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

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Short title:

Numerical deficiency in B regulatory cells in AAV

ABSTRACT

Objective

B cells are central to the pathology of ANCA-associated vascultitis (AAV), a disease characterised 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), naïve 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 to healthy controls, the frequency of Breg ($CD24^{hi}$ $CD38^{hi}$) was significantly reduced during disease remission and during acute disease in patients with PR3-ANCA, while the frequency of memory cells (Bmem, $CD24^{hi}$ $CD38^{lo}$) was reduced during active disease, and restored during remission. Breg cell frequency showed a positive correlation, whilst 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.

KEYWORDS

ANCA-associated vasculitis, B lymphocyte subsets, B regulatory cells

INTRODUCTION

Anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitidies (AAV) are characterised by autoantibodies against myeloperoxidase (MPO-ANCA) or proteinase 3 (PR3-ANCA). Autoreactive B cells class switch and undergo somatic hyper-mutation within secondary lymphoid tissues, producing high affinity antibodies that directly contribute to pathogenesis (1-3). ANCA activate neutrophils and monocytes, promoting adherence to endothelial cells and degranulation, resulting in release of proteolytic granule proteins, cytokines and chemokines, culminating in further leukocyte recruitment and vascular damage (4). The importance of B cells in disease pathogenesis is further demonstrated by use of B cell depleting agents such as rituximab, shown to induce clinical remission with similar efficacy to cyclophosphamide (5, 6).

B cells can modulate immunity independently of antibody production. They are effective antigen presenting cells (7), and a potent source of cytokines, shaping the CD4 T cell response (8). Some B cell subsets may act to limit inflammation, this function has been attributed to the CD19+ $CD38^{hi}$ $CD24^{hi}$ population of B regulatory cells (Bregs) (9-11). Bregs suppress T cell proliferation and production of pro-inflammatory cytokines *in vitro*, partly mediated by interleukin 10 (IL-10) and dependent on direct T cell contact (11). Other inhibitory mechanisms may include production of TGF-beta and inhibitory antibodies (12). In this study we set out to assess the balance of the different B cell subsets, Breg ($CD38^{hi}$ $CD24^{hi}$), B memory (Bmem, $CD38^{lo}$ $CD24^{hi}$) and naïve B cells (Bnaive, $CD38^{int}$ $CD24^{int}$ (10, 11), in an AAV cohort during different disease states.

MATERIALS AND METHODS

Subjects

Samples were obtained in accordance with the 1975 Declaration of Helsinki, after informed patient consent and under local ethical approval (05/Q0508/6). All patients fulfilled the Chapel Hill definitions for granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA) (13). The study group comprised 19 healthy controls and 58 patients, categorised by disease activity. Acute samples were obtained at the time of initial presentation. Remission was defined as the complete absence of clinical disease attributable to vasculitis, minimum of one 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 (PBMC) were isolated from venous blood by gradient centrifugation on lymphoprep (Alere, Stockport, UK). B cell subsets were isolated from PBMC by cell sorting on a BD FACSAria (BD Biosciences). Cells were selected on the basis of DAPI (4', 6-diamidino-2-phenylindole) exclusion (Sigma-Aldrich, Dorest, UK) and relative expression of CD19, CD24 and CD38.CD4+CD25- T cells were isolated by serial CD25 and CD4 magnetic bead isolation on MS columns (Miltenyi Biotec, Surrey, UK).

B cell immunophenotyping

PBMC were stained with CD19 [HIB19], CD24 [eBioSN3] and CD38 [HIT2] antibodies (eBioscience Ltd, Hatfield, UK). Acquisition was performed on an LSRFortessa instrument (BD Bioscience). Flow cytometry 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, previously described (14, 15).

B cell IL10 and TNF-α production

B cell IL-10 production was assessed in a subset of individuals from the main cohort: 12 remission patients (5 male, 8 PR3-ANCA, 3 MPO-ANCA, 1 ANCA negative); 4 rituximab-treated patients (2 male, 4 PR3-ANCA) and 8 controls (4 male). PBMCs were cultured in RPMI 1640 supplemented with 2mM L-glutamine (Life Technologies Ltd, Paisley, UK) and 10% FCS (Sigma-Aldrich), for 48 hours at 37ºC and 5% CO_2. Untreated cells were compared with CpG- stimulated cells(40µg/ml ODN 2006-G5 (InvivoGen, San Diego, CA, USA)), with or without CD154(4µg/ml CD154 and 10 µg/ml cross-linking antibody (R&D systems, Abingdon, UK)). For the last 5 hours Golgi-Plug (BD Bioscience) was added, with 50ng/ml phorbol myristate acetate (PMA) and 1µg/ml inomycin (Sigma-Aldrich).

Viability was assessed with BD Horizon™ Fixable Viability Stain (BD Biosciences). CD19 cell surface staining was performed and intracellular staining conducted according to the manufacturer's instructions (eBioscience fixation and permeablisation kit), with IL-10 [JES3-9D7] (Biolegend, London, UK) and TNF-α [MAb11] (eBioscience) antibodies. Sample acquisition as described.

B cell co-cultures

Effects on T cell activation were assessed in a subset of individuals from the main cohort: 5 remission patients (1 male, 2 PR3-ANCA, 3 MPO-ANCA) and 5 controls (4 male). CD4+

CD25- T cells were cultured alone or with B cell subsets, at a fixed ratio of 4:1 T:B cells in RPMI 1640, supplemented with: 2mM L-glutamine, 10% FCS, NEAA (Fisher, Loughborough, UK), 1mM sodium pyruvate (Sigma-Aldrich) and penicillin/streptomycin (Life Technologies Ltd).

T cells were stimulated with soluble anti-CD28 [CD28.8] at 2µg/ml (eBioscience) and plate bound anti-CD3 [HIT3a] (BD Biosciences, 10µg/ml). Unstimulated T cells were included as a control. Cells were cultured for 5 days at 37ºC and 5% CO_2. For the last 4 hours, 50ng/ml PMA and 1µg/ml inomycin (Sigma-Aldrich) were added to CD3/28 stimulated cells and Golgi-Plug and Stop added to all wells (BD Bioscience).

Viability was assessed and staining conducted for CD4 [SK3] (Biolegend), as previously described. Cells were fixed in 4% PFA and permeablised in 0.5% saponin (Sigma Aldrich). Staining was conducted for IFN-γ [4S.B3] (Biolegend) and TNF-α [MAb11] (eBioscience), for 40 minutes on ice, in 0.1% saponin (Sigma Aldrich). Sample acquisition and analysis as described; results expressed as percentage change, relative to T cells cultured alone (normalised to zero)

Statistical analysis

Statistical analyses were performed in GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA). Chi-Square was performed for discrete variables; 1-way ANOVA to compare single parameters in multiple test groups; 2-way ANOVA to compare multiple parameters in patients and controls. Mann Whitney U test was used when comparing two groups, for a single parameter. Regression analyses were performed, as indicated; correlation was assessed by Spearman rank.

RESULTS

AAV and control subjects

The control group comprised 10 males and 9 females, and did not differ from the AAV cohort. The median age of the controls was 50 years (IQR 40-60), which differed only from the remission group at 65 years (52-75) (p=0.0083). The characteristics of 51 patients are summarised 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, due to the profound effects of therapy on B cell homeostasis.

TABLE 1 **Comparison of patients, according to disease activity**

Diagnosis and ANCA specificity did not differ and there was no difference in total lymphocyte count between the groups. The remission group contained a higher proportion of women and had lower serum creatinine than active or tolerant groups. With regards time from diagnosis, samples were taken from acute patients at time of initial presentation, but there was no difference between remission and tolerant patients. Standard induction therapy in acute patients was high dose prednisolone and cyclophosphamide. Maintenance therapy in remission comprised azathioprine or mycophenolate mofetil, in combination with low dose prednisolone.

Differences in B cell subset distribution, according to disease activity

Breg percentage frequency was reduced in remission but not in tolerant or acute patients, relative to controls (P=<0.0001, Figure 1A). We observed reduced Bmem in acute disease (P=0.0145) but no decline in Bmem in remission and a trend towards lower Bmem frequency in tolerant patients (P=0.0698), compared to controls (Figure 1B). We represented the imbalance in effector and regulatory subsets by a memory: regulatory ratio, denoted $M:R_n$. 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 significantly increased in remission subjects (19.8, IQR 6.6-45.2), compared to controls (5.1, IQR 2.9-9.4)(P=0.0008, Figure 1C). While we cannot exclude the influence of treatment on B cell subsets, the total lymphocyte counts did not differ between AAV and control subjects (Table 1), and we found no significant differences in B cell subsets according to immunosuppressive treatment (Supplementary figure 1), in agreement with recent data, in which cyclophosphamide did not affect CD19+ $CD38^{hi}$ $CD24^{hi}$ B cell frequency (16).

B cell subsets in PR3- and MPO-ANCA disease

When we examined distribution of B cell subsets according to ANCA specificity, we found Bmem frequency was higher in remission patients with PR3-ANCA (37.4, IQR 27.7-52.8), than MPO-ANCA patients (27.7, IQR 9.7-41.0) (P=0.0382, Figure 1B). However, $M:R_n$ did not differ between these groups (Figure 1C). In acute disease, there was no significant difference in Bmem frequency according to ANCA subtype, but frequency of Breg was reduced in PR3-ANCA patients (2.7, IQR 2.0-2.9) compared with MPO-ANCA (5.9, IQR 4.2-9.6) (P=0.0101, Figure 1A).

FIGURE 1 **Disruption of B cell homeostasis in AAV**

Outcome data

Within the remission group, 12 month clinical follow up was available for 24 patients; 3 relapses were recorded, all 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 and frequency of CD19 cells within the lymphocyte gate, were statistically higher in those who relapsed, compared to those who did not (P=0.0238 and 0.0476 respectively). We observed a tendency towards lower Breg frequency in relapsing patients (IQR 0.4-1.7), compared to those in stable remission (IQR 1.4-3.5), but this did not reach statistical significance. In addition 5 out of the 6 patients in stable remission had an $M:R_n$, below the lower 95% confidence interval for whole PR3-ANCA remission cohort(<15.2), whereas all those who went on to relapse had an $M:R_n$ above the upper 95% confidence interval ($\geq$37.8).

Effects of rituximab treatment on B cell subsets

The median time from treatment with rituximab was 16 months, ranging from 6 months to 7 years (Table 2). Flow cytometry plots before and after rituximab illustrate the profound changes that occur within the B cell populations (Figure 2A). The reduction in Bmem and increase in Breg were statistically significant compared with controls (Figure 2B). These changes continued after B cell repopulation within the lymphocyte gate. Effects were sometimes long-lasting, with increased Breg frequency observed in one patient 5 years after rituximab treatment, longer than previously reported (9).

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

FIGURE 2 **Effects of rituximab on B cell subsets**

B cell cytokine profile in AAV

In CPG-treated PBMC there was no difference in induction of IL10 positive B cells. When PBMC were treated with CD154 in addition to CPG, there was a dramatic increase in the frequency of IL10+ B cells compared to CPG alone (fold-increase 4.9, IQR 3.2-7.7). There remained no significant difference between controls and remission patients, but rituximab patients had a significantly higher frequency of IL10+ B cells than controls (P=0.0090) or remission patients (P=0.0347).

B cell IL-10 induction upon CPG and CD154 stimulation was proportionate to Breg and inversely proportionate to Bmem frequency (Figure 3C). Rituximab-treated patients had higher frequency of Breg (29.4, IQR 15.1-44.5) and lower frequency of Bmem (5.7, IQR 3.1-11.2), compared to controls or remission patients (1-way ANOVA, P=0.0140 and P=0.0108), accounting for the increased frequency of IL10+ B cells.

The stimulation used to induce IL-10, also resulted in strong TNF-α expression within the B cell population (Figure 3A and B). On CPG treatment, TNF-α expression was lower in rituximab treated patients (Median MFI 668, IQR 415-986) compared to remission patients (Median MFI 1370, IQR 917-1827) or controls (1112, IQR 868-1238), but this effect was lost upon CD154 stimulation (Figure 4B).

FIGURE 3 **B cell cytokine profile**

Effects of B cell subsets on Th1 activation

B cell subsets used in the T cell co-cultures, ranged in purity from 83-100%, with a median value of 93% (Figure 4A). Inhibition of IFN-γ or TNF-α cytokine production by CD4+ T cells following addition of Breg at a ratio of 1:4 did not reach statistical significance, but was in keeping with previously reported results in which a Breg dose dependent effect was demonstrated, with maximum inhibition at a 1:1 ratio (11). 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 results as percentage difference relative to CD4+ CD25- T cell stimulation alone (Figure 4B and 4C). Importantly, no difference was observed in suppression between remission patients and healthy controls, (2-way ANOVA); this enabled patient and control data to be combined (n=10) for subset analysis

Within this combined data set, there was a significant difference between the B cell subsets (results expressed as mean $\pm$ standard deviation). The percentage of TNF-α positive T cells was increased in Bmem (5.40 $\pm$6.223) and Bnaive (6.28 $\pm$3.500) co-cultures, relative to Breg (-5.13 $\pm$2.652), (P= 0.0362). The frequency of IFN-γ positive CD4 cells was also increased in Bmem (25.30 $\pm$5.515) and Bnaive (18.03 $\pm$2.510) co-cultures, compared to Breg (-4.23 $\pm$5.621), (P= 0.0219). The results indicate that Bmem and Bnaive cells promote Th1 cell differentiation *in vitro*, whereas Breg have an opposite effect.

FIGURE 4 **Effects of B cell subsets on Th1 differentiation**

DISCUSSION

The overall balance of B effector and regulatory cells is likely to be important in determining clinical outcome in AAV. Bmem have increased ability to stimulate T cells and can readily differentiate into plasmablasts on re-encountering antigen (14). The profile of cytokines produced by Bmem also differs, with higher lymphotoxin and TNF-α (17). In contrast, Breg produce IL-10 and TGF-beta (18), limiting differentiation and proliferation of pro-inflammatory cells (11, 15, 19-21).

We found Breg to be profoundly diminished in clinical remission, with restoration of Bmem; this imbalance was summarised by the $M:R_n$. Ratio. $M:R_n$ was increased in remission patients, reduced following rituximab therapy and in patients who regained immunological tolerance. In patients who relapsed within 12 months of initial immunophenotyping, $M:R_n$ was greater than the upper 95% confidence interval. The overall balance of B cell subsets determined 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*, whilst Breg did not.

We found no difference in $M:R_n$ during acute disease relative to controls; this might be due to B cell recruitment to the site of inflammation, terminal B cell differentiation with loss of CD19 expression or existence of a Bmem population with a non-classic phenotype. CD27-IgD- double negative Bmem have previously described in systemic lupus erythematous, with low expression of CD38 and CD24 (22).

We utilised CD24 and CD38 to define Breg, however suppressive activity has also been attributed to CD5+ $CD1d^{hi}$ cells, CD25+ and CD27+ B cells (12, 23-25).This study adds to

the evidence that regulatory B subsets are diminished in AAV, but includes functional characterisation, previously lacking (16, 26-28). Eriksson *el al* found CD25+ B cells to be increased in clinical remission relative to acute disease (26), but they did not detect any deficit in Bmem cells (Bm5 or CD27+ cells), in contrast to Tadema *et al* (29). Bunch *et al* described a numerical deficiency in CD5+ B cells compared to controls, most profound during active disease (27). Although enriched for $CD24^{hi}$ $CD38^{hi}$ cells, CD5 B cells are present in the periphery at a higher frequency than Breg, and have previously shown to overlap with Bmem and Bnaive (30). When we segregated results according to antibody specificity, we found Breg reduced in acute PR3-ANCA patients, compared to both MPO-ANCA patients and controls. The discrepancy between Bunch's results and our own might therefore be due to a higher proportion of PR3-ANCA patients in their active cohort.

We found no difference in IL-10 production in patients and controls, in contrast to Wilde *et al.* However, this cohort consisted of 78% PR3-ANCA subjects (28), in which we found lower Breg and higher Bmem, correlating with diminished IL-10 production. IL-10 production in B cells may also be accompanied by pro-inflammatory cytokine production, including IL-4, IL-6, IL-12 and IFN-γ (21, 31). We demonstrate global expression of TNFα on CPG treatment. Thus, some IL-10 positive B cells may have a net pro-inflammatory effect, necessitating a combination of phenotypic and functional studies to define their role.

In our suppression assays, there was a modest reduction Th1 cytokine production on addition of Breg. We conducted co-cultures over 5 days, with anti-CD28 and anti-CD3 stimulation, a stronger stimulus than that employed by Blair *et al* (11). Although the suppressive capacity of Breg on global T cell stimulation was limited, they remained unable to augment Th1 differentiation. In contrast, we found addition of Bmem or Bnaive cells increased the

production of proinflammatory cytokines by T cells. Augmentation of Breg frequency in tolerant patients and following rituximab treatment, may therefore limit B cell mediated Th1 activation *in vivo.*

Breg are thought to act in an antigen specific manner. B cell receptor (BCR) signalling is dependent on CD19 and is followed by increase in intracellular calcium. In $CD19^{-/-}$ mice IL-10 production is reduced and experimental autoimmune encephalomyelitis (EAE) exacerbated (32, 33). Mice deficient for the endoreticular calcium sensors also have increased EAE severity, with altered splenocyte cytokine profile *ex vivo* (34). Addition of anti-IgM has been shown to increase B cell IL-10 production *in vitro (21),* with infusion of treated B cells protecting mice from diabetes (35). Furthermore, in an animal model of contact hypersensitivity, B cell mediated protection was only conferred when mice were re-challenged with the same stimulus (36).

In vitro studies with human cells provide further evidence that Breg are antigen specific and require direct T cell contact as suppressive capacity is reduced in transwell cultures (11, 20). Allergen specific B cells have 30.8 fold higher 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), possibly indicating that Breg inhibit antigen specific T cell proliferation more potently than CD3 induced division. In AAV the antigen is known in the vast majority of patients (MPO or PR3); induction of antigen specific Breg *ex vivo* might therefore be possible, and could represent an effective therapeutic strategy for AAV.

KEY MESSAGES

1. B cell homeostasis is disturbed in AAV, with imbalance in memory and regulatory cells
2. The balance of regulatory and memory subsets, determines frequency of IL-10 positive B cells
3. Regulatory cells limit Th1 differentiation, whilst effector subsets augment TNFα and IFN γ production

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Table 1 Comparison of patients, according to disease activity

Patient characteristics		Remission	Acute	Tolerant	P-value
Number		33	14	4	
Age	Median	65	64	73	0.3200
	IQR	52-75	49-71	52-83	
Sex (n)	Male:Female	9:24	8:6	3:1	0.0495
Lymphocyte count	Median ($x10^9$/L)	1.4	1.0	1.5	0.5629
	IQR	0.8-1.8	0.7-1.7	1.1-1.8	
Serum creatinine	Median (mg/dL)	98	152	220	0.0038
	IQR	63-155	124-503	134-316	
Diagnosis	MPA:GPA(n)	19:14	10:4	3:1	0.5811
Point of diagnosis	Median (years)	6	0	11	0.0010
ANCA specificity	PR3 (n)	18	7	1	0.5343
	MPO (n)	12	6	3	
Treatment (n)	None	2	3		0.1181
	Cyclophosphamide	1	6		0.0005
	Mycophenolate	6	0		0.0876
	Methotrexate	2	2		0.8541
	Azathioprine	18	1		0.0025
	Corticosteroids	21	11		0.3151
Oral prednisolone	Median dose (mg)	5.0	37.5		<0.0001

Statistical analyses were performed to compare patient groups: Chi-square for discrete variables and 1-way ANOVA for continuous variables, significance assumed at $P \leq 0.05$.

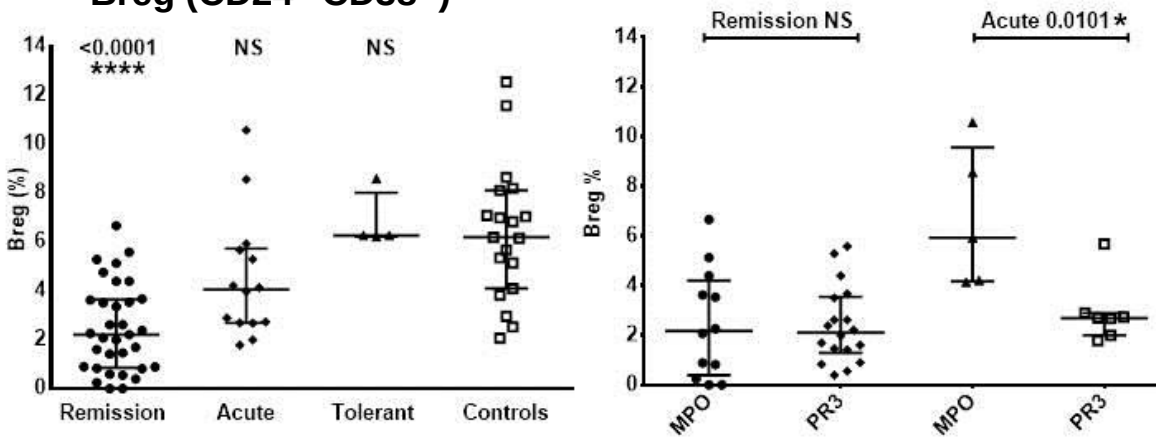
Table 2 B cell subsets following rituximab therapy

Age	Sex	ANCA	Time Since RTX	CD19 %	Bmem %	Breg %	M:R_n
48	F	PR3	1.2	0.359	2.6	27.7	0.092
34	M	PR3	0.6	0.095	1.5	42.0	0.036
71	M	PR3	5	1.75	4.7	27.5	0.171
65	M	PR3	1.2	4.2	6.9	31.0	0.223
47	M	PR3	8	4.61	12.6	10.9	1.161
52	F	PR3	1	5.77	4.6	48.2	0.095
16	F	MPO	7	7.89	20.8	6.8	3.052

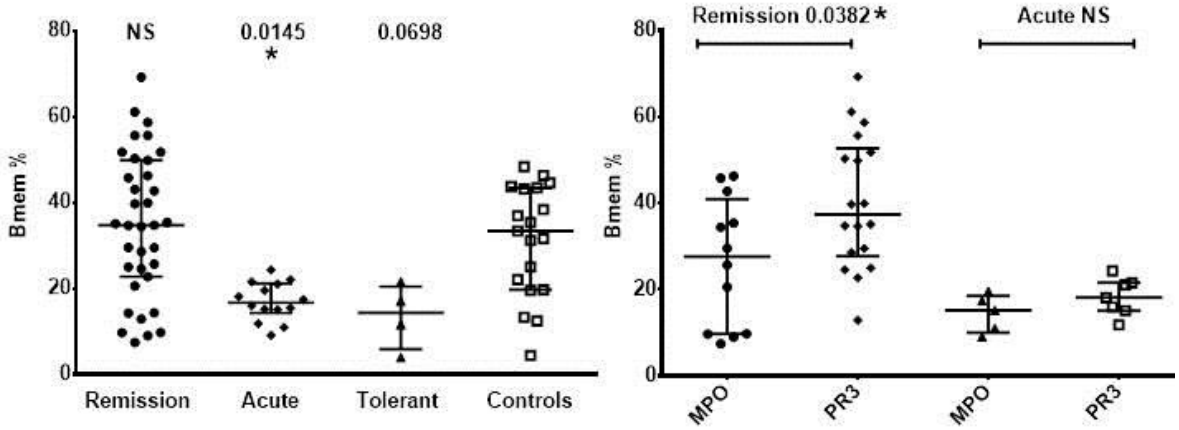
Time since last rituximab (RTX) infusion shown in years, median 16 months. CD19 percentage corresponds to frequency of B cells within the lymphocyte gate. Bmem and Breg shown as corrected percentages, M:R_n calculated as previously described.

Figure 1

A Breg ($CD24^{hi}$ $CD38^{hi}$)



B Bmem ($CD24^{hi}$ $CD38^{lo}$)



C Ratio of Bmem to Breg ($M:R_n$)

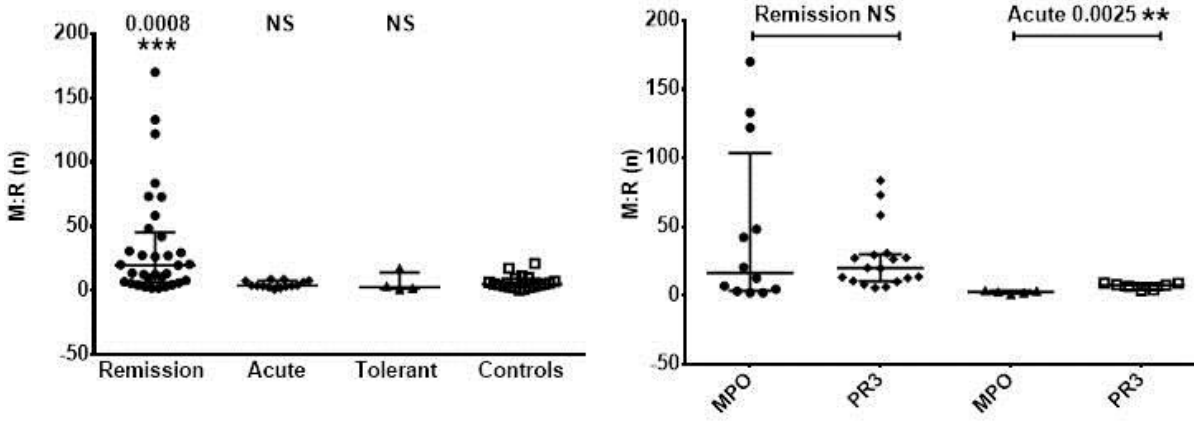
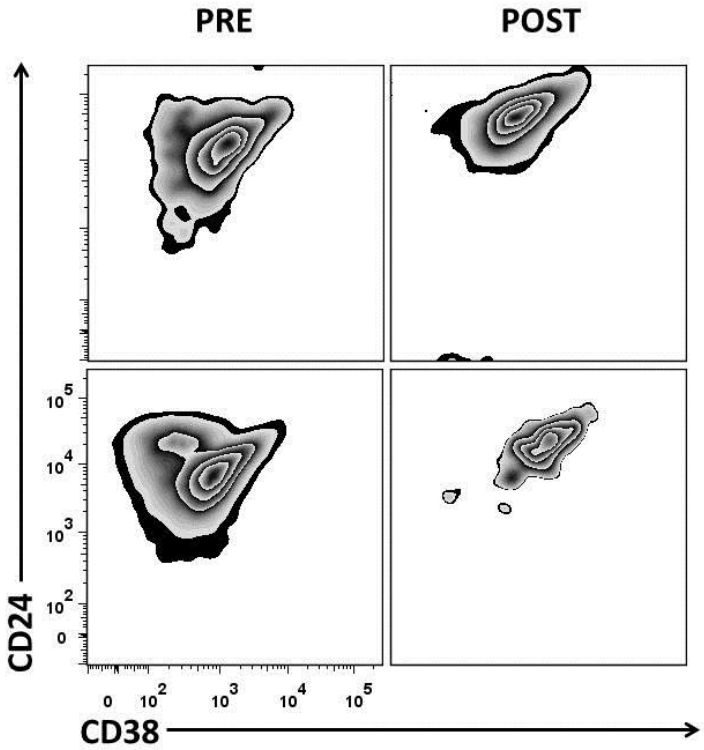


Figure 2

A **B cell profile after rituximab**



B **Percentage frequency of Breg and Bmem**

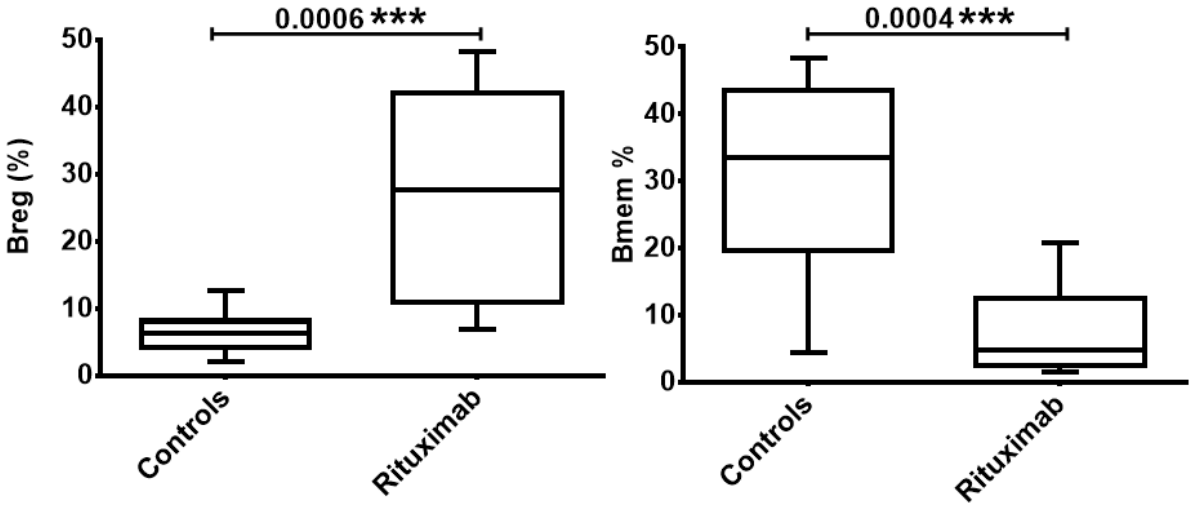


Figure 3

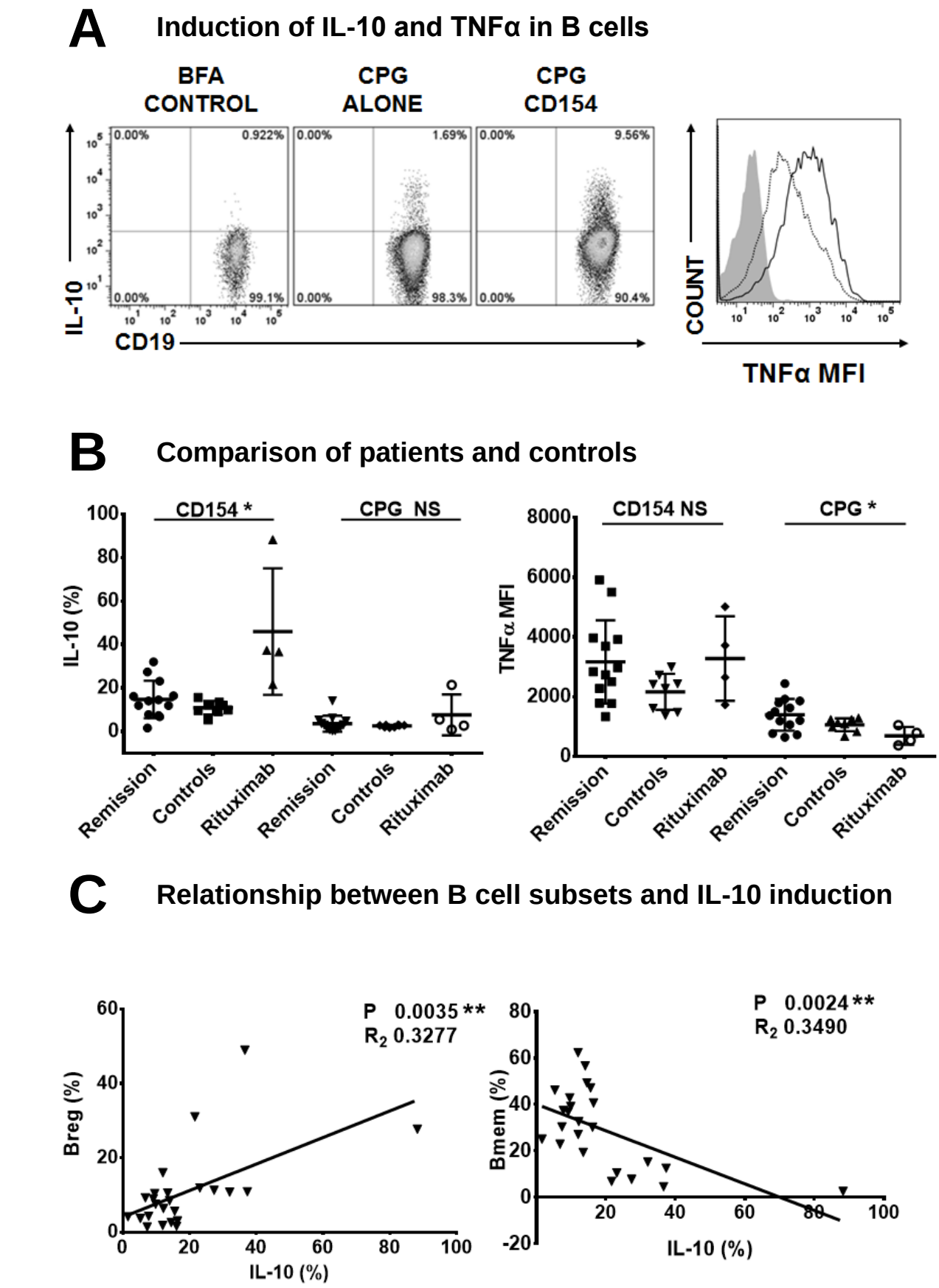
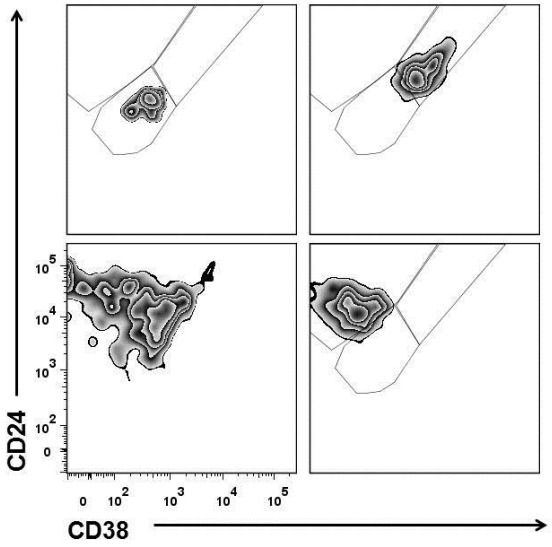
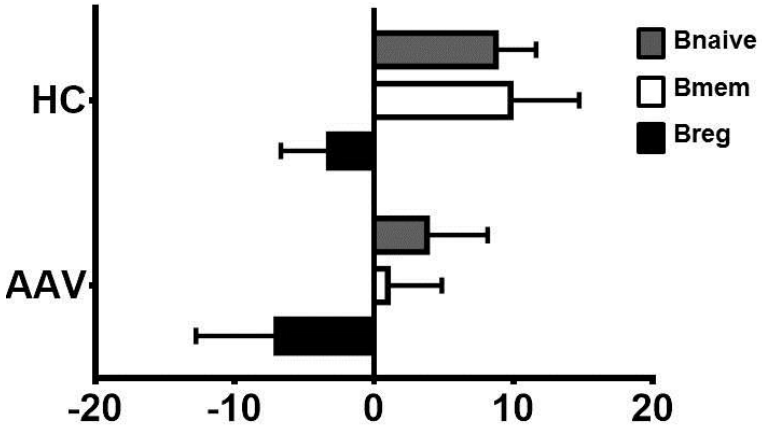

A
Induction of IL-10 and TNFα in B cells
BFA CONTROL
CPG ALONE
CPG CD154
0.00%
0.922%
0.00%
99.1%
0.00%
1.69%
0.00%
98.3%
0.00%
9.56%
0.00%
90.4%
IL-10
CD19
COUNT
TNFα MFI
B
Comparison of patients and controls
CD154 *
CPG NS
IL-10 (%)
Remission
Controls
Rituximab
CD154 NS
CPG *
TNFα MFI
C
Relationship between B cell subsets and IL-10 induction
P 0.0035 **
R2 0.3277
Breg (%)
IL-10 (%)
P 0.0024 **
R2 0.3490
Bmem (%)

Figure 4

A **Purity of FACS isolated B cell subsets**



B **Percentage change in TNFα positive CD4 cells**



C **Percentage change in IFNɣ positive CD4 cells**

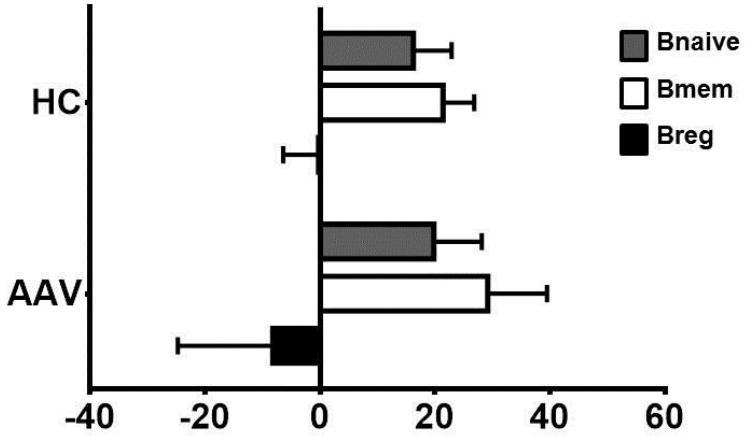


FIGURE LEGENDS

Figure 1. Disruption of B cell homeostasis in AAV

[**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 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) (* $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

Figure 2. Effects of rituximab on B cell subsets

[**A**] Flow cytometry plots from 2 patients before (left hand side) and after rituximab treatment (right hand side). After B cell repopulation Bmem (CD24hi CD38lo) and Bnaive cells are diminished (CD24int CD38int); the majority of B cells have a regulatory phenotype (CD24hi CD38hi). [**B**] Rituximab treated patients have statistically lower frequency of Bmem (P=0.0004) and higher frequency of Breg (P=0.0006) than controls (n=19); Mann Whitney U test (*** $P \leq 0.001$). Box and whiskers plots show minimum and maximum values, rituximab data points also provided in Table 2 (n=7).

Figure 3. B cell cytokine profile in CPG and CD154 treated PBMC

[**A**] Quadrants drawn so ≤1% positivity in control (BFA, Brefeldin A). Treatment resulted in global production of TNFα: BFA (filled), CPG (dashed line), CPG and CD154 (black line). [**B**] Frequency of IL10 positive B cells did not differ on CPG treatment; on addition

of CD154 frequency was higher in rituximab group than controls (P=0.0071) or remission(P=0.0191). TNFα MFI was lower in rituximab group upon CPG stimulation, than remission(P=0.0097), statistical significance lost on addition of CD154 [**C**] Frequency of IL10positive B cells upon CPG and CD154 stimulation, was proportionate to Breg (P=0.0043)and inversely correlated with Bmem (P=0.0027) (* $P \leq 0.05$, ** $P \leq 0.01$).

Figure 4. Effects of B cell subsets on Th1 differentiation

The cohort comprised 10 individuals, however a single data point was missing for 2 of the controls (Bnaive and Breg, n=9). **[A]** There was modest decrease in cytokine positive CD4 cells with Breg and increase, in Bmem and Bnaive co-cultures (2-way ANOVA, TNFα P=0.0362 and IFNγ P=0.0219, * $P \leq 0.05$). [**B]** MFI was also reduced in Breg and increased, in Bmem and Bnaive co-cultures (2-way ANOVA, TNFα and IFN-γ ****$P<0.0001$). The change in MFI differed between patients and controls for IFNγ, but not TNFα. The increase in IFNγ MFI was less in patient Bmem (P=0.0310) and Bnaive (P=0.0175) co-cultures(Holm-Šidak's multiple comparison)