REGULATORY B CELLS IN ANCA ASSOCIATED VASCULITIS: THEIR ROLE IN RESTORING IMMUNE TOLERANCE

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Introduction

The anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitidies (AAV) are characterised by autoantibodies, directed against myeloperoxidase (MPO) or proteinase 3 (PR3). Although antibodies are considered the principal effector molecules of the autoreactive B cells, B cells can also act to suppress harmful autoimmune responses. B cell deficiency in mice can result in exacerbated autoimmune responses [Mauri et al., 2003; Fillatreau et al., 2002; Mizoguchi et al., 1997]. Protection has been attributed to a subset of B cells, which produce IL10. These B regulatory cells, express high levels of CD5, CD10, CD38, CD24 and CD1d surface antigens in man and are functionally impaired in SLE [Blair et al., 2010]. The balance of B cell subpopulations in AAV remains unknown; the aim of this study was to determine whether there was any difference in the relative frequency of B cell subsets in AAV compared to controls.

Patients and controls

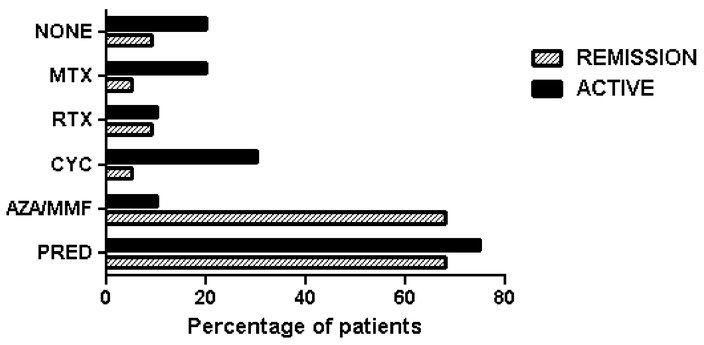
The AAV cohort comprised patients with active disease; those in remission and patients who had regained immune tolerance. Tolerant patients were defined as those with a history of active AAV, who subsequently became autoantibody negative and remained free from pathology after withdrawal of immunosuppression (minimum of 2 years). B cell frequency was assessed in AAV cohorts as well as healthy and immunosuppressed controls (renal transplant recipients).

Table 1. Demographics and ANCA-specificity.

		Gender		Age		Autoantibody specificity			
	N	M	F	Mean ±SEM	Range	PR3	MPO	Atypical	None
AAV - All patients	48	25	23	59.3 ± 2.4	17-85	25	18	1	4
AAV- Tolerant	6	5	1	67.3 ± 6.8	46-85	1	4	0	1
Active disease	20	12	8	53.0 ± 2.9	32-72	12	5	1	2
Remission	22	8	14	62.8 ± 3.9	17-85	12	9	0	1
Transplant recipients	11	4	7	62.3 ± 2.6	49-75	0	0	0	0
Healthy controls	9	6	3	40.9 ± 3.7	21-57	0	0	0	0

Figure 1. AAV treatment

AAV patients were treated with a combination of therapies, summarised in figure 1. All transplant recipients were on combined AZA/MMF and FK/CsA therapy (100%). Healthy controls and tolerant patients received no treatment.



Abbreviations: predinsolone (PRED), tacrolimus (FK), Ciclosporin A (CsA) , methotrexate (MTX), azathioprine (AZA), mycophenolate (MMF), cyclophosphamide (CYC), mycophenolic mofetil (MMF) and rituximab (RTX).

Methods

Mononuclear cells were isolated from peripheral blood by density gradient centrifugation on Lymphoprep (Axis-Shield, Dundee, UK); viability was 95% or greater, assessed by trypan blue exclusion (Invitrogen, Paisley, UK).

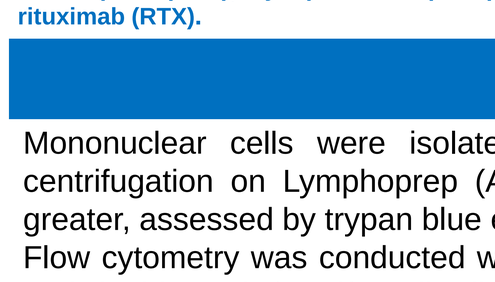
Flow cytometry was conducted with: CD19 PE-Cy7 (HIB19), CD24 PE (eBioSN3) and CD38 FITC (HIT2) antibodies (eBioscience, Hatfield, UK). A healthy control was included in each analytical run and staining conducted, alongside isotype and single-colour compensation controls. Data was acquired on an LSRFortessa instrument (BD Biosciences, Oxford, UK) and analysis conducted with FlowJo software (Treestar, Inc., San Carlos, CA).

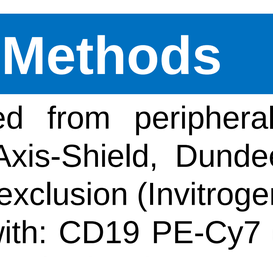
Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA) using one-way ANOVA and student's unpaired, two-tailed t-test.

Gating strategy

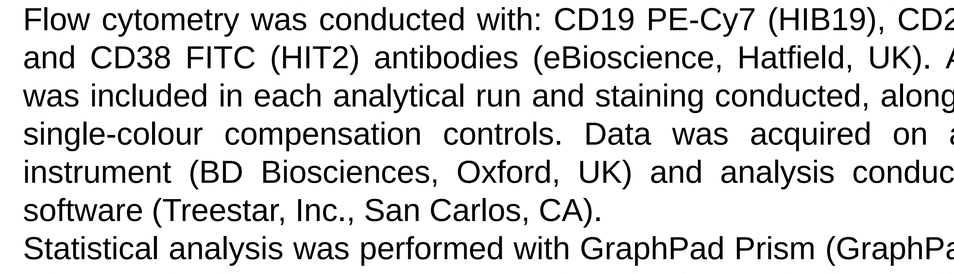
The combination of CD19, CD24 and CD38 antibodies, permits definition of regulatory ($CD19^+CD38^{hi}CD24^{hi}$), memory ($CD19^+CD24^{hi}CD38^-$) and mature ($CD19^+CD38^{int}CD24^{int}$) B cell subsets [Carsetti *et al*, 2004; Sims *et al,* 2005].

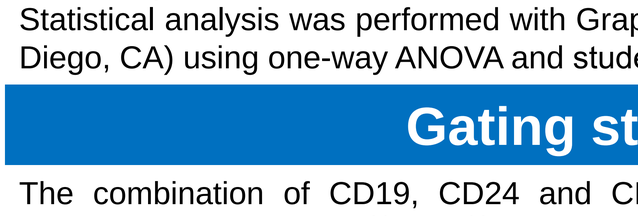
Figure 2. Schematic of gating strategy and sample data from a healthy control





Initial gating for lymphocytes, based on intrinsic physical properties (forward and side scatter); followed by selection of B cells, by expression of CD19 cell surface antigen.

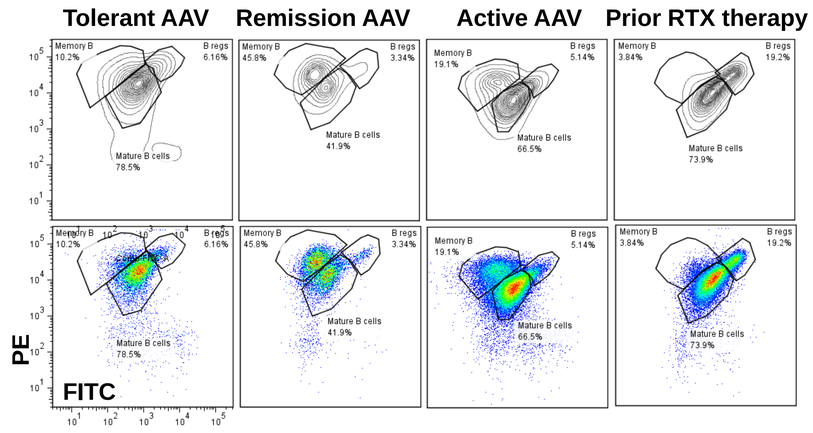




Additional flow cytometry staining showed that the B regulatory cells were CD5 high and B memory cells CD27 positive; confirming phenotype assigned by CD19, CD24 and CD38 labelling (eBioscience, clones UCHT2 and O323).

Results

Figure 3. Representative B cell plots, paired contour and dot plots



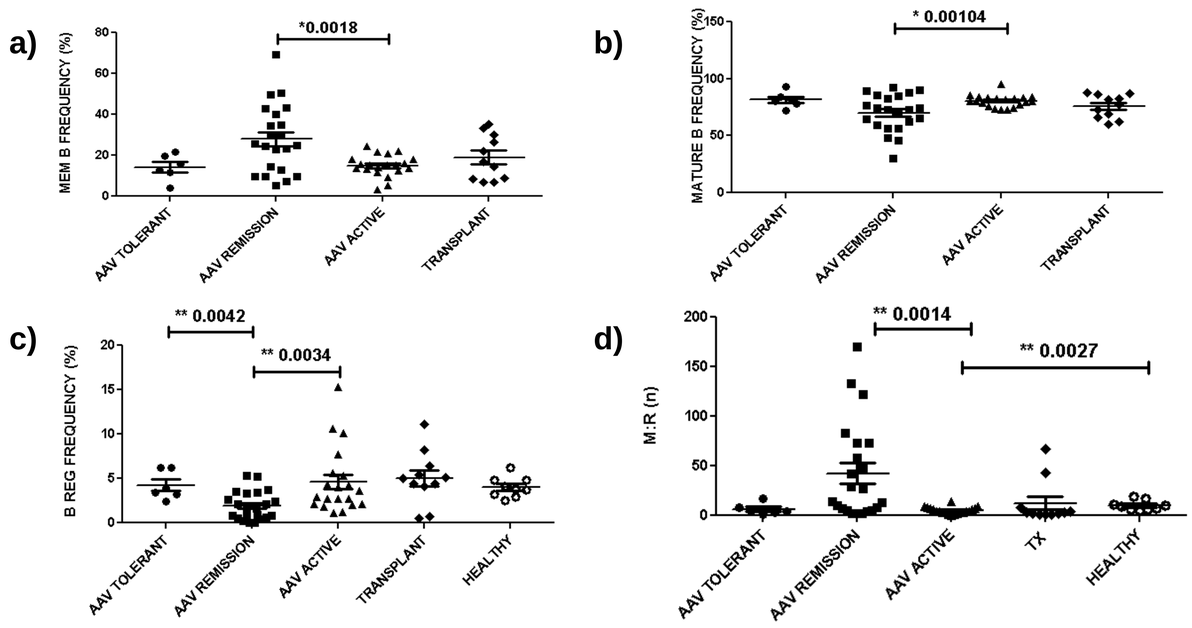
Plots show variation in the frequency of B cell subpopulations in the AAV patient groups.

The image on the far right hand side shows enrichment of B regulatory cells following rituximab treatment, as previously described [Palanichamy *et al*, 2009].

Table 2. Results from B cell immunophenotyping

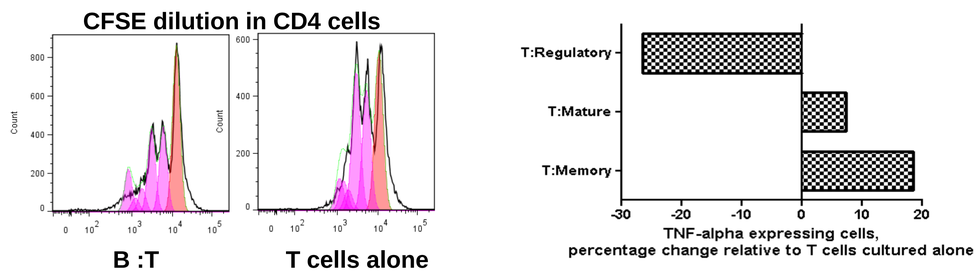
	Regulatory B cells frequency (%)	Memory B cells frequency (%)	Mature B cells frequency (%)	Memory:Regulatory cell ratio
AAV - All patients	3.33 ± 0.42	20.79 ± 1.95	75.89 ± 1.85	22.37 ± 5.42
AAV- Tolerant	4.24 ± 0.66	14.18 ± 2.59	81.58 ± 2.79	6.37 ± 2.43
Active disease	4.61 ± 0.82	14.94 ± 1.18	80.46 ± 1.20	5.06 ± 0.76
Remission	1.92 ± 0.34	27.90 ± 3.54	70.18 ± 3.47	42.47 ± 10.33
Transplant recipients	5.05 ± 0.91	19.06 ± 3.29	75.89 ± 3.09	12.47 ± 6.59
Healthy controls	4.02 ± 0.38	38.69 ± 5.14	57.29 ± 5.17	10.26 ± 1.66
1 way ANOVA	0.0042**	< 0.0001****	***0.001	0.0011**

Figure 4. Summary of flow cytometry data, SEM and statistical significance shown (unpaired, two-tailed Student's t-test)



Graphs (a-c) show frequencies of B cell subsets, expressed as a percentage of total CD19+ events. Significant differences were found in the proportion of memory and mature B cells in active, tolerant and transplant patients, compared to healthy controls (not shown). B cell frequencies also differed according to disease activity in AAV; there were more memory B cells and fewer regulatory and mature B cells in remission. Ratio of memory to regulatory cells was calculated by dividing the number of cells in the memory cell gate by the number in regulatory B cell gate M:R(n). This ratio differed according to disease activity in AAV and was lower in patients with active disease, compared to healthy controls; graph (d).

Figure 5. Suppressive ability of $CD19^+CD38^{hi}CD24^{hi}$ population



To confirm suppressive ability of B regulatory cells, co-cultures were set up at a ratio of 1:4 with untouched CD4+ CD25- T cells (B regs were isolated by FACS sort and T cells were isolated using MS columns and microbeads, Miltenyi Biotech, Surrey, UK). These studies showed reduced proliferation in T:B co-culture, compared to T cells alone ; there was 33% less division of the CD4 cells as assessed by CFSE dilution at 72 hours (CD4 clone RPA-T4, Biolegend, San Diego; CFSE, Invitrogen, Paisley, UK). This was accompanied by subsequent reduction in T cell differentiation at 5 days; 27% fewer TNF-alpha positive CD4 cells detected (eBioscience clone, MAb11).

Conclusions

Remission AAV patients have fewer B regulatory cells than active or tolerant patients. This is not likely to be solely due to immunosuppression as transplant recipients have a greater immunosuppressive load and higher frequency of B regulatory cells.

The B regulatory cells characterized in this study by high expression of CD24 and CD24, are functionally suppressive, inhibiting T cell proliferation and cytokine production. Further investigation of B regulatory function in tolerant and remission patients is currently underway.

B regulatory cells are restored in those AAV patients who regain immunological tolerance. Harnessing B regulatory cells may be a novel therapeutic strategy for relapsing remitting AAV.

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