>9:21</td>
<td>8:4</td>
<td>3:1</td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td>Median, ×10^9/l</td>
<td>1.4</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>IQR</td>
<td>0.8–1.9</td>
<td>0.6–1.5</td>
<td>1.1–1.8</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>Median, mg/dl</td>
<td>98</td>
<td>216</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>IQR</td>
<td>62–165</td>
<td>144–522</td>
<td>134–316</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>MPA:GPA, n</td>
<td>17:13</td>
<td>9:3</td>
<td>3:1</td>
</tr>
<tr>
<td>Point of diagnosis</td>
<td>Median, years</td>
<td>6</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>ANCA specificity</td>
<td>PR3, n</td>
<td>18</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Treatment, n</td>
<td>MPO, n</td>
<td>12</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CYC</td>
<td>1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMF</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MTX</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AZA</td>
<td>16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corticosteroids</td>
<td>19</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Oral prednisolone</td>
<td>Median dose, mg</td>
<td>5.0</td>
<td>40.0</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analyses were performed to compare patient groups: chi-squared test for discrete variables and one-way analysis of variance for continuous variables. Significance assumed at $P \leq 0.05$. IQR: interquartile range; MPA: microscopic polyangiitis; GPA: granulomatosis with polyangiitis.
disease and positively skewed in remission (1.711, \(d’\text{Agostino and Pearson test, } P=0.0003\)).

Increased memory:regulatory ratio in remission

We represented the imbalance in effector and regulatory subsets by a memory:regulatory ratio, denoted M:R\(_n\) (Fig. 1C). This was derived by dividing the absolute number of cells within the Bmem gate by the number within the Breg gate. M:R\(_n\) was increased in remission (IQR 7.77–43.75) compared with controls (IQR 2.9–9.4, \(P<0.0001\)). When separated according to ANCA and disease activity, the highest M:R\(_n\) values were observed in MPO-ANCA remission (IQR 3.496–103.60; Fig. 1C), however, the median was only statistically higher than controls in PR3-ANCA remission patients (IQR 10.38–29.85, \(P=0.0006\)).

We also observed differences between MPO-ANCA and PR3-ANCA patients with similar disease activity and treatment. Bregs were reduced in acute PR3-ANCA but not acute MPO-ANCA patients. In remission, Bmem frequency was higher in PR3-ANCA patients than in MPO-ANCA patients. Our results suggest that the role of B cells in pathogenesis may differ between PR3- and MPO-ANCA disease. This is perhaps not surprising, with variations in genetics, pathology and outcome also reported [14, 15].

Outcome data

Within the remission group, 12-month clinical follow-up was available for 22 patients; three relapses were recorded in PR3-ANCA patients, enabling a comparison of PR3-ANCA-positive patients who relapsed (\(n = 3\)) with those who did not (\(n = 6\)). Bmem numbers were statistically higher in those who relapsed compared with those who did not (\(P = 0.0238\)). We also observed a tendency towards lower Breg frequency in relapsing patients (IQR 0.4–1.7) compared with those in stable remission (IQR 1.4–3.5), but this did not reach statistical significance. In addition, five of the six patients in stable remission had an M:R\(_n\) below the lower 95% CI for the entire PR3-ANCA remission cohort (<15.2), whereas all those who went on to relapse had an M:R\(_n\) above the upper 95% CI (\(>37.8\)).

### Effects of rituximab treatment on B cell subsets

Flow cytometry plots before and after rituximab illustrate the profound changes that occur within the B cell populations (Fig. 2A). The reduction in Bmem and the increase in Breg were statistically significant compared with controls (Fig. 2B). The median time from treatment with rituximab was 16 months (range 6 months–7 years) (Table 2). Effects were sometimes long-lasting, with increased Breg frequency observed in one patient 5 years after rituximab treatment. At the point of sampling, the relative B cell numbers in rituximab-treated patients did not differ significantly from other patient groups (one-way ANOVA, \(P=0.5689\); see supplementary Fig. S1, available at Rheumatology Online).

### B cell cytokine profile in AAV

Treatment of PBMCs with CpG alone or in combination with CD154 induced IL-10 expression within CD19\(^+\) cells (Fig. 3A). In CpG-treated PBMCs there was no difference in the frequency of IL-10-positive B cells between patients and controls (Fig. 3B). When PBMCs were treated with CD154 in addition to CpG, the frequency of IL-10-positive B cells was increased compared with CpG alone (fold increase 4.9, IQR 3.2–7.3). There remained no significant difference between controls and remission patients, but rituximab patients had a significantly higher frequency of IL-10-positive B cells than controls (\(P=0.0071\)) or remission patients (\(P=0.0191\)). Statistical significance was retained, even when the strongest responder in the rituximab cohort was removed from analysis (controls, \(P=0.0185\); remission, \(P=0.0471\)).

IL-10 induction upon CpG and CD154 stimulation was proportionate to Breg and inversely proportionate to Bmem frequency (Fig. 3C). In addition, IL-10-positive B cells induced by CpG and CD154 were enriched within the Breg gate, expressing high levels of CD24 and CD38 (see supplementary Fig. S2, available at Rheumatology Online). Rituximab-treated patients had a higher frequency of Breg and a lower frequency of Bmem, accounting for the increased frequency of IL-10-positive B cells.

---

**Table 2** B cell subsets following rituximab therapy

<table>
<thead>
<tr>
<th>ANCA</th>
<th>RTX</th>
<th>CD19, n</th>
<th>Bmem, n</th>
<th>Breg, n</th>
<th>Bmem, %</th>
<th>Breg, %</th>
<th>M:R(_n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR3(^a)</td>
<td>1.2</td>
<td>1.62E+06</td>
<td>2.49E+04</td>
<td>6.83E+05</td>
<td>2.6</td>
<td>27.7</td>
<td>0.092</td>
</tr>
<tr>
<td>PR3</td>
<td>0.6</td>
<td>2.55E+06</td>
<td>6.19E+04</td>
<td>6.70E+05</td>
<td>1.5</td>
<td>42.0</td>
<td>0.036</td>
</tr>
<tr>
<td>PR3</td>
<td>5</td>
<td>2.43E+07</td>
<td>1.12E+06</td>
<td>6.59E+06</td>
<td>4.7</td>
<td>27.5</td>
<td>0.171</td>
</tr>
<tr>
<td>PR3(^a)</td>
<td>1.2</td>
<td>1.68E+07</td>
<td>1.13E+06</td>
<td>5.04E+06</td>
<td>6.9</td>
<td>31.0</td>
<td>0.223</td>
</tr>
<tr>
<td>PR3(^a)</td>
<td>8</td>
<td>6.27E+07</td>
<td>7.34E+06</td>
<td>6.23E+06</td>
<td>12.6</td>
<td>10.9</td>
<td>1.161</td>
</tr>
<tr>
<td>PR3</td>
<td>1</td>
<td>6.87E+07</td>
<td>3.03E+06</td>
<td>3.20E+07</td>
<td>4.6</td>
<td>48.2</td>
<td>0.095</td>
</tr>
<tr>
<td>MPO</td>
<td>7</td>
<td>2.11E+08</td>
<td>3.98E+07</td>
<td>1.30E+07</td>
<td>20.8</td>
<td>6.8</td>
<td>3.052</td>
</tr>
</tbody>
</table>

Bmem: B memory cells; Breg: B regulatory cells; M:R\(_n\): memory:regulatory ratio; RTX: rituximab; PR3: proteinase 3. Time since last RTX infusion shown in years; median 16 months. Relative CD19, Bmem and Breg cell numbers per litre of blood (derived from the lymphocyte count and raw percentages within flow cytometry gates). Bmem and Breg also shown as corrected percentages. M:R\(_n\), calculated as previously described. *Indicates the subset of RTX-treated patients tested in the cytokine assays.
Fig. 1 Disruption of B cell homeostasis in ANCA-associated vasculitis

(A) Breg reduced during remission relative to controls (P = 0.0001). Breg also reduced in acute PR3-ANCA disease relative to acute MPO-ANCA (P = 0.0101) or controls (P = 0.0017). No significant difference (NS) between MPO-ANCA and PR3-ANCA patients during remission. (B) Bmem reduced in acute disease relative to controls (P = 0.0121), with a trend in tolerant subjects (P = 0.0624). No significant difference in Bmem during remission relative to controls, but Bmem higher in PR3-ANCA than MPO-ANCA (P = 0.0382). (C) M:Rn increased in remission, with high values observed in both MPO-ANCA and PR3-ANCA. M:Rn higher in acute PR3-ANCA than MPO-ANCA (P = 0.0025). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Breg: B regulatory cells; PR3: proteinase 3; Bmem: B memory cells; M:Rn: memory:regulatory ratio.
The patients selected for IL-10 assays were B cell replete. Although they still had a tendency towards lower Breg than controls, the median did not differ significantly (4.16% and 8.27%, Mann–Whitney U-test, \( P = 0.1285 \)). Based on the regression analysis, we predict that remission patients with the greatest reduction in Breg frequency would have diminished IL-10 competency.

The stimulation used to induce IL-10 also resulted in strong TNF-\( \alpha \) expression within the B cell population (Fig. 3A and B). On CpG treatment, TNF-\( \alpha \) expression was lower in rituximab-treated patients (IQR 415–986).

**Fig. 2** Effects of rituximab on B cell subsets
Fig. 3 B cell cytokine profile in CpG- and CD154-treated peripheral blood mononuclear cells

(A) Quadrants drawn as ≤1% positivity in control (BFA: brefeldin A). Treatment resulted in global production of TNF-α: BFA (filled), CpG (dashed line), CpG and CD154 (black line). (B) Frequency of IL-10-positive B cells did not differ on CpG treatment. On addition of CD154, frequency was higher in the rituximab group than controls (P = 0.0071) or remission (P = 0.0191). TNF-α MFI was lower in the rituximab group upon CpG stimulation than remission (P = 0.0097). Statistical significance lost on addition of CD154. (C) Frequency of IL-10-positive B cells upon CpG and CD154 stimulation was proportionate to Breg (P = 0.0043) and inversely correlated with Bmem (P = 0.0027). *P < 0.05, **P < 0.01. MFI: mean fluorescence intensity; Breg: B regulatory cells; Bmem: B memory cells.
compared with remission patients (IQR 1096–1843), but this effect was lost upon CD154 stimulation (Fig. 3B).

Effects of B cell subsets on Th1 activation

In our co-culture system, the addition of Breg at a ratio of 1:4 did not statistically reduce the frequency of IFN-γ or TNF-α T cells. Effects were modest and in keeping with previously reported results in which a Breg dose-dependent effect was demonstrated, with maximum inhibition at a 1:1 ratio [8]. We were unable to increase the proportion of Breg further due to limited numbers of B cells. However, we were able to assess whether there was any variation in CD4 cytokine production between the different B cell subset co-cultures. We did so by expressing the difference in frequency [mean fluorescence intensity (MFI)] as a percentage relative to T cells alone (normalized to zero). In T cells cultured alone, the frequency of TNF-α-positive cells ranged from 35% to 84% and that of IFN-γ-positive cells from 5% to 29%; TNF-α MFI ranged from 1569 to 8770 and IFN-γ from 179 to 11,367.

The change in TNF-α and IFN-γ T cell frequency did not differ between patients and controls, but there was a difference between B cell subsets (Fig. 4A). Results show a modest decrease in Th1 differentiation with Breg and an increase in Bmem and Bnaive co-cultures (two-way ANOVA, TNF-α P = 0.0362 and IFN-γ P = 0.0219). The median reduction in cytokine-positive cells with Breg was −4.23% for IFN-γ and −5.13% for TNF-α. When Tukey’s multiple comparison was conducted, IFN-γ-positive CD4 cells were significantly lower in Breg co-cultures compared with Bmem (P = 0.0180), and Bnaive showed a similar trend (P = 0.0896). TNF-α-positive CD4 cells also tended to be lower in Breg than in Bmem (P = 0.0614) or Bnaive co-cultures (P = 0.0531).

MFI was also reduced in Breg and increased in Bmem and Bnaive co-cultures relative to T cells alone (two-way ANOVA, P < 0.0001; Fig. 4B). The median reduction in MFI in Breg co-cultures was −15.7% for TNF-α and −18.9% for IFN-γ. Tukey’s multiple comparison test was conducted for TNF-α, and Breg co-cultures differed significantly from Bnaive (P = 0.0006) and Bmem (P < 0.00001). Holm-Šídák’s multiple comparison testing was conducted for IFN-γ, since patients and controls also differed (P = 0.0002). IFN-γ MFI in Breg co-cultures differed significantly from Bnaive (P = 0.0004) and Bmem (P = 0.0002). The increase in IFN-γ MFI was lower in Bmem (P = 0.031) and Bnaive (P = 0.0175) co-cultures than controls. The difference observed might be due to higher basal activation in patient T cells, limiting further increases with the addition of Bmem or Bnaive cells [16].

The addition of Bmem or Bnaive cells augments Th1 cytokine production relative to T cells cultured alone. When Breg cells are added at the same ratio, there is a reduction in IFN-γ and TNF-α production that is comparable in patients and controls. Results indicate that although there is a numerical deficiency of Breg in remission, when the frequency is comparable to healthy controls, there is no functional impairment.

Discussion

The overall balance of B effector and regulatory cells is likely to be important in determining clinical outcome in AAV. Bmem have an increased ability to stimulate T cells and can readily differentiate into plasmablasts on re-encountering antigen [11]. The profile of cytokines produced by Bmem also differs, with higher lymphotokin and TNF-α [17]. In contrast, Breg produce IL-10 and TGF-β [18], limiting inflammation [8, 12, 19–21].

We found Breg diminished in remission for both MPO- and PR3-ANCA, with restoration of Bmem; this imbalance was summarized by the M:Rn ratio (Fig. 1C). The M:Rn ratio was increased in remission patients (statistically significant only for PR3-ANCA) and decreased following rituximab therapy and in patients who regained immunological tolerance. In patients who relapsed within 12 months of initial immunophenotyping, M:Rn ratio was greater than the upper 95% CI. The overall balance of B cell subsets determined the frequency of IL-10 B cells, central to suppression of harmful Th1 and Th17 autoimmune responses in vivo [19]. In addition, Bmem and Bnaive augmented Th1 differentiation in vitro, while Breg did not (Fig. 4).

We found no difference in M:Rn ratio during acute disease relative to controls. Breg frequency was comparable in acute MPO-ANCA patients and controls, while Bmem frequency was reduced universally (Fig. 1). The reduction in Bmem might be due to B cell recruitment to the site of inflammation, terminal B cell differentiation with loss of CD19 expression or the existence of a Bmem population with a non-classic phenotype [22].

We utilized CD24 and CD38 to define Breg, however, suppressive activity has also been attributed to CD5+ CD1dhi cells and CD25+ and CD27+ B cells [9, 23–25]. This study adds to the evidence that regulatory B cell subsets are diminished in AAV, but includes functional characterization that was previously lacking [26–29]. Eriksson et al. [26] found CD25+ B cells to be increased in clinical remission relative to acute disease, but they did not detect any deficit in Bmem cells (Bm5 or CD27+ cells), in contrast to Tadema et al. [30]. Bunch et al. [28] described a numerical deficiency in CD5+ B cells compared with controls, which was most profound during active disease. Although enriched for CD24hiCD38hi cells, CD5 B cells are present in the periphery at a higher frequency and phenotypically overlap with Bmem and Bnaive cells [31]. When we segregated results according to antibody specificity, we found Breg cells were reduced in acute PR3-ANCA patients but not acute MPO-ANCA patients. The discrepancy between Bunch et al.’s results and our own might therefore be due to a higher proportion of PR3-ANCA patients in their active cohort.

We found a positive correlation between Breg and IL-10-positive B cells and the inverse for Bmem (Fig. 3C), supported by back-gating of IL-10-positive cells, which demonstrated that they are predominantly CD24hi and CD38hi (see supplementary Fig. S2, available at Rheumatology Online). Our results indicate that there is
no intrinsic defect in IL-10 production in AAV, but when Breg cells are diminished, IL-10 competency is reduced. This is in keeping with the findings of Wilde et al. [29], who demonstrated that IL-10 competency is diminished in AAV patients during remission and active disease. In contrast, when Breg frequency is increased following rituximab treatment, this is accompanied by an increase in IL-10 competency.

IL-10 production in B cells may also be accompanied by proinflammatory cytokine production, including IL-4, IL-6, IL-12 and IFN-γ [21, 32]. We demonstrated global expression of TNF-α on CpG treatment (Fig. 3A). Thus
some IL-10-positive B cells may have a net proinflammatory effect, necessitating a combination of phenotypic and functional studies to define their role.

In our suppression assays there was a modest reduction in Th1 cytokine production on addition of Breg cells (Fig. 4). In contrast, we found the addition of Bmem or Bnaive cells increased the production of proinflammatory cytokines by T cells (frequency and MFI). Augmentation of Breg frequency in tolerant patients and following rituximab treatment may therefore limit B cell–mediated Th1 activation in vivo.

Breg cells are thought to act in an antigen-specific manner. B cell receptor signalling is dependent on CD19 and is followed by an increase in intracellular calcium. In CD19−/− mice IL-10 production is reduced and experimental autoimmune encephalomyelitis (EAE) is exacerbated [33, 34]. Mice deficient for endoreticular calcium sensors also have increased EAE severity, with an altered splenocyte cytokine profile ex vivo [35]. The addition of anti-IlgM has been shown to increase B cell IL-10 production in vitro [21], with infusion of treated B cells protecting mice from diabetes [36]. Furthermore, in an animal model of contact hypersensitivity, protection was only conferred when mice were rechallenged with the same stimulus [37].

In vitro studies with human cells provide additional evidence that Breg cells are antigen specific and require direct T cell contact [8, 20]. Allergen-specific B cells have a 30-fold greater expression of IL-10 mRNA than non-antigen-specific cells and are highly effective at reducing T cell division induced by a recall antigen [24]. This raises the possibility that PR3- or MPO-specific B cells may be even more potent in vivo than our non-antigen-specific functional tests demonstrate and implies that if induction of PR3- or MPO-specific Breg ex vivo is possible, it could represent an effective therapeutic strategy for AAV.

While we cannot exclude the influence of treatment on B cell subsets, circumstantial data suggest that the differences observed do not seem to be due to immunosuppression alone, as there was no significant difference in lymphocyte counts (Table 1) or relative B cell numbers (see supplementary Fig. S1, available at Rheumatology Online) between any of the patient groups. In addition, Breg frequency was previously shown to be increased in treated SLE patients receiving similar immunosuppressive agents as our current AAV remission group (71% AZA and 58% prednisolone) [8].

We also observed differences between MPO-ANCA and PR3-ANCA patients with similar disease activity and treatment. Breg cells were reduced in acute PR3-ANCA patients but not acute MPO-ANCA patients. In remission, Bmem frequency was higher in PR3-ANCA patients than MPO-ANCA patients. Our results suggest that the role of B cells in pathogenesis may differ between PR3- and MPO-ANCA disease. This is perhaps not surprising, with variations in genetics, pathology and outcome also reported [14, 15].

Our study is limited by the small cohort size and should be validated in other larger cohorts, but we feel it highlights a potential avenue for further research and possible therapeutic manipulation. We had few patients off all immunosuppression, and the impact treatment may have had on B cell subsets needs to be better determined in AAV patients.

### Rheumatology key messages

- B cell homeostasis is disturbed in ANCA-associated vasculitis, with an imbalance in memory and regulatory cells.
- The balance of regulatory and memory subsets determines the frequency of IL-10-positive B cells in ANCA-associated vasculitis.
- Regulatory cells limit Th1 differentiation while effector subsets augment TNF-α and IFN-γ production in ANCA-associated vasculitis.

### Funding

This work was supported by grants from Kidney Research UK (grant number RP32/2011) and The Wellcome Trust (grant number 090048/B/09/Z).

### Disclosure statement

The authors have declared no conflicts of interest.

### Supplementary data

Supplementary data are available at Rheumatology Online.

### References

9. Iwata Y, Matsushita T, Horikawa M et al. Characterization of a rare IL-10-competent B-cell subset in humans that...
Numerical deficiency in B regulatory cells in AAV


19 Carter NA, Rosser EC, Mauri C. Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 regulatory cells and re-differentiation of collagen-induced arthritis. Arthritis Res Ther 2012;14:R32.


34 Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. J Immunol 2009;182: 7459–72.

