B Cell Abnormalities in Wegener's Granulomatosis and Microscopic Polyangiitis: Role of CD25+-expressing B Cells

PER ERIKSSON, CHRISTINA SANDELL, KARIN BACKTEMAN, and JAN ERNERUDH

ABSTRACT. ***Objective.*** The use of rituximab in vasculitis has increased interest in B cell biology. A subpopulation of B cells expressing CD25 shows antigen-presenting properties and may have regulatory functions. We assessed subpopulations of B cell maturation (Bm) and markers related to activity and antigen presentation, and related the findings to disease activity.

Methods. Multiparameter flow cytometry was used to assess numbers and proportions of circulating lymphocytes from 34 patients with vasculitis (16 remission, 18 active) and 20 controls.

Results. Active vasculitis samples showed decreased proportions of Bm1 (7.8% vs 11%; p = 0.041), Bm2' (0.2% vs 0.7%; p = 0.002), and Bm3/Bm4 (0.1% vs 0.3%; p = 0.006), compared with controls; Bm2 cells were the most frequently occurring B cells but they were not significantly different in active vasculitis (74% vs 62%; p = 0.083). In patients with remission the proportion of CD25+ B cells was increased compared to controls (48% vs 29%, respectively; p = 0.006) and also compared to active vasculitis (23%; p = 0.006). The proportion of CD86+ B cells was also increased (31%) compared to active vasculitis (8%; p = 0.001), and to controls (6%; p = 0.0003). In multivariate analysis, Bm2' cells and CD25+27– B cells were independently influencing the patient group.

Conclusion. In active vasculitis, a lower proportion of Bm1 cells may indicate activated B cells. Patients in remission had higher proportions of CD25+ (α-chain of interleukin 2 receptor) and CD86+ (costimulatory molecule) B cells. We suggest that these B cells may have a regulatory role, or alternatively may result from previous treatment. (J Rheumatol First Release August 15 2010; doi:10.3899/jrheum.100074)

Key Indexing Terms:
VASCULITIS ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES B CELLS CD25

Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA) are characterized by vasculitis in small and medium-size vessels, as well as by antineutrophil cytoplasm antibodies (ANCA). Rituximab has been successfully used in patients with vasculitis[1,2], further strengthening the interest in B lymphocytes, not only as precursors to antibody-secreting plasma cells, but also as cells involved in antigen presentation and immune regulation[3].

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B cells are generated and differentiated in the bone marrow. The differentiation from stem cells to mature B cells (Bm) has been thoroughly characterized, and use of the surface markers CD38 and IgD has been crucial in classifying developmental stages from naive to memory cells (Bm1 to Bm5)[4,5]. By using additional cell-surface markers, including the memory B cell marker CD27, circulating blood cells could be grouped into Bm1, Bm2, early Bm5, and Bm5 cells, and also small amounts of germinal center founder cells (Bm2' and Bm3∂-Bm4∂ cells)[6,7].

In addition to their role in antibody production, B cells have prominent roles in immune regulation, e.g., as cytokine-secreting and antigen-presenting cells (APC). Subpopulations of B cells display different functions. Interestingly, B cells expressing CD25, i.e., the alpha chain of the interleukin 2 (IL-2) receptor, have been ascribed important potentially regulatory functions[8,9]. The CD25+ B cell population does not secrete immunoglobulins, while its antigen-presenting ability is increased compared with conventional CD25– B cells[8].

Although much more premature than for T cells, there is evidence for the existence of B cell subsets with regulatory properties[3,10], accomplished for example by the production

of the cytokines IL-10 and transforming growth factor ß (TGF-ß) and by the ability of B cells to interact with T cells. Antibodies themselves may also suppress immune responses, for example by interacting with inhibitory Fcγ RIIB[10]. However, the phenotype of regulatory B cells is uncertain. Human CD25+ B cells have been shown to produce IL-10 upon stimulation, suggesting that these cells may participate in regulatory B cell responses[11], although Tretter, *et al* found that T cell suppression by CD25+ B cells is dependent on cell contact and is not influenced by IL-10 or TGF-ß[9]. CD25 is also a marker of regulatory T cells (Treg), but it is important to distinguish CD25+ B cells from CD25+ Treg, since CD25+ B cells do not express the typical and functionally important transcription factor Foxp3, closely associated with Treg.

APC present antigen to T cells through the T cell receptor. Costimulatory molecules CD80 and CD86 are also necessary for T cell activation, and thus CD80 and CD86 expression on B cells may suggest antigen-presenting activity and capacity. CD86 and MHCII are upregulated after activation of B cell receptors and may therefore be considered markers of B cell activation[12].

Studies in mice have designated CD5-expressing B cells as B1 cells, in contrast to conventional B2 cells. In humans as well, B1 cells produce natural antibodies of broad specificity, including autoantibodies that may be associated with autoimmune diseases such as systemic lupus erythematosus (SLE)[12,13]. CD5+ B cells have also been ascribed specific properties such as being the main source of IL-10. However, human data are more controversial[3] and CD5 can be upregulated on B cells following activation[14].

In contrast to T cells[15], information on B cell subpopulations in vasculitis is limited, although Popa, *et al* made interesting observations using CD38 expression as a marker of B cell activation in WG[16].

In our study of patients with WG and MPA, the aims were to assess aberrations in B cell subpopulations with regard to different developmental stages, expression of costimulatory markers on B cells, and in particular, the recently described CD25-expressing B cells, a subpopulation with possible immunoregulatory capacity.

MATERIALS AND METHODS

Patients. Patients with WG or MPA according to the Chapel Hill definitions[17] were included. Blood samples were drawn from 16 patients with vasculitis in remission [median age 75 years (range 27–85), 7 men and 9 women, 12 WG and 4 MPA], 18 patients with active vasculitis [median age 67 years (range 25–83), 12 men and 6 women, 12 WG and 6 MPA], and 20 healthy controls [median age 70 years (range 22–83), 12 men and 8 women]. The healthy controls were blood donors or current and previous personnel at the hospital. They were all free from diseases such as autoimmune diseases and were not on continuous medication. Clinical characteristics of individual patients are given in Tables 1 and 2. The study was approved by the regional ethics committee in Linköping, and was in compliance with the Helsinki Declaration.

In the remission group, cyclophosphamide (CYC) was not given within at least 11 months before blood sampling but had previously been given to 14 of 16 subjects. In the group with active vasculitis, 8 of 18 subjects had never been given CYC, 6 subjects had stopped CYC at least 13 months prior to our study, and 4 additional patients received CYC during the month before our study (Table 2). Two of the relapsing patients (Cases 10A and 18A) had been treated with rituximab 17 and 54 months prior to blood sampling. In the remission group, 3 subjects were treated with methotrexate 12.5–20 mg/week just before blood sampling, and in the group with active vasculitis, 3 were treated with methotrexate 15–25 mg/week, 2 with azathioprine 50–150 mg/day, and 3 with mycophenolate mofetil 2 g/day. In the active disease group, 6 patients (3A, 5A, 6A, 8A, 9A, and 13A) taking a daily dose ≤ 10 mg prednisolone were defined as a low-prednisolone subgroup within the active group (vs the remaining active patients constituting a high-prednisolone group).

WG was localized to upper airways (not generalized) in 2/12 in the remission group and in 3/12 in the active group. In the group with active disease, 11 of 18 presented with vasculitis and 7 had relapse at the time of blood sampling.

The Birmingham Vasculitis Activity Score (BVAS)[18] was used for assessment of disease activity at blood sampling. Relapse was defined as the return of clinical signs or symptoms or laboratory evidence of vasculitis activity sufficient to warrant a sustained increase in immunosuppresive therapy[19].

ANCA were analyzed with capture enzyme-immunoassays (Wielisa, Euro-Diagnostica AB, Malmö, Sweden) for antibodies to myeloperoxidase and proteinase (PR)-3. The blood samples were drawn at the time of our study and analyzed directly.

Flow cytometry for assessment of B cell subpopulations. Blood samples were collected into EDTA tubes and analyzed with 6-color flow cytometry. All monoclonal antibodies (mAb) were purchased from BD Biosciences or BD Pharmingen (San Jose/San Diego, CA, USA).

Cells from whole blood were stained in 3 tubes with the following combinations of mAb for human cell-surface markers: (1) CD19-PE-Cy7 (clone SJ25C1), CD20-APC-Cy7 (clone H7-L27)/APC-H7 (clone H7-L27), anti-human IgD-FITC (clone IA6-2), CD27-PE (clone –L128), CD38-PerCP-Cy5.5 (clone HIT2), CD23-APC (clone EBVCS-5); (2) CD19-PE-Cy7 (clone SJ25C1), CD45-FITC (clone 2D1), CD27-PE (clone L128), CD80-PE-Cy5 (clone L307.4), CD86-APC (clone 2331,FUN-1), anti-HLADR-APC-Cy7 (clone L243); and (3) CD19-PE-Cy7 (clone SJ25C1), CD45-FITC (clone –2D1), CD27-PE (clone L128), CD5-PE-Cy5 (clone UCHT2), CD25-APC (clone 2A3), and anti-HLADR-APC-Cy7 (clone L243). CD25 expression on B cells was also checked by mAb (clone 2A3, same as for APC) conjugated with phycoerythrin (PE) and PerCP-Cy5.5, and also with another clone (clone M-A251) conjugated with APC-H7. To determine the number of leukocytes/μl, a Trucount tube (BD Biosciences) containing an exact number of lyophilized beads was used. In this tube the numbers and proportions of lymphocytes were also assessed, based on CD45 and side-scatter characteristics. The absolute number of B cells from this tube was then applied in all other tubes. B cell subsets were expressed both as numbers of cells and as proportions (percentage) of B cells.

Blood cells were incubated with mAb for 15 min at room temperature in darkness. Then erythrocytes were lyzed with FACS lyzing solution (BD Biosciences) for 15 min at room temperature in darkness. Tubes without beads were washed and resuspended in phosphate buffered saline containing bovine serum albumin, before collection of data.

The stage of B cell maturation was characterized by a combination of cell-surface markers: Bm1 (CD19+20+IgD+38–/+23–), Bm2 (CD19+20+IgD+38–/+23+), Bm2' (CD19+20+IgD+38++), Bm3/Bm4 (CD19+20+IgD–38++27+), Bm5 (CD19+20+IgD–38–/+27+), and early Bm5 (CD19+20+IgD–38+27+). Plasma cells were characterized by CD19–20low-IgD–38++27+.

In order to consistently gate populations with continuous expression from positive to negative, we used a negative population as reference. In the case of CD25 expression on B cells, a standardized gate set on granu-

Table 1. Clinical characteristics of each patient in remission (R) (Birmingham Vasculitis Activity Score 0 by definition).

Patient	Sex/ Age, yrs	Disease Duration, yrs	Ltd WG, WG, MPA	Anti-PR3 or MPO*	CRP, mg/l	Prednisolone Month Before Study, mg/day	Current Immune Therapy**, mg/wk	Last CYC (Months Before Study)/Accum. CYC, g
1R	F 79	5	WG	PR3 67	10	0	0	43/20.4
2R	M 73	6	WG	PR3 30	10	2.5	0	71/8.3
3R	M 58	15	Ltd WG	PR3 0	10	0	0	Never
4R	F 36	3	MPA	MPO 1	10	2.5	0	32/9.6
5R	F 61	2	MPA	MPO 1	10	0	0	26/12.5
6R	M 80	9	WG	PR3 > 200	16	2.5	0	42/24.2
7R	M 84	1	MPA	MPO 85	10	5	0	11/2.5
8R	F 78	2	WG	MPO 2	10	2.5	MTX 12.5	24/5.4
9R	F 61	14	WG	PR3 60	10	0	0	154/73.5
10R	F 85	5	WG	PR3 0	10	2.5	0	33/23.5
11R	F 76	15	WG	PR3 168	10	0	0	187/15.5
12R	M 72	9	Ltd WG	PR3 61	10	0	0	104/5.6
13R	F 65	8	MPA	PR3 80	10	0	0	Never
14R	M 78	20	WG	Unknown 0	10	1.25	0	123/231.9
15R	M 28	9	WG	PR3 194	10	5	MTX 20	36/7.2
16R	F 81	17	WG	PR3 4	10	0	MTX 15	165/27.8

* Positive anti-PR3 or anti-MPO any time plus levels at study. ** During month before study. WG: Wegener's granulomatosis; MPA: microscopic polyangiitis; PR3: proteinase 3; MPO: myeloperoxidase; CYC: cyclophosphamide; MTX: methotrexate; CRP: C-reactive protein.

Table 2. Clinical characteristics of patients with active (A) vasculitis.

Patient	Sex/ Age, yrs	Disease Duration, yrs	Ltd WG, WG, MPA	Anti-PR3 or MPO*	Relapse or 1st Event	BVAS	CRP, mg/l	Steroids Month Before Study	Immune Therapy Month Before Study	Last CYC (Months Before Study)/Accum. CYC, g
1A	M 73	0	MPA	MPO 49	1st	15	53	MP 500+ 500 mg	0	Never
2A	F 41	0	Ltd WG	PR3 43	1st	7	15	Pred 60 mg/day 3 days, not last days	MTX 15 mg once	Never
3A	F 81	2	MPA	MPO 4	Relapse	3	197	Pred 2.5 mg/day	MTX 15 mg/wk 14 days	13/6
4A	M 68	0	WG	PR3 164	1st	21	69	Pred 30-60 mg/day 45 day	0	Never
5A	M 53	0	Ltd WG	PR3 126	1st	6	10	0	0	Never
6A	M 71	0	MPA	MPO 79	1st	15	17	Pred 10 mg/day 10 day	0	Never
7A	M 63	1	MPA	PR3 43	Relapse	10	94	MP 250+ 250+ 24 mg, 3 day	MYC 2 g/day	1/13
8A	M 55	9	WG	PR3 152	Relapse	6	10	Pred 10 mg/day	MYC 2 g/day	87/12
9A	F 67	13	Ltd WG	PR3 > 200	Relapse	6	10	Pred 5 mg/day	MYC 2 g/day	65/106
10A	M 62	7	WG	PR3 59	Relapse	6	150	Pred 80 + 60 mg/day 2 day	0	18/103
11A	M 83	0	MPA	MPO > 200	1st	15	15	MP 500 + 250 + 250 + 100 mg 4 day	CYC 500 mg IV day before	0/0.5
12A	F 25	0	WG	PR3 77	1st	5	157	Pred 40 mg/day 6 day	MTX 25 mg once	Never
13A	M 80	0	WG	PR3 > 200	1st	36	68	0	0	Never
14A	M 77	0	MPA	MPO > 200	1st	15	14	MP 500 mg day before	0	Never
15A	F 66	8	WG	PR3 > 200	Relapse	14	22	Pred 20 mg/day 45 day	AZA 150 mg/day	61/19
16A	M 77	0	WG	PR3 2	1st	23	47	MP 125 + 125 mg 2 day	CYC IV 7 day before	0/2
17A	M 58	0	WG	PR3 142	1st	32	10	Pred 60 mg/day 7 day	CYC 50 mg/day 7 day	0/0.4
18A	F 54	6	WG	PR3 > 200	Relapse	19	37	Pred 30 mg/day, 11 days	AZA 50 mg/day	56/77

* Positive anti-PR3 or anti-MPO any time plus levels at study. WG: Wegener's granulomatosis; MPA: microscopic polyangiitis; PR3: proteinase 3; MPO: myeloperoxidase; BVAS: Birmingham Vasculitis Score; CYC: cyclophosphamide; AZA: azathioprine; MP: methylprednisolone; MTX: methotrexate; Pred: prednisolone; MYC: mycophenolate mofetil; CRP: C-reactive protein; IV: intravenous.

locytes was used as a negative population (Figure 1), and the same procedure was used for CD80- and CD86-expressing B cells. Isotype control was found to show a slightly lower background expression than the granulocyte population. Fluorescence minus one (FMO) testing (i.e., testing the effect of excluding 1 antibody while the others remained) was used to ascertain that no false-positive staining occurred.

During the first part of our study (May 2006-September 2006), data were collected with FACS Canto A (BD Biosciences), and thereafter (October 2006-November 2007) data were collected with FACS Canto II. Parallel runs of samples ensured that the change of flow cytometer did not affect results. Analyses were performed with FACSDiva software (BD Biosciences). Compensation settings and status of tandem-dyes were checked regularly and adjusted accordingly. The laboratory participates in external quality programs from UK-Nequas, UK, and Equalis, Sweden.

Statistics. Median and 25%–75% percentiles (interquartiles) are given. To compare the 3 groups (remission, active vasculitis, and healthy controls), the nonparametric Kruskal-Wallis test was used, followed by the Mann-Whitney U test to compare 2 of the groups.

Univariate multinominal logistic regression was used to look for any influence of different B cell subpopulations on the 3 groups of subjects. Independent variables giving a p value < 0.05 were then entered into a multivariate stepwise backward multinominal logistic regression analysis.

Univariate linear regression analysis was used to look for any influence of clinical measures [age, sex, WG vs MPA, systemic vs localized WG, BVAS, anti-PR-3 vs antimyeloperoxidase, C-reactive protein (CRP) at blood sampling, daily dose of prednisolone < 10 mg vs > 10 mg, methotrexate or azathioprine or mycophenolate mofetil previous month vs none of these drugs, CYC last month, rituximab ever, CYC ever] on B cell subpopulations shown to significantly influence patient groups in the multivariate multinominal logistic regression analysis. Independent variables giving a p value < 0.1 were entered into a multivariate stepwise backward linear regression analysis.

RESULTS

Blood leukocytes and lymphocytes. The total counts of blood leukocytes were significantly higher in the group with active vasculitis [median 12926 × 10^6/l (9803–14549 × 10^6/l)] compared with the group in remission [median 6809 × 10^6/l (5480–8448 × 10^6/l); p = 0.0002] and compared with controls [median 5406 × 10^6/l (3674–7908 × 10^6/l); p < 0.0001]. On the other hand, patients with active vasculitis had significantly lower numbers of lymphocytes [median 1087 × 10^6/l, 10% (4.0%–16%) of leukocytes] compared with patients in remission [median 1610 × 10^6/l, 26%

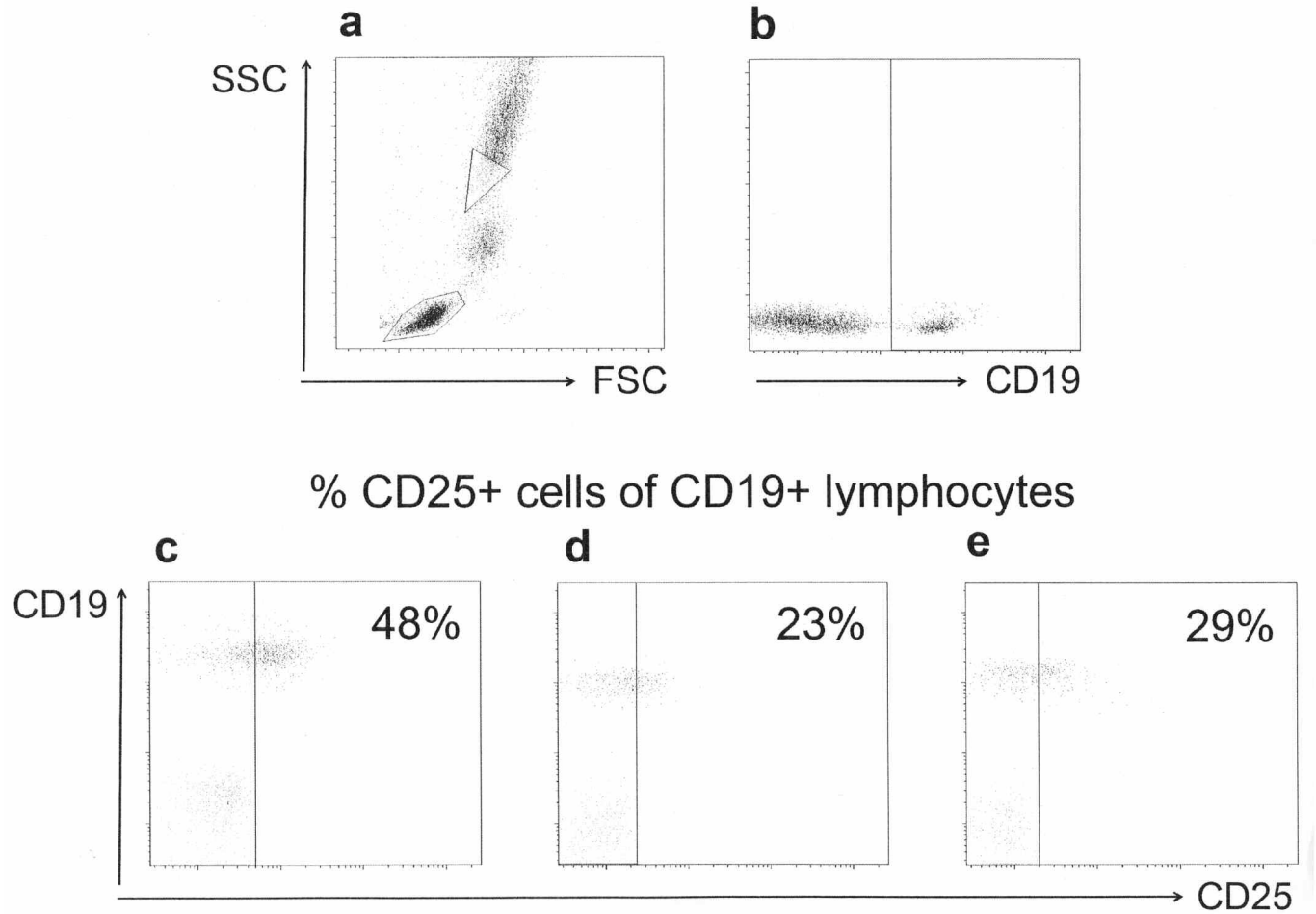

Figure 1. Principle of the gating strategy for CD25+ B cells: Lymphocytes were identified according to forward (FSC) and side-scatter characteristics (SSC; panel a, lower population). B cells were identified by expression of CD19 (b), and the B cell population was transferred to a dot-plot for further analysis of CD25 expression (c, d, e). To establish a reference population for CD25 expression, a standardized gate was set on granulocytes (a, upper population) and this population was used to set the negative gate for CD25 expression (c, d, e, lower populations). Typical plots are shown: (a) patients in remission, median 48%; (b) patients with active disease, median 23%; (c) controls, median 29%.

(16%–35%); p = 0.0007] and controls [median 1881 × 10^6/l, 36% (32%–43%); p < 0.0001]. No significant differences were found for total counts or proportions of B cells [12% of lymphocytes (7.9%–20%) in active vasculitis, 9% (5.9%–12%) in remission, 10% (7.2%–14%) in controls].

B cell maturation. As shown in Table 3, Bm2 cells were the most abundant subset of B cells in all 3 groups. In active vasculitis, a tendency to a lower proportion of Bm1 cells (median 7.8% vs 11% in controls) was found, while Bm2 cells were the most frequently occurring B cells, but not significantly different, in active vasculitis (median 74% vs 62% in controls). Significantly lower proportions of Bm2' (median 0.2% vs 0.7% in controls) and Bm3/Bm4 cells (median 0.1% vs 0.3% in controls) were also found in active vasculitis.

Patients in remission did not differ from controls or patients with active vasculitis regarding proportion of different Bm1-Bm5 subsets of B cells. The only exception was the proportion of Bm2' cells, which was higher in patients with remission than in patients with active vasculitis. Proportions of early Bm5, Bm5, and plasma cells did not differ between the groups.

CD25+ B cells, CD27+ B cells, and CD5+ B cells. As shown in Table 4, Figure 1 c-e, and Figure 2, the proportion of B cells expressing CD25 was significantly higher in patients with remission compared to patients with active vasculitis and controls. Further, the same pattern was found for the subpopulation of CD25-expressing B cells not expressing CD27 (i.e., CD19+25+27–; Figure 3 and Table 4). To check the consistency of CD25 expression, we also used mAb conjugated with other fluorochromes and found that APC, PerCP-Cy5.5, and PE gave the same results, while APC-H7, also involving another mAb clone, resulted in a CD25+ population that was difficult to distinguish from the negative population, leading to varying interobserver and intraobserver results.

The proportion of CD27+ memory B cells did not differ between the groups (Table 4). However, the proportion of CD27+ memory B cells also expressing CD5 (CD27+5+) was lower in remission, as well as in active vasculitis, compared with controls (Table 4).

The proportion of CD5+ B cells did not differ between the groups, while the proportion of B cells expressing both CD25 and CD5 was higher in remission compared to controls and active disease (Table 4).

Costimulatory molecules and HLA-DR expression on B cells. The proportion of B cells expressing CD86 was increased in remission (median 31%, interquartile range 23%–49%) compared to active disease (7.8%, 4.1%–23%; p = 0.001) and controls (6.2%, 2.7%–13%; p = 0.0003; Kruskal-Wallis p = 0.0003), as shown in Figure 4.

The proportion of B cells expressing CD80 did not differ between the 3 groups (Kruskal-Wallis p = 0.787). Median proportions of CD80+ B cells were 24% (11%–34%) in

Table 3. Proportions of Bm1, Bm2, Bm2', Bm3/Bm4, early Bm5, and Bm5 cells expressed as percentage of B cells. Plasma cells are expressed as percentage of lymphocytes. Medians (interquartile range) are given. Significant differences shown in bold type.

Type	Remission, n = 16	Active, n = 18	Controls, n = 20	KW p	Remission vs Active, M W p	Remission vs Controls, M W p	Active vs Controls, M W p
Bm1	9.1 (4.3–19)	7.8 (3.5–11)	11 (6.9–21)	0.097	NS	NS	**0.041**
Bm2	63 (48–81)	74 (63–78)	62 (51–76)	0.232	NS	NS	0.083
Bm2'	1.1 (0.4–1.9)	**0.2** (0.0–0.5)	0.7 (0.5–1.4)	**0.001**	**0.001**	NS	**0.002**
Bm3/4	0.2 (0.0–0.5)	**0.1** (0.0–0.2)	0.3 (0.1–0.6)	**0.026**	NS	NS	**0.006**
Early Bm5	7.3 (2.3–12)	5.7 (3.3–10)	5.6 (3.5–8.5)	0.990	NS	NS	NS
Bm5	13 (6.5–21)	11 (6.7–16)	13 (8.8–23)	0.462	NS	NS	NS
Plasma cells	0.2 (0.1–0.4)	0.2 (0.1–0.3)	0.1 (0.1–0.2)	0.120	NS	NS	NS

K W: Kruskal-Wallis test for comparison of 3 groups. M W: Mann-Whitney U test for comparison between 2 groups. NS: not significant.

Table 4. Proportions of B cells (%) with or without expression of CD25, CD27, and CD5. Medians (interquartiles) are given. Significant differences shown in bold type.

CD19+ B Cells Expressing	Remission	Active	Controls	K W p	Remission vs Active, M W p	Remission vs Controls, M W p	Control vs Active, M W p
25+	**48** (34–61)	23 (17–29)	29 (20–38)	**0.001**	**0.006**	**0.006**	0.228
25+27–	**38** (19–46)	18 (10–20)	13 (10–22)	**0.001**	**0.001**	**0.002**	0.867
27+	19 (11–26)	17 (11–24)	23 (14–30)	0.385	—	—	—
5+	20 (16–30)	18 (13–27)	24 (18–34)	0.239	—	—	—
25+5+	**15** (11–24)	4.5 (2.2–9.8)	7.4 (5.4–10)	**0.007**	**0.004**	**0.013**	0.357
27+5+	0.6 (0.0–1.1)	0.6 (0.3–1.7)	**1.6** (0.8–3.0)	**0.007**	0.334	**0.003**	**0.028**

K W: Kruskal-Wallis test. M W: Mann-Whitney U test.

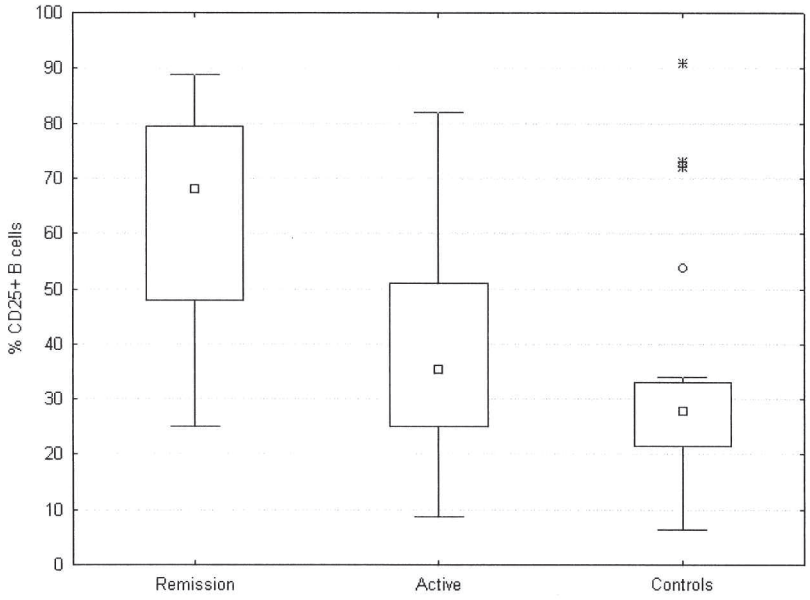

Figure 2. The proportion of CD25+ B cells was significantly higher in remission compared with controls (p = 0.006) and with patients having active vasculitis (p = 0.006).

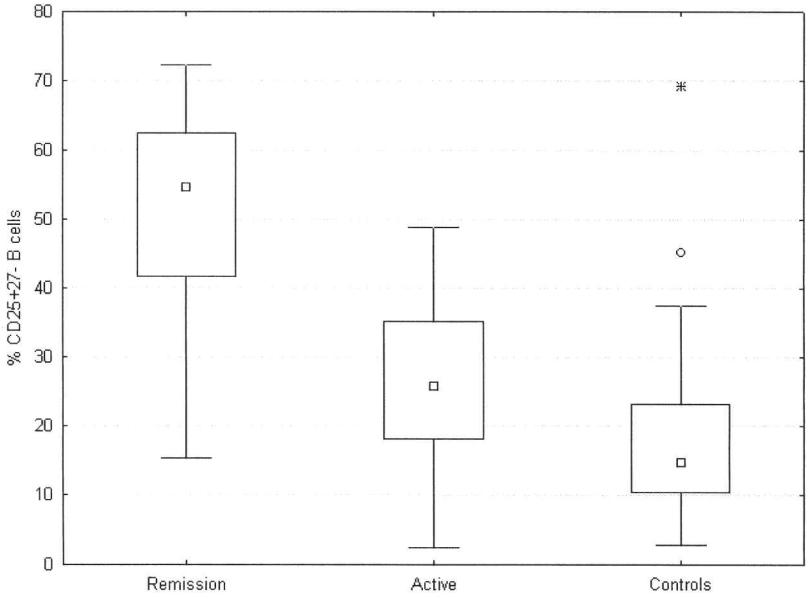

Figure 3. Further subdividing the CD25+ B cells into a CD25+27– B cell population also showed a higher proportion of these cells in remission compared with controls (p = 0.002) and patients with active vasculitis (p = 0.001).

remission, 14% (12%–28%) in active disease, and 18% (12%–26%) in controls.

The median proportion of B cells expressing HLA-DR was 100% (100%–100%) in all 3 groups (Kruskal-Wallis p = 0.617).

Relationship among different B cell subpopulations and the remission, active vasculitis, and control groups. Statistical analyses were performed to establish the relative effect of different B cell populations on the dependent variable "patient group." In univariate multinominal logistic regression analysis, the following B cell subpopulations were related to the group ($p < 0.05$): CD25+ (p = 0.0075),

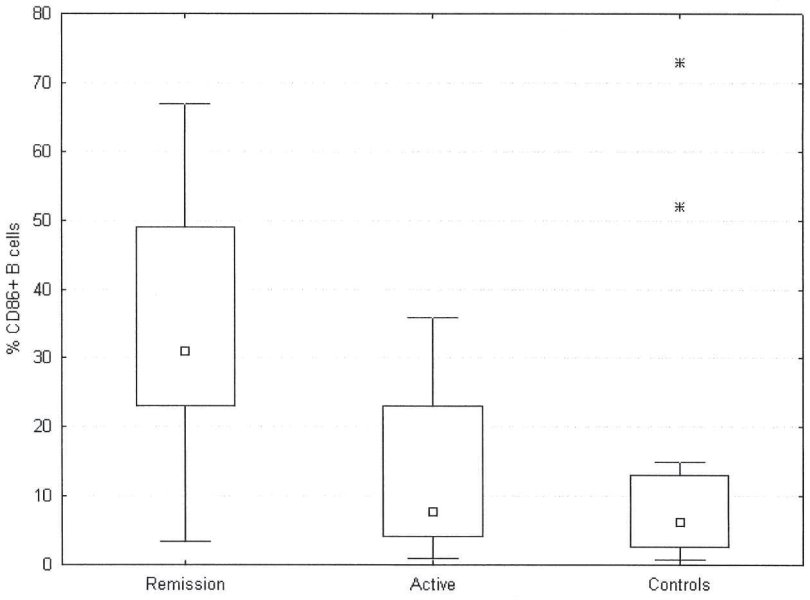

Figure 4. Proportion of B cells expressing CD86 in remission, active vasculitis, and controls.

CD25+27– (p = 0.0031), CD25+5+ (p = 0.0152), CD25+27–5+ (p = 0.0466), CD25– (p = 0.0106), CD25–27– (p = 0.0094), CD27+5+ (p = 0.0320), CD86+ (p = 0.0046), CD86+27– (p = 0.005), and Bm2' cells (p = 0.0078). Using multivariate analysis, only CD25+27– B cells (p = 0.0045) and Bm2' cells (p = 0.0151) had significant independent effects on the groups.

Clinical correlates to CD25+ B cells and to subsets of B cells with independent effects on the remission, active vasculitis, and control groups. In univariate linear regression analysis, proportions of Bm2' cells were inversely related with anti-PR3 (p = 0.0032), disease activity assessed by BVAS (p = 0.0351), and male gender (p = 0.0428). In multivariate analysis, the results of anti-PR3 (p = 0.0033) and BVAS (p = 0.0320) were still significant.

In univariate analysis, proportions of CD25+ B cells were inversely related with BVAS (p = 0.0008), prednisolone (daily dose ≥ 10 mg; p = 0.0027), and CYC therapy in the last month (p = 0.0094). Multivariate analysis could detect only BVAS (p = 0.046) as an explaining factor.

A similar pattern was found for CD25+27– B cells. In univariate analysis, CD25+27– B cells were inversely related to BVAS (p = 0.0016), prednisolone dose ≥ 10 mg (p = 0.0028), CYC in the last month (p = 0.0128), and male gender (p = 0.0310). In multivariate analysis, male gender (p = 0.0088), CRP (p = 0.0214 in multivariate and p = 0.0880 in univariate analysis), and CYC in the last month (p = 0.0241) were still significant.

DISCUSSION

Our study shows several highly significant aberrations of B cell subpopulations, suggesting their involvement in vasculitis. The most striking finding relates to CD25-expressing B cells occurring at significantly higher frequency in patients in remission (median 48% of B cells) compared to patients with active vasculitis (23%) and healthy controls (29%). In multivariate analysis, CD25+27– B cells (B cells expressing CD25 but not the memory marker CD27) and Bm2' cells were the only B cell populations independently influencing the dependent variable "group" (remission, active vasculitis, or healthy subjects). In further multivariate analysis of clinical factors related to B cell populations, CD25+ B cells were inversely related to disease activity as assessed by BVAS, and the CD25+27– B cells were inversely related to CRP and CYC in the last month. Thus, high levels of CD25+27– B cells seemed to be confined to patients in remission and may therefore be a marker of successful mastering of disease, and perhaps denote a population with immunoregulatory properties. However, it cannot be excluded that levels of these cells were influenced by CYC treatment. More data are needed to confirm any role of the CD25+ or CD25+CD27– B cell populations as possible markers of disease activity in vasculitis. Disease activity of vasculitis may be preceded by changes in biological markers (e.g., ANCA levels) over a period of several months. Accordingly, it would be interesting to evaluate different B cell populations longitudinally in patients with vasculitis.

CD25 constitutes the alpha-chain of the IL-2 receptor, and the default explanation of its role in B cells would be that CD25+ B cells are activated (able to respond to IL-2 secreted from activated T cells). However, CD25+ B cells have recently been characterized as a unique phenotypical

and functional subpopulation displaying antigen-presenting properties rather than being involved in antibody production[8,9]. Blocking of CD25 on B cells caused an almost total abrogation of antigen presentation as assessed by allogeneic mixed lymphocyte reactions[8]. Further, Toll-like receptor activation rapidly converts CD25– B cells to CD25+ B cells[8]. Consequently, CD25 expression on mature B cells may be considered either as a marker of B cell activation[9,20] or as a marker of antigen-presenting capacity. Although this B cell population must be characterized further, its presence in our patients with remission is in line with possible immunoregulatory functions.

CD25+ B cells have been investigated in SLE and rheumatoid arthritis (RA) and were found at similar levels as in healthy controls[21]. CD25 expression was not related to disease activity in RA, while CD25+ B cells were increased in patients with SLE nephritis. No relationship was detected between treatment and CD25 expression[21]. CD25+ B cells have also been found at increased levels in ophthalmopathy associated with thyroid disease[22]. It is possible that different disease mechanisms implicate different patterns of CD25+ B cells, and also it is notable that our material was strictly divided into active disease and remission of disease.

We also found a higher proportion of B cells expressing the costimulatory molecule CD86 in patients in remission compared to those with active vasculitis and controls, while no differences were found concerning CD80 expression on B cells. There is no obvious explanation for our discrepant results regarding CD86+ B cells being associated with remission, and CD80+ B cells occurring at similar frequency across groups. Both CD80 and CD86 bind CD28 and cytotoxic T lymphocyte antigen (CTLA)-4 on T cells and have overlapping but distinct functions, which is of interest concerning the new anti-RA treatment CTLA-4-Ig. The first-generation product, abatacept, shows lower avidity to CD86 compared with CD80[23]. In addition, interesting data indicate that CD80/CD86 on B cells can signal bidirectionally[24]. For example, CD86 stimulates CD28 on T cells, and in parallel, transduces positive signals into B cells affecting Ig production[25]. Thus, CD86 expression on B cells may indicate a putative immunoregulatory role that requires further investigation. Moosig, *et al* studied CD86 expression on B cells in vasculitis, but found no differences between remission and active vasculitis. However, in that study T cells were first depleted and the B cells were analyzed after stimulation (24 and 48 hours later)[26], which may have affected the results. Increased expression of CD86 on B cells has been found in SLE[27], as well as on CD25+27– B cells in RA[11]. However, these studies did not distinguish patients in remission versus active disease. We did not analyze CD80/CD86 and CD25 expression on B cells from the same blood tubes. Thus, we have no information on any possible coexpression of CD25 and CD86 on B cells.

In active vasculitis we found a tendency to a lower proportion of Bm1 cells (median 7.8% vs 11.0% in controls), a finding that supports B cell activation. Lower proportions of Bm2' (median 0.2% vs 0.7% in controls) and Bm3/Bm4 germinal center founder cells (median 0.1% vs 0.3% in controls) were also found. Multivariate analysis revealed an independent relationship between Bm2' cells and disease group. Interestingly, Tretter, *et al* reported that tonsillar IgD+CD38++ B cells (Bm2' cells) reduced T cell proliferation more profoundly than other subpopulations of B cells, suggesting a possible regulatory effect of these B cells (on T cells)[9], a finding in line with the diminished Bm2' population we observed in active vasculitis. The observed changes of circulating Bm cell populations of different maturation further indicate involvement of B cells in the pathogenesis of vasculitis.

Popa, *et al* found a higher proportion of CD38++ B cells in active WG (0.82% vs 0.31% in controls and 0.40% in the remission group)[16]. However, CD38 was not related to expression of surface IgD and therefore it seems difficult to interpret CD38 alone as a marker of either B cell maturation or B cell activity. IgD+CD38++ expressing cells may represent germinal center founder cells (Bm2' cells) and also young naive B cells exiting the bone marrow[28]. These cells are also characterized by expression of CD10 and CD24[29]. Bm3 cells, Bm4 cells, and plasma cells all express CD38 extensively, but not IgD[30]. Summarizing the percentages of Bm2', Bm3, and Bm4 cells, our results disagree with those of Popa, but the data are not completely comparable. Although B cell populations in different stages of maturation show a high heterogeneity of surface markers, the classification so far has mainly relied on the expression of CD38, mIgD, and CD27[30], as did our data. Thus, this is the first report of Bm1-Bm5 cells in patients with primary vasculitis.

Several observations on B cell subsets in different stages of maturation have been reported in other immune-related diseases, some associated with secondary vasculitis. In SLE, naive B cell lymphopenia, increase of circulating germinal center founder Bm2' cells, and expansion of circulating CD27++ plasmablasts and transitional cells have been found[31,32,33,34]. A subset of memory B cells, expressing CD95 but neither CD27 nor IgD, has been shown to correlate with disease activity in SLE[35]. Patients with primary Sjögren's syndrome exhibit an increase in Bm2 and Bm2' cells[6] and a decrease in Bm5 memory cells[36]. Thus, although both SLE and primary Sjögren's syndrome are associated with Bm cell abnormalities, their profile seems to differ from that in ANCA-associated vasculitis.

The proportion of CD5-expressing B cells did not differ between the groups (around 20%). After activation of Bm2 cells, CD5 expression is induced, but is later lost after V(D)J-rearrangement of the B cells[12], possibly explaining why quite a few CD27+ memory cells were CD5-positive and why CD5 may suggest B cell activation as an alternative

model to B1 cells constitutively expressing CD5. Different markers of B cell activation have been proposed, such as CD86, HLA-DR, CD38[35], CD5[14], and CD25[9], but the distinction between cell activation, maturation, costimulation, and other specific properties may be difficult. It remains to be settled whether our findings are of primary importance, or perhaps more likely, are secondary to the disease process.

One concern when evaluating distribution of cell subpopulations is that the total numbers of cells may differ between patient and control groups, thus influencing the actual numbers of cells in a subpopulation, e.g., a low number of the "mother" population may lead to a low actual number of a subpopulation despite a high proportion of the very same subpopulation. Importantly, in our study there were no differences across groups regarding the number of B cells. We therefore chose to display B cell subpopulations as proportions of the "mother" population, i.e., B cells. Another important methodological aspect is that the choice of fluorochrome and mAb clone may influence the appearance of some subpopulations. Because of our findings related to proportions of CD25+ B cells, we tested different fluorochromes and found that PE, PerCP-Cy5.5, and APC gave similar results, while APC-H7 and a different mAb clone resulted in a CD25+ population that was difficult to distinguish from the negative population, hence leading to inconsistent results. Finally, we want to reinforce the importance of defining a negative population as a reference for proper gating. The expression of CD25 on a variable proportion of and with variable density on T cells and natural killer cells, perhaps with differences across groups, made these populations less suitable as reference populations for the setting of a positive gate. Instead, we found that using a standardized proportion of the granulocytes to define a negative population led to consistent and reliable data.

Significant abnormalities in B cell populations were found, indicating the involvement of B cells in the process of ANCA-associated vasculitis. A lower proportion of Bm1 cells may indicate activated B cells in active vasculitis. Patients in remission had higher proportions of B cells with a presumed regulatory function: CD25+ (α-chain of IL-2 receptor) B cells, CD86+ (costimulatory molecule) B cells, and Bm2' cells. We suggest that these B cells may have a regulatory role, or alternatively may result from previous treatment.

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