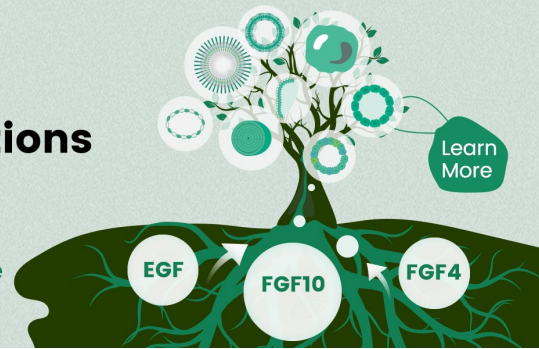


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## Bm1–Bm5 Classification of Peripheral Blood B Cells Reveals Circulating Germinal Center Founder Cells in Healthy Individuals and Disturbance in the B Cell Subpopulations in Patients with Primary Sjögren's Syndrome<sup>1</sup> ✓

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# Bm1–Bm5 Classification of Peripheral Blood B Cells Reveals Circulating Germinal Center Founder Cells in Healthy Individuals and Disturbance in the B Cell Subpopulations in Patients with Primary Sjögren's Syndrome<sup>1</sup>

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Analyses of B cells in the bone marrow and secondary lymphoid tissues have revealed a broad range of cell surface markers defining B cell subpopulations, but only a few of these have been used to analyze B cell subpopulations in peripheral blood (PB). We report here the delineation of circulating PB B cell subpopulations by staining for CD19, CD38, and IgD in combination with CD10, CD44, CD77, CD95, CD23, IgM, and the B cell memory marker CD27. The utility of this approach is shown by the demonstration of disturbances of circulating B cell subpopulations in patients with autoimmune disease. Five mature B cell (Bm) subpopulations were identified in normal PB that were comparable with the tonsillar Bm1, Bm2, early Bm5, Bm5 subpopulations and, surprisingly, to the germinal center (GC) founder cell subpopulation (Bm2' and Bm3δ–4δ), suggesting that some GC founder cells are circulating. No PB B cells resembled the Bm3 and Bm4 GC cells. Remarkably, some cells with the CD38<sup>−</sup>IgD<sup>+</sup> phenotype, previously known as naive Bm1 cells, expressed CD27. The CD38<sup>−</sup>IgD<sup>+</sup> subpopulation therefore includes both naive Bm1 cells and IgD<sup>+</sup> memory B cells. This new classification of B cell developmental stages reveals disturbances in the proportions of B cell subpopulations in primary Sjögren's syndrome (pSS) patients compared with healthy donors and rheumatoid arthritis patients. Patients with pSS contained a significantly higher percentage of B cells in two activated stages, which might reflect a disturbance in B cell trafficking and/or alteration in B cell differentiation. These findings could be of diagnostic significance for pSS. *The Journal of Immunology*, 2001, 167: 3610–3618.

In humans, B cells are generated throughout life in the bone marrow (1), and the mechanisms and molecules involved in the maturation of stem cells to mature B cells (Bm)<sup>3</sup> have been well characterized (2–6). Mature B cells leave the bone marrow and migrate via the peripheral blood (PB) to secondary lymphoid tissues such as spleen, lymph nodes, and Peyer's patches where further development takes place (7–9). In secondary lymphoid tissues, Ag-specific memory B cells or plasma cells are formed (10), through well-defined developmental stages (11–14). Although B cells are continuously recirculating among PB, bone marrow, and secondary lymphoid organs (15), the relationship between B cell subpopulations in the different compartments has been less well studied.

Analyses of B cells in the bone marrow and secondary lymphoid tissues have revealed a broad range of cell surface markers defin-

ing B cell subpopulations, but only a few of these (mainly IgM and IgD) have been used to analyze B cell subpopulations in PB. Mutational analyses of PB B cell subpopulations defined by IgM and IgD have led to the conclusion that PB B cells consist of unmutated naive B cells (~60%) and somatically mutated memory B cells (~40%; Ref. 16). Recently, CD27 was found to be a useful marker for PB B cells that have gone through somatic hypermutation and was proposed as a new marker for memory B cells (17–21). This led to division of memory B cells into four subpopulations, all expressing CD27 but differing in their expression of IgM and IgD (19).

In secondary lymphoid tissue, CD38 and IgD have been useful in classifying important developmental stages from naive to memory B cells (Bm1–Bm5) (11, 12). Cell surface markers such as CD10, CD44, CD77, CD23, IgM, and CD95 have been used in combination with CD38 and IgD for defining seven tonsillar B cell subpopulations (13, 14, 22). Analyses of mutational frequency, functional properties, and Ig class switching have shown that the Bm1–Bm5 classification is an apposite method of defining peripheral B cell developmental stages (11, 13, 14, 22). Immunohistochemical staining of tonsillar sections have confirmed that the Bm1–Bm5 B cell subpopulations are localized in different functional areas of the tonsil (14). Thus, using this Bm1–Bm5 classification to recognize B cell developmental stages in PB would be of a great advantage. Furthermore, the use of CD27 could possibly solve a limitation of the Bm1–Bm5 classification in not having a positive marker for memory B cells.

The aim of this study was to analyze both PB and tonsillar B cells using the Bm1–Bm5 classification system in combination with CD27 to compare the proportion of B cell subpopulations in

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<sup>3</sup> Abbreviations used in this paper: Bm, mature B cell; PB, peripheral blood; pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; GC, germinal center; MNC, mononuclear cells; AET, aminoethylisothiuronium bromide; RF, rheumatoid factor; NSAID, nonsteroidal anti-inflammatory drugs.

Table I. Clinical, laboratory, and medication data for the pSS patients<sup>a</sup>

Patient	RF	ANA	ENA	SSA	SSB	SR	CRP	WBC	Drugs
S1	+	2048	+	+	–	28	8	4.1	None
S2	+	2048	+	–	+	56	ND	7.4	None
S3	+	2048	+	+	+	ND	<5	4.3	NSAID
S4	+	2048	+	+	+	57	<5	4.2	None
S5	+	2048	+	–	+	50	<5	3.2	Other <sup>b</sup>
S6	+	2048	+	–	+	96	<5	4.4	Other <sup>c</sup>
S7	+	>2048	+	–	+	20	<5	6.0	None
S8	+	2048	+	–	+	32	<5	5.9	NSAID <sup>b,d</sup>
S9	+	–	+	+	–	7	<5	3.8	None
S10	+	1024	+	–	+	7	<5	3.4	None
S11	+	512	–	–	–	22	<5	11.6	None

<sup>a</sup> Antinuclear Ab (ANA) number refers to titer of ANA; ENA, extractable nuclear Ag; SSA, Sjögren's syndrome A; SSB, Sjögren's syndrome B; SR, sedimentation ratio (millimeters/hour), CRP, C-reactive protein (milligrams/liter); WBC, white blood cells ( $\times 10^9$ /liter).

<sup>b</sup> Hydroxychloroquin.

<sup>c</sup> Trionetta (contraceptive pill).

<sup>d</sup> Thyroxin.

these two compartments. The generation of Ag-specific memory B cells and plasma cells takes place in several B cell differentiation stages (13). We were thus particularly interested in comparing the normal PB B cell subpopulations with those seen in autoimmune diseases such as rheumatoid arthritis (RA) and primary Sjögren's syndrome (pSS). Possible alterations in the PB B cell subpopulations in these diseases could be of use as diagnostic aids and provide information on the disease pathology.

## Materials and Methods

### Patients and control samples

PB samples were collected from 12 healthy donors (10 female, 2 male; mean age 53 years; range 27–66 years). Tonsils were obtained from five patients with recurring tonsillitis after routine surgical removal at the Lovisenberg Hospital, Oslo, Norway.

PB from 11 patients (9 female, 2 male; mean age 49 years; range 20–73 years) with pSS were collected during routine examination at The National Hospital (Oslo, Norway). All these patients fulfilled the European criteria for pSS (23, 24), and clinical, laboratory, and medication data are included in Table I. Finally, PB were obtained from nine patients with RA (eight female, one male; mean age 45 years; range 25–73 years), fulfilling the revised American College of Rheumatology criteria for RA (25). The laboratory, clinical, and medication data for each RA patient are shown in Table II. Most of the PB samples were taken in connection to routine examination at the Center for Rheumatic Disease, at the National Hospital, or in connection to orthopedic surgery (patients R2 and R3) at the Dia-konhjemmet Hospital (Oslo, Norway).

### Cell preparation

Heparin or citrate peripheral blood samples were collected from the donors and centrifuged at  $560 \times g$  for 25 min. The buffy coat layer was diluted

in a 10% solution of citrate-phosphate-dextrose (Sigma, St. Louis, MO) in PBS (Life Technologies, Paisley, U.K.) and centrifuged for 10 min at  $450 \times g$ . The cells were diluted in PBS and layered on Isopaque-Ficoll separation medium (Lymphoprep; Nycomed, Oslo, Norway) and centrifuged for 25 min at  $450 \times g$ . T cells were depleted from purified mononuclear cells (MNC) by rosetting with aminoethylisothiuronium bromide (AET)-treated SRBC and a second round of Isopaque-Ficoll separation (26).

The tonsils were minced, diluted in PBS, and filtered through a 70- $\mu$ m pore size nylon filter (BD Biosciences, San Jose, CA) to deplete larger cells. The filtered cells were washed twice in PB and diluted in PBS. The cell suspension was layered on Isopaque-Ficoll separation medium (Lymphoprep) and centrifuged for 25 min at  $450 \times g$ . T cells were depleted from the accumulated MNC by rosetting with AET-treated SRBC and a second round of Isopaque-Ficoll separation.

### Monoclonal Abs

Cells were stained with mAb specific for the following human cell surface markers: CD19 (B cell specific: allophycocyanin, IgG1, J4.119), CD38 (R-PE linked to cyanin (PC5), IgG1, 679.1 Mc7), CD95 (Fas; FITC, IgG1, UB2), CD23 (FITC, IgG1, 9P25), IgG1 (allophycocyanin, PC5, and PE, isotypic control, 679.1 Mc7), rat anti-human CD77 (unconjugated, IgM, 38-13) followed by goat anti-rat IgM (FITC, F(ab')<sub>2</sub>), all from Immunotech (Luminy, France); CD3 (T cell specific: FITC, IgG2a, 33-2A3), IgM (PE, IgG2a, IIE2), IgG1 (FITC, isotypic control, 1B9), IgG2a (PE, isotypic control, 5A7), all from Diatec (Oslo, Norway); IgD (PE, IgG2a, IA6-2); CD27 (PE, IgG1, M-T271), IgG2a (FITC, isotypic control, G<sub>155-178</sub>), all from BD PharMingen (San Diego, CA); IgD (FITC, F(ab')<sub>2</sub>, 107), CD10 (PE, IgG1, SS2/36), CD44 (FITC, IgG1, DF1485) from DAKO (Glostrup, Denmark).

### Multiparameter flow cytometric analysis

Isolated and T cell-depleted MNC were stained for CD19, CD38, and IgD in combination with the following cell surface markers: CD10, CD44,

Table II. Clinical, laboratory, and medication data for RA patients<sup>a</sup>

Patient	RF	ANA	ENA	SR	CRP	WBC	NSAID	Corticosteroid	Methotrexate
R1	–	–	–	16	<5	8.2	Yes <sup>b</sup>	None	None
R2	+	–	ND	44	ND	14.5	None	Yes	Yes
R3	+	–	ND	36	ND	13.5	None	Yes	Yes
R4	+	128	–	21	<5	10.4	Yes <sup>b</sup>	None	None
R5	+	128	–	24	14	7.2	None	None	Yes <sup>c</sup>
R6	+	–	–	26	33	4.4	Yes <sup>b</sup>	None	None
R7	+	32	–	22	36	15.2	Yes	Yes	Yes
R8	+	512	–	24	6	7.0	Yes	None	Yes
R9	+	32	–	29	6	7.1	Yes	None	Yes

<sup>a</sup> Antinuclear Ab number refers to titer of ANA; ENA, extractable nuclear Ag; SR, sedimentation ratio (millimeters/hour); CRP, C-reactive protein (milligrams/liter); WBC, white blood cells ( $\times 10^9$ /liter).

<sup>b</sup> Also receiving Salazopyrin (sulfasalazin).

<sup>c</sup> Also receiving Thyroxin.

Table III. Summary of the CD markers used in the study and their function and expression on peripheral B cells

CD Marker	Ligand	Function on B Cells	Expression on Peripheral B Cell Subpopulations
CD10	Peptidase	Unknown	GC founder and GC B cells
CD19	(Associates with CD21/CD81/Leu13)	Involved in signal transduction	All peripheral B cells
CD23	IgE, FcεRII	Ag presentation	Activated naive B cells
CD27	CD70	CD27-CD70 interactions regulate B cell proliferation and differentiation	Somatically mutated cells; GC, memory, and plasma cells
CD38	CD31 Hyaluronic acid	The biological role is controversial but seems to act as a channel that delivers activation signals in B cells	During early differentiation and activation
CD44	Hyaluronic acid	Attachment to and rolling on endothelial cells, homing to peripheral lymphoid organs and to sites of inflammation	Naive and memory B cells Not on GC B cells
CD77	CD1 (possibly)	Might play a role in apoptosis	Centroblasts
CD95 (Fas)	CD95 ligand	Mediation of apoptosis-inducing signals	On some activated B cells

CD77, CD23, IgM, CD27, and CD95 (the CD markers with their expression and function on B cells are summarized in Table III). To assure there were no T cells in the CD19-positive B cell populations, we also stained for CD3 in combination with CD19. The cells were incubated with mAb (20  $\mu$ l) for 30 min, washed twice in PBS containing FCS (2%), and fixed with paraformaldehyde (1%). Data were collected using a FACSCalibur analytical instrument, and four-color analyses were performed by CellQuest software (BD Biosciences) or WinMDI (<http://tacs.scripps.edu/>).

#### Sorting of CD27<sup>+</sup> and CD27<sup>-</sup> PB B cells and of the Bm1 (CD38<sup>-</sup>IgD<sup>+</sup>) and Bm2' (CD38<sup>++</sup>IgD<sup>+</sup>) B cell subpopulations from a healthy donor

T cells were depleted from the isolated MNC suspension by rosetting with magnetic Dynabeads coated with a primary mAb specific for human CD3 (DynaL Biotech, Oslo, Norway). The procedure was performed as recommended in the product protocol. The cell suspensions were stained for CD19 and CD27 or CD19, CD38, and IgD, as described above, but the cells were kept on ice and in medium (RPMI containing 10% FCS) instead of fixation. The CD19<sup>+</sup> B cells were sorted into CD27<sup>+</sup> and CD27<sup>-</sup> subpopulations and collected into two tubes on a FACS Vantage (BD Biosciences).

#### Molecular analysis of V<sub>H</sub> region from CD27<sup>+</sup>, CD27<sup>-</sup>, Bm1 (CD38<sup>-</sup>IgD<sup>+</sup>), and Bm2' (CD38<sup>++</sup>IgD<sup>+</sup>) PB B cells

The V<sub>H</sub> region sequences were obtained by extracting mRNA from the sorted B cells with oligo(dT)<sub>25</sub>-coated magnetic Dynabeads (mRNA direct kit; Dynal). First-strand cDNA was prepared by using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ) and a primer from the IgM constant region priming the 3' end (5'-AAGGGTTGGGGCG GATGC-3').

Second-strand synthesis and amplification of V<sub>H</sub>1, V<sub>H</sub>3, V<sub>H</sub>4, and V<sub>H</sub>6 regions were done by PCR using 2  $\mu$ l of the single-stranded cDNA product in a final volume of 50  $\mu$ l containing 200  $\mu$ M (0.2 mM) concentrations of each dNTP, 40 pmol of the C $\mu$  primer, 40 pmol of one of the V<sub>H</sub> primers, 5  $\mu$ l of the 10 $\times$  cloned PFU DNA polymerase reaction buffer, and 1.25 U PfuTurbo DNA polymerase (Stratagene, La Jolla, CA). Oligonucleotides specific for the 5' end of the V<sub>H</sub> families were used (V<sub>H</sub>1, 5'-CCATGGACT GGACCTGGAGG-3'; V<sub>H</sub>3, 5'-CCATGGAGTTGGGCTGAGC-3'; V<sub>H</sub>4, 5'-ATGAAACACCTGTGGTTCTT-3'; V<sub>H</sub>6, 5'-ATGTCTGTCTCCTTC CTCAT-3') (Eurogentec, Brussels, Belgium). PCR was conducted for 41 cycles under the following conditions: 2 cycles (98°C for 30 s, 48°C for 30 s, and 72°C for 45 s); 3 cycles (98°C for 30 s, 50°C for 30 s, and 72°C for

45 s); 3 cycles (94°C for 30 s, 51°C 30 s, and 72°C for 30 s); 33 cycles (94°C for 30 s, 53°C 30 s, and 72°C for 30 s); and a final extension of 72°C for 15 min by using GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). PCR products were analyzed on a 1.2% agarose gel; bands were cut out of the gel and concentrated by QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The blunt end PCR products of V<sub>H</sub> genes (2  $\mu$ l) were cloned by using a Zero blunt TOPO PCR cloning kit for sequencing with chemically competent *Escherichia coli* (Invitrogen, Groningen The Netherlands). Plasmid DNA was purified from ampicillin-selected clones by a Wizard cap. Plus SV minipreps DNA purification system (Promega, Madison, WI). Plasmid DNA (1  $\mu$ l) was checked for insertion of the V<sub>H</sub> PCR product by restriction analysis by cutting with EcoRI enzyme (10 U; Stratagene). An aliquot (4  $\mu$ l) of positive restriction analyzed plasmid DNA was sequenced by using BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems), and data were collected by a 377 DNA Sequencer and PBI prism software (Applied Biosystems). Nucleotide sequences were analyzed by Lasergene99 software (DNASTAR), Blast search (<http://www.ncbi.nlm.nih.gov/>), and V base (<http://www.mrc-cpe.cam.ac.uk/>).

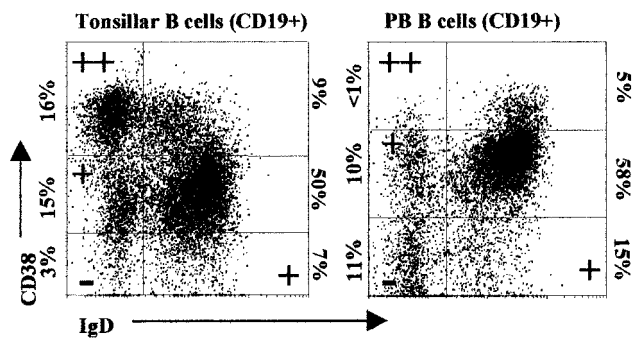
#### Statistical analyses

Percentages of cells expressing cell surface markers were described as means of the individuals in each group. Variation in each group was defined by SEM. Differences between healthy donors, pSS, and RA patient groups were compared by an unpaired two-tailed *t* test. When the SD was significantly different between the groups, a Welch-corrected *t* test was used. A value of *p* < 0.05 was considered to be statistically significant. The statistical calculations were performed with GraphPad InStat.

## Results

#### Five of the six tonsillar B cell subpopulations defined by membrane expression of CD38/IgD are represented in PB

Staining for CD38 and IgD gave a subdivision of PB B cells into five subpopulations compared with six subpopulations among the tonsillar B cells (Fig. 1). These subpopulations, named Bm1–Bm5, have been described in tonsils based on the surface expression of CD38 and IgD in conjunction with CD10, CD44, CD77, CD23, IgM, and CD95 (11, 13). We therefore compared the expression of these markers in addition to CD27 on PB B cells with that on tonsillar B cells, to determine which peripheral B cell differentiation stages are also found in PB (Figs. 1–3). The germinal center



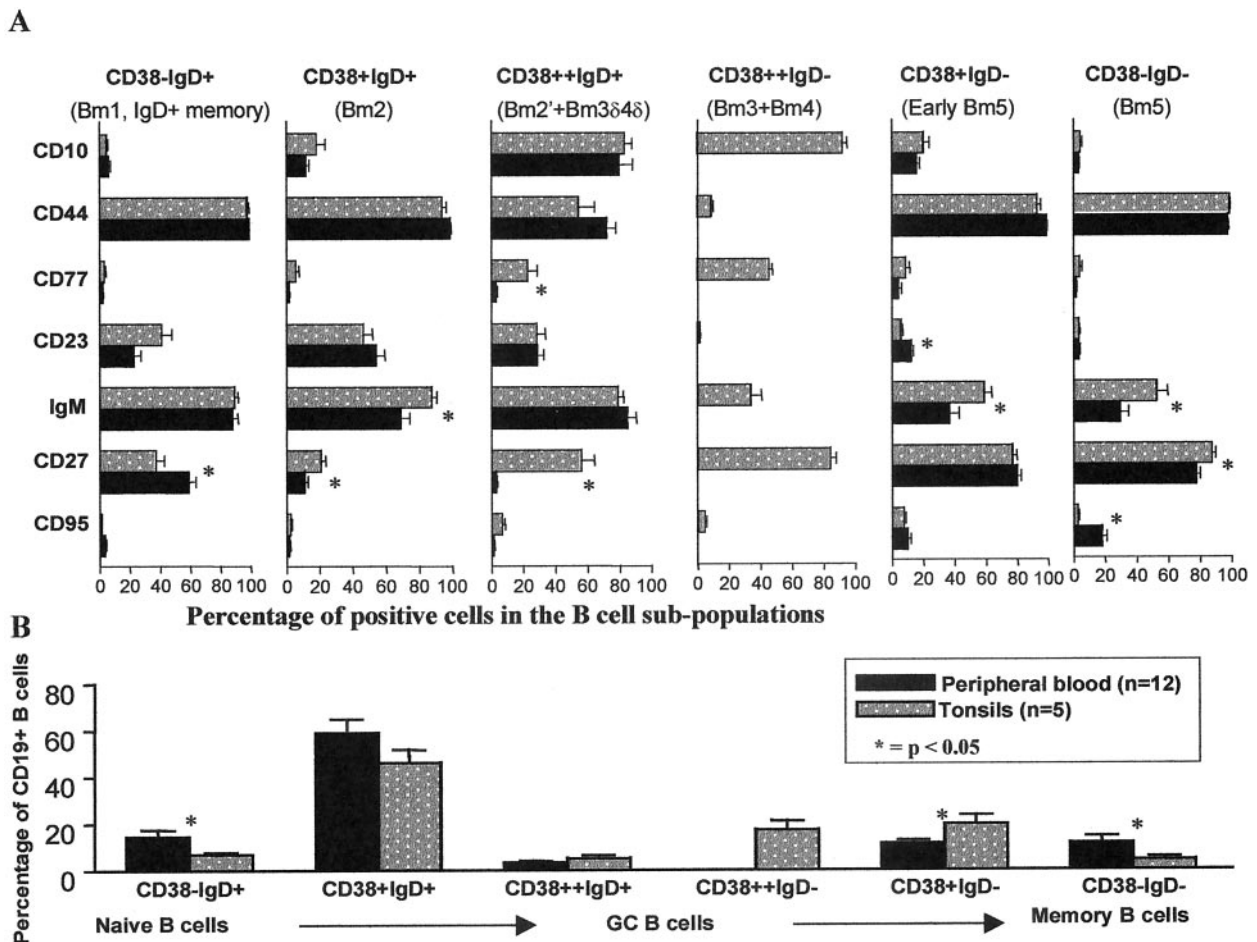
**FIGURE 1.** CD19<sup>+</sup> tonsillar, and PB B cells stained for CD38 and IgD and divided into B cell subpopulations according to the expression level of the cell surface markers. The lower left quadrant separating negative and positive subpopulations is based on the isotype control (not shown), and the upper horizontal line separating positive and double positive subpopulations is based on the expression pattern for CD77, CD10, and CD44 shown in Fig. 3. The percentage distribution of the cells in the subpopulations is shown.

(GC) B cell subpopulation (CD38<sup>++</sup>IgD<sup>-</sup>) is undetectable or absent in PB. However, surprisingly, a small proportion of GC founder cells (CD38<sup>++</sup>IgD<sup>+</sup>) was found circulating in the PB. In addition, the staining of CD27 revealed new information on the previously defined peripheral B cell subpopulations.

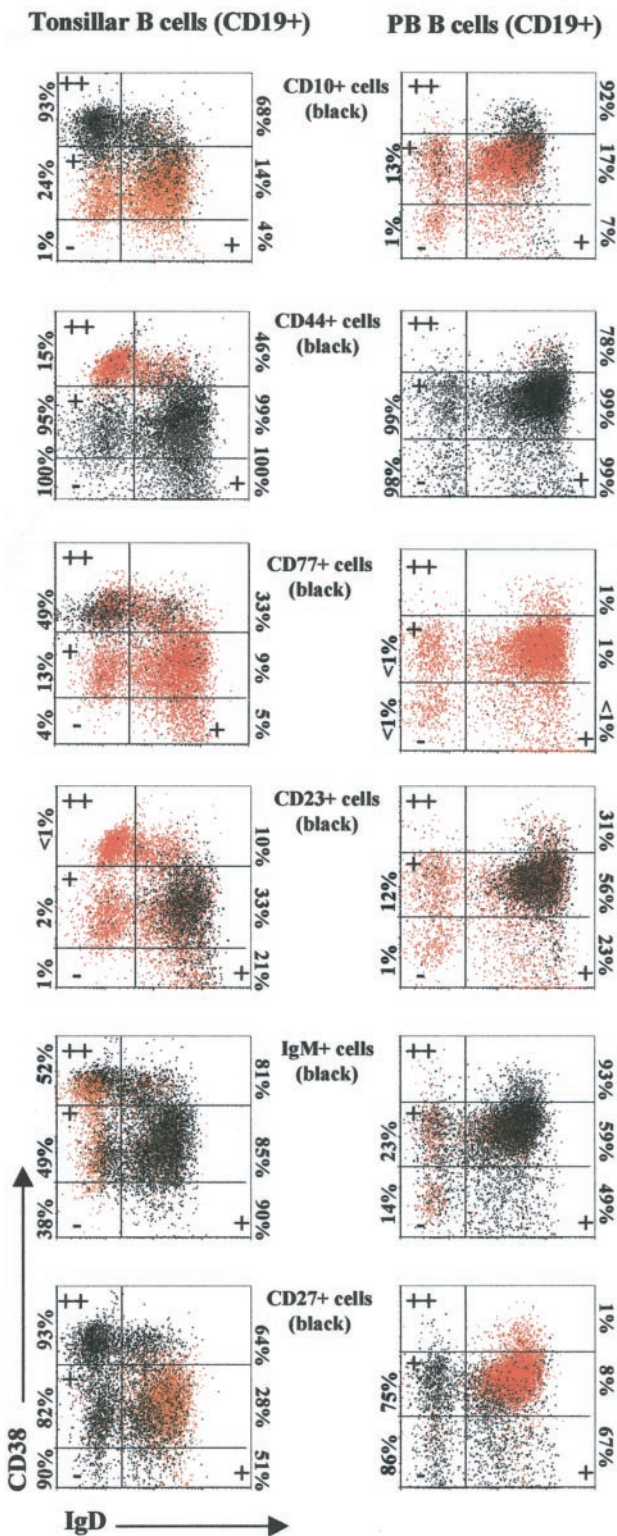
Overall PB B cells were found to consist of ~40% resting B cells (Bm1, early Bm5, and Bm5) and a surprisingly high percentage (60%) of activated B cells expressing IgD in addition to CD23 and/or CD38 (Bm2 and Bm2' + Bm3δ4δ). Tonsils consisted of ~30% resting B cells (Bm1, early Bm5, and Bm5) and 70% activated B cells (Bm2, Bm2' + Bm3δ4δ, Bm3, and Bm4) (Fig. 2).

*The CD38<sup>-</sup>IgD<sup>+</sup> B cell subpopulation contains a significant IgD<sup>+</sup> memory B cell compartment in addition to naive Bm1 cells, whereas the large, activated naive Bm2 subpopulation consists of almost all naive B cells*

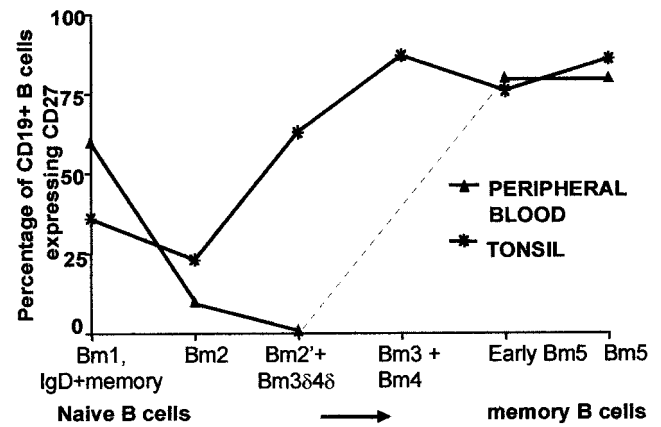
The CD38<sup>-</sup>IgD<sup>+</sup> subpopulation, previously known as naive Bm1 cells, was found in both PB and tonsils. Analysis of CD27 expression on these cells suggested that this subpopulation might also include an IgD<sup>+</sup> memory B cell component, in both blood (59%) and tonsil (37%) (Figs. 2–4). We confirmed that these were memory cells by showing that CD27<sup>+</sup> B cells were clearly somatically mutated by analyzing the sequences from expressed V<sub>H</sub> region genes of seven IgM<sup>+</sup>CD27<sup>+</sup> PB clones and eight IgM<sup>+</sup>CD27<sup>-</sup> PB clones. All CD27<sup>+</sup> V<sub>H</sub> sequences showed somatic mutations (Table IV). The mean somatic mutation frequency of the IgM<sup>+</sup>CD27<sup>+</sup> B cells was 3.1% (range 0.7–7.4%). Only one of the eight CD27<sup>-</sup> B cell clones showed evidence of somatic mutation. In addition, three sequences obtained from the CD38<sup>-</sup>IgD<sup>+</sup> B cell subpopulation (IgM<sup>+</sup>, CD27 unknown) were all mutated (mean 5.6%, range 1.0–12.9%; Table V).



**FIGURE 2.** A, Percentages of CD19<sup>+</sup> B cell subpopulations expressing the cell surface markers CD10, CD44, CD77, CD23, IgM, CD27, and CD95. B, Percentage of each subpopulation of CD19<sup>+</sup> B cells from PB and tonsil. Bars represent the mean percentages of data from tonsils of five individuals (gray spotted bars) and PB of 12 healthy donors (black filled bars); error bars define SEM.



**FIGURE 3.** Two-dimensional illustration of CD19<sup>+</sup> (red dots) tonsillar B cells from one donor and CD19<sup>+</sup> (red dots) PB B cells from a healthy donor stained for CD38, IgD. Cells expressing one of the following cell surface markers, CD10, CD44, CD77, CD23, IgM, and CD27, are visualized (black dots) in the CD38/IgD plot. The amounts of cells expressing the selected cell surface marker (black dots) are shown as percentages of the cells in each quadrant. The cell surface markers are specific for different Bm subpopulations; CD23 is mainly expressed on activated naive B cells (Bm2), CD10 on GC founder cells (Bm2' + Bm384δ), and GC B cells (Bm3 + Bm4) and CD77 on centroblasts (Bm3). CD44 is expressed on naive (Bm1 and Bm2), memory (Bm5), and on some GC founder cells. CD27 is a marker for somatically mutated cells/memory B cells.



**FIGURE 4.** Percentage of CD27<sup>+</sup> cells in the Bm1–Bm5 population. The dot line between Bm2' + Bm384δ and early Bm5 indicates that no Bm3- or Bm4-like cells were found in PB.

The activated naive Bm2 population characterized by the phenotype CD38<sup>+</sup>IgD<sup>+</sup> was the largest subpopulation in both PB and tonsils (PB 59%; tonsil 46%). In comparison with the CD38<sup>+</sup>IgD<sup>+</sup> subpopulation, CD27 was expressed at a much lower frequency (Figs. 2–4), with PB cells expressing a significantly lower percentage than tonsil cells (PB 11%; tonsil 21%;  $p = 0.016$ ).

*GC founder cells circulate in the PB, whereas GC cells do not circulate*

Surprisingly, we identified a small subpopulation (3%) of CD38<sup>+</sup>IgD<sup>+</sup> or GC founder cells (Bm2' + Bm384δ) in 9 of the 12 PB samples. A high percentage of the CD38<sup>+</sup>IgD<sup>+</sup> cells expressed CD10 (80% in PB; 83% in tonsil;  $p = \text{NS}$ ) as expected for GC founder and GC cells. In contrast to the tonsillar subpopulation, the expression of CD27 and CD77 was almost absent on the PB GC founder population (CD77 PB 3% vs tonsil 23%,  $p = 0.032$ ; CD27 PB 3% vs tonsil 56%,  $p = 0.003$ ) (Figs. 2A and 4). In addition to the previously described sequencing analysis of CD27<sup>-</sup> and CD27<sup>+</sup> PB B cells discriminating between unmutated and mutated cells, two sequences from the PB Bm2' subpopulation were found to be unmutated (Table V), excluding these cells from being memory B cells re-entering the GC. The absence of CD38<sup>+</sup>IgD<sup>-</sup> B cells in PB, confirms that GC cells do not circulate.

*Two IgD<sup>-</sup> memory B cell subpopulations exist in both PB and tonsil (early Bm5 and Bm5), but a higher percentage of these cells are class switched and express CD95 in PB*

The IgD<sup>-</sup> memory B cells could be divided into two subpopulations, one subpopulation still expressing CD38 (CD38<sup>+</sup>IgD<sup>-</sup>, early Bm5) and one that had down-regulated the CD38 expression (CD38<sup>-</sup>IgD<sup>-</sup>, Bm5). The percentages of CD27<sup>+</sup> cells were high in these two subpopulations from both tonsil and PB (Figs. 2 and 4). In addition, CD10 expression was lost, and CD44 expression was up-regulated in both PB and tonsil (Fig. 2A). More circulating IgD<sup>-</sup> memory B cells are class switched than the tonsillar IgD<sup>-</sup> memory B cells, in that IgM was expressed on a significantly lower percentage of both IgD<sup>-</sup> memory B cell subpopulations from PB. The early Bm5 subpopulation contained 37% IgM<sup>+</sup> cells in PB vs 59% in tonsils ( $p = 0.046$ ) and for Bm5 the percentage of IgM expressing cells was 29% in PB vs 53% in tonsil ( $p = 0.029$ ). Moreover, the percentage expression of the apoptosis related marker, CD95, was higher in the circulating Bm5 subpopulation (PB = 18%, tonsil = 3%,  $p = 0.0001$ ).

Table IV. Somatic mutation in  $V_H$  region genes from  $IgM^+CD27^+$  and  $IgM^+CD27^-$  PB ( $CD19^+$ ) B cells from healthy donors<sup>a</sup>

CD27 <sup>+</sup>					CD27 <sup>-</sup>				
Clone	$V_H$ segment	D segment	J segment	Mutations (n)	Clone	$V_H$ segment	D segment	J segment	Mutations (n)
HC1+6	1-8	4-17	JH5b	2 (0.7%)	HC3-5	3-30.3	2-15	JH6b	0 (0.0%)
HC1+2	1-8	5-12	JH4b	21 (7.4%)	HC1-2	3-72	4-17	JH2	29 (10.2%)
HC3+6	$V_H3-8^b$	2-2	JH4b	3 (1.1%)	HC1-1	6-1	2-15	JH5b	0 (0.0%)
HC4+5	4-34	1-26	JH5b	4 (1.4%)	HC3-2	6-1	3-10	JH6b	0 (0.0%)
HC4+9	4-59	NF	JH4b	9 (3.3%)	HC3-1	1-2	NF	JH4b	0 (0.0%)
HC4+8	4-34.2 <sup>b</sup>	NF	JH4b	6 (2.1%)	HC4-8	6-1	6-19	JH6b	0 (0.0%)
HC4+2	4-4	NF	JH4a	16 (5.6%)	GE4-7 <sup>c</sup>	4-30.1	2-21	JH3b	0 (0.0%)
					GE6-10 <sup>c</sup>	3-53	4-17	JH6b	0 (0.0%)
Total mean (Range)				3.1% (0.7–7.4%)	Total mean (Range)				1.3% (0.0–10.2%)

<sup>a</sup> The numbers of base pairs sequenced from each  $V_H$  gene segment were between 282 and 284 bp, and the mutation frequencies were calculated by dividing the number of nucleotide exchange mutations with the total base pairs analyzed.

<sup>b</sup> For these  $V_H$  segments, a different gene nomenclature was used instead of the gene locus. NF, Not found.

<sup>c</sup> This sequence is from a different healthy donor.

#### Expression of CD27 on the Bm1–Bm5 subpopulations from PB and tonsil

CD27-expressing B cells were present in both PB and tonsils but at significantly different percentages in all except one subpopulation (the early Bm5) (Figs. 2A and 4). The percentage of  $IgD^+$  memory B cells, expressing CD27, within the  $CD38^-IgD^+$  subpopulation was higher in PB than in tonsil, but in PB the percentages were dramatically lower in the next two B cell subpopulations (Bm2 and Bm2' + Bm3848). In contrast, the tonsillar B cells showed almost the same percentage of  $IgD^+CD27^+$  memory B cells in the  $CD38^-$  and the  $CD38^+$  subpopulations, whereas in the Bm2' + Bm3848 subpopulations the percentages of  $CD27^+$  cells were high. The percentages of  $CD27^+$  B cells were high in both PB and tonsil for the early Bm5 and the Bm5 subpopulations.

#### A significantly higher proportion of activated B cells is found in pSS patients than in RA patients and healthy donors

The CD38 and IgD staining pattern showed that the Bm2 subpopulation was the most abundant PB B cell subpopulation in pSS, RA patients, and healthy donors (54–75%) (Fig. 5). The four other subpopulations each accounted for between 2 and 17% of  $CD19^+$  PB B cells. There were remarkable and significant differences in the relative sizes of the subpopulations among the pSS patients compared with both healthy donors and RA patients. The Bm2 subpopulation was significantly larger for patients with pSS than for healthy donors (pSS 75%, healthy donors 59%;  $p = 0.031$ ). In contrast, the  $CD38^-IgD^+$  (including Bm1 and the  $IgD^+$  memory B cells) and the early Bm5 subpopulations comprised a significantly lower percentage of PB B cells in pSS patients

( $CD38^-IgD^+$  6%, early Bm5 7%) than in healthy donors ( $CD38^-IgD^+$  15%,  $p = 0.013$ ; early Bm5 14%,  $p = 0.031$ ). There were no significant differences in the sizes of the PB B cell subpopulations between RA patients and healthy donors. However, the GC founder, Bm2' + Bm3848 subpopulation was observed in only 5 of 9 RA patients and 9 of 12 healthy donors, whereas it was seen in all 11 patients with pSS.

Comparisons of pSS and RA showed significant differences between all PB B cell subpopulations (Fig. 5). The percentages of  $CD38^-IgD^+$  (pSS = 6%, RA = 17%), early Bm5 (pSS = 7%, RA = 13%), and Bm5 (pSS = 5%, RA = 13%) subpopulations were significantly lower in pSS than in RA ( $p = 0.0001$ , pSS =  $p = 0.026$  and  $p = 0.002$ , respectively), whereas the Bm2 (pSS = 75%, RA = 54%) and the Bm2' + Bm3848 (pSS = 7%, RA = 2%) subpopulations were significantly larger in pSS than in RA ( $p = 0.001$ ,  $p = 0.033$ , respectively). Overall there was a higher percentage of activated B cells (Bm2 and Bm2' + Bm3848) in pSS patients than in healthy donors and RA patients.

## Discussion

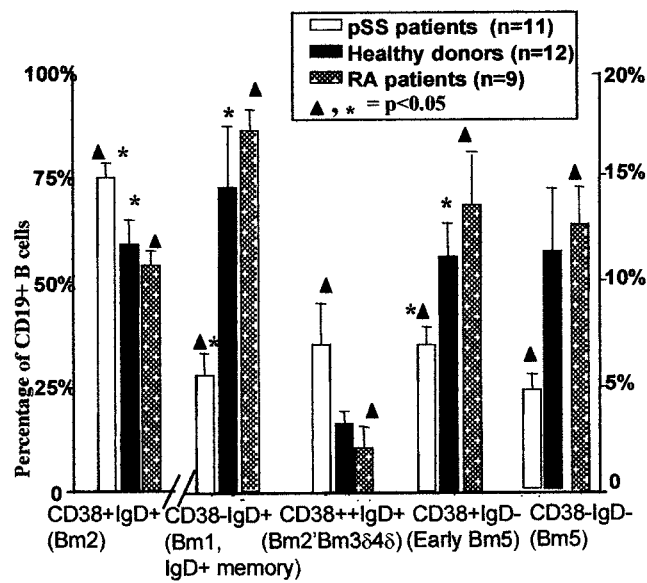
### PB B cell subpopulations comparable with those in tonsil

Earlier studies of PB B cells using costaining of IgD and IgM have identified only naive and memory subpopulations ( $IgM^-IgD^-$ ,  $IgM^+IgD^-$ , and  $IgM^+IgD^+$ ) (19, 27). Different surface markers have been used to identify B cell differentiation stages in secondary lymphoid tissue (tonsil). The tonsillar B cell subpopulations were characterized in a system based on staining of CD38 and IgD in conjunction with CD10, CD44, CD77, CD23, IgM, and CD95 (11, 13) and named from Bm1 (naive) to Bm5 (memory). We have

Table V. Somatic mutation in  $V_H$  region genes from  $IgM^+IgD^+CD38^{++}$  (Bm2') and  $IgM^+IgD^+CD38^-$  (Bm1 cells) PB ( $CD19^+$ ) B cells from healthy donors<sup>a</sup>

CD38 <sup>++</sup> IgD <sup>+</sup> (Bm2')					CD38 <sup>-</sup> IgD <sup>+</sup> (Bm1)				
Clone	$V_H$ segment	D segment	J segment	Mutations (n)	Clone	$V_H$ segment	D segment	J segment	Mutations (n)
JO3.3	6-1	5-24	JH4b	0 (0.0%)	JO4.3	4-34	3-10	JH4	3 (1.0%)
JO3.6	6-1	3-10	JH5	0 (0.0%)	JO4.12	4-31	4-23	JH4	9 (3.0%)
					JO4.14	4-28	3-9	JH3	38 (12.9%)
Total mean				0.0%	Total mean (range)				5.6% (1.0–12.9%)

<sup>a</sup> The Bm2' subpopulation contains 3%  $CD27^+$  B cells, whereas 59% of the cells in the  $IgD^+CD38^-$  subpopulation are  $CD27^+$ . The numbers of base pairs sequenced from each  $V_H$  gene segment were between 282 and 284 bp, and the mutation frequencies were calculated by dividing the number of nucleotide exchange mutations with the total base pairs analyzed.



**FIGURE 5.** Distribution of PB B cell subpopulations in pSS, RA, and healthy donors. Percentages represent mean data from 11 pSS patients (white), 12 healthy donors (black), and 9 RA patients (gray spotted); error bars define SEM. The significances of the differences between the groups were calculated by an unpaired two-tailed *t* test and Welch correction was applied when necessary. \*, Significant difference ( $p < 0.05$ ) between pSS and healthy donors. ▲, Significant difference ( $p < 0.05$ ) between pSS and RA. There were no significant differences between RA and healthy donors.

used this Bm1–Bm5 classification of B cell subpopulations to identify and compare circulating B cell differentiation stages in PB with those in tonsil. The CD38/IgD staining by itself revealed five PB B cell subpopulations comparable with five of the six CD38/IgD-defined B cell subpopulations detected in tonsil. The GC cell subpopulation (CD38<sup>+++</sup>IgD<sup>-</sup>) was missing in PB. In 1994, Pascual et al. (11) defined four subpopulations only based on CD38/IgD expression, and later Grammer et al. (28) could divide tonsillar B cells into the same six subpopulations, as we observed, in addition to two plasma cell populations (CD38<sup>+++</sup>). New mAb used for the staining of CD38 give a better differentiation of CD38 expression and thus reveal more subpopulations. The present comparisons using additional surface markers within the CD38/IgD-defined subdivisions revealed PB B subpopulations not previously known to circulate. In addition, except for some minor differences in expression of cell surface markers, the B cell subpopulations in PB and tonsil seem to be highly homologous (Tables VI and VII).

#### CD27 expression reveals a memory compartment within the CD38<sup>-</sup>IgD<sup>+</sup> subpopulation

The lack of a specific memory B cell marker has been a limitation of the Bm1–Bm5 classification system. CD27 has recently been

Table VII. Bm1–Bm5 B cell subpopulations in PB and secondary lymphoid tissue

Differentiation Step	PB	Secondary Lymphoid Tissue
Naive B cells		
CD38 <sup>-</sup> IgD <sup>+</sup> <sup>a</sup>	Bm1 <sup>a</sup>	Bm1 <sup>a</sup>
CD38 <sup>+</sup> IgD <sup>+</sup>	Bm2	Bm2
GC founder cells		
CD38 <sup>+++</sup> IgD <sup>+</sup>	Bm2' + Bm3&4&6	Bm2' + Bm3&4&6
CD38 <sup>++</sup> IgD <sup>weak</sup>		Early Bm3
GC cells		
CD38 <sup>++</sup> IgD <sup>-</sup>		Bm3 + Bm4
Memory B cells		
CD38 <sup>+</sup> IgD <sup>-</sup>	Early Bm5	Early Bm5
CD38 <sup>-</sup> IgD <sup>-</sup>	Bm5	Bm5

<sup>a</sup> This subpopulation also contains some CD27<sup>+</sup>IgD<sup>+</sup> memory B cells.

defined as a marker for somatically mutated B cells, and thus memory B cells, by two independent groups (17–20). Therefore, we analyzed the expression of CD27 on the Bm1–Bm5 classified cells. Surprisingly, CD27 was expressed on more than one-half of the CD38<sup>-</sup>IgD<sup>+</sup> PB B cells, previously defined as unmutated naive Bm1 cells. Sequencing analysis of V<sub>H</sub> gene segments from CD27<sup>+</sup> and CD27<sup>-</sup> PB B cells of healthy donors confirmed that the CD27 marker discriminates between memory and naive B cells. This indicates that CD38<sup>-</sup>IgD<sup>+</sup> B cells include memory/somatically mutated IgD<sup>+</sup> B cells in addition to naive B cells. Moreover, three V<sub>H</sub> gene sequences obtained from the sorted CD38<sup>-</sup>IgD<sup>+</sup> PB B cells were all mutated. We should also have obtained unmutated sequences from the V<sub>H</sub> genes analyzed as this population also contains the naive Bm1 cells. However, because CD27<sup>+</sup> B cells are over-represented in the PB CD38<sup>-</sup>IgD<sup>+</sup> subpopulation, the sequences are likely to have been obtained only from the CD27<sup>+</sup> B cells. Previously, the CD38<sup>-</sup>IgD<sup>+</sup> (Bm1) subpopulation has been found to contain only unmutated cells (11, 12). This discrepancy may be explained by the use of different mAb for CD38 staining in the two studies. The large CD38<sup>-</sup>IgD<sup>+</sup> subpopulation examined in the previous study probably included both the CD38<sup>-</sup>IgD<sup>+</sup> and the CD38<sup>+</sup>IgD<sup>+</sup> (Bm2) subpopulations found in the present analysis. This combined population would therefore have a very low percentage of CD27<sup>+</sup> cells. Thus the sequences obtained might only come from CD27<sup>-</sup> cells, due to the low percentage of CD27<sup>+</sup> cells. IgD<sup>+</sup> memory or somatically mutated B cells have previously been described in both PB (19) and tonsils (21, 29), and these IgD<sup>+</sup> memory B cells have possibly been “hidden” in the CD38/IgD classification system.

#### Some GC founder cells circulate

Surprisingly, some GC founder cells (CD38<sup>+++</sup>IgD<sup>+</sup>) were identified in PB, indicating that these cells are circulating, contradictory to previous impressions (16). The expression of CD10 on

Table VI. Expression of selected surface markers on tonsillar CD19<sup>+</sup> B cells and Bm1–Bm5 subpopulations<sup>a</sup>

	CD38 <sup>-</sup> IgD <sup>+</sup>	CD38 <sup>+</sup> IgD <sup>+</sup>	CD38 <sup>++</sup> IgD <sup>+</sup>	CD38 <sup>+++</sup> IgD <sup>-</sup>	CD38 <sup>+</sup> IgD <sup>-</sup>	CD38 <sup>-</sup> IgD <sup>-</sup>
CD10	-	- (+)	+ (-)	+ (-)	- (+)	-
CD44	+	+	+/-	-	+	+
CD77	-	-	+/-	+/-	-	-
CD23	+/-	+/-	+/-	-	-	-
IgM	+	+	+ (-)	+/-	+/-	+/-
Subpopulation	Bm1	Bm2	Bm2' + Bm3&4&6	Bm3 + Bm4	Early Bm5	Bm5

<sup>a</sup> -, All cells are negative; +, all cells are positive; +/-, ~50% positive cells; (-) or (+), few negative/positive cells.



most of the cells in this PB subpopulation corroborates the GC founder cell phenotype, because CD10 is a marker for GC and GC founder cells (22). In addition, all the CD38<sup>+</sup>IgD<sup>+</sup> PB B cells were negative for CD27, confirming that they are not yet GC B cells or somatically mutated memory B cells re-entering the germinal center. Furthermore, two V<sub>H</sub> gene sequences obtained from Bm2'-sorted cells were found to be unmutated. In contrast, many of the tonsillar CD38<sup>+</sup>IgD<sup>+</sup> B cells express CD27 and CD77, which suggests that these cells are starting to develop into GC cells (CD38<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup>CD77<sup>+</sup>/early Bm3). The presence of circulating GC founder cells may reflect the activation stage of an ongoing immune response. Moreover, this suggests that GC founder cells may be able to migrate between distinct secondary lymphoid tissues via PB. The identification of memory B cells still expressing CD38 (early Bm5) in both PB and tonsil suggests that B cells may also be able to leave GC and secondary lymphoid tissues at an earlier differentiation stage than previously believed.

*The GC cells are not circulating, and the IgD<sup>-</sup> memory B cell subpopulations show some differences in the two compartments*

Our data indicate that GC B cells are the only peripheral B cell differentiation stage that does not circulate, consistent with other studies showing that GC B cells down-regulate expression of chemokine receptors or lose responsiveness to certain chemokines (30, 31). CD77 is believed to be a regulator of BCR-induced apoptosis (32). Significantly, CD77 was not found on circulating B cells, suggesting that this regulation mechanism is required only in secondary lymphoid tissue. More memory B cells were class switched (IgM<sup>-</sup>) in PB than in tonsil, suggesting that memory B cells that are not class switched are more prone to die and/or more likely to be activated for a second round of GC reaction. Moreover, a surprisingly high fraction of the memory (Bm5) subpopulation in PB expressed the apoptosis-related marker, CD95 (33, 34), compared with the tonsillar Bm5 cells, suggesting that apoptosis may also be important in the circulating memory B cell compartment. Altogether, our analyses have revealed differentiation stages of peripheral B cells not previously known to circulate. This new knowledge gives an important basis for further analysis on peripheral B cell development in healthy and disease.

*Disturbed proportions of PB B cell subpopulations in pSS patients, but not in RA patients*

The utility of an expanded classification of PB B cell subpopulations can be addressed by analyses of patients with immunological diseases. RA and pSS are autoimmune rheumatic diseases characterized by chronic inflammation and infiltration of T and B cells in the affected joints of RA patients and the exocrine glands of pSS patients. Ectopic GC-like structures that might play a role in the generation of autoimmune B cells have been identified in the affected tissue of RA (35) and pSS (36) patients.

Remarkably, pSS patients showed different proportions of B cell subpopulations compared with healthy donors and RA patients. GC founder cells (Bm2' + Bm3δ4δ) were detectable in all pSS patients but only in 5 of the 9 RA patients and 9 of 12 healthy donors. The elevated presentation of this subpopulation in pSS might be a result of a frequent B cell activation in this disease. Furthermore, pSS patients had a higher percentage of activated naive B cells (Bm2) and a lower percentage of resting B cells (Bm1, the newly identified IgD<sup>+</sup>CD27<sup>+</sup> memory B cells, early Bm5, and Bm5) than both healthy donors and RA patients. An increase of memory B cells (37, 38) and a decrease of activated B cells (39) in PB during aging has been described, but this could not explain the difference between the groups given that the mean age and range were approximately the same for all groups.

Rheumatoid factor (RF) is an important diagnostic marker for both pSS (23, 24) and RA (25), and elevated plasma levels of RF are associated with a severe disease prognosis for pSS (40) and RA (41). Recently, we found that PB B cells from patients with pSS produce RF and anti-Ro/Sjögren's syndrome A with a much lower degree of somatic mutation (42–44) than RF from RA patients and healthy donors (45). These findings might be explained by the higher percentage of activated naive B cells (Bm2) and GC founder cells (Bm2' + Bm3δ4δ) found circulating in pSS, resulting from an hyperactivation of the peripheral B cells. Furthermore, disturbances in chemokine levels and the expression of their receptors on B cells, important for GC formation and B cell migration, may influence peripheral B cell homeostasis in pSS (30, 31, 46–49).

No significant differences were found in the Bm1–Bm5 B cell subpopulations between RA patients and healthy donors. However, all RA patients in the present study were on at least one of the following drugs: nonsteroidal anti-inflammatory drugs (NSAID); corticosteroids, methotrexate; and Salazopyrin. Corticosteroids can affect the expression of cell surface markers (50), and the therapeutic regimens may have masked disturbances in the proportions of B cell subpopulations in the RA group, although we could not see a systematic difference in the three RA patients not receiving corticosteroids or methotrexate. Two of the pSS patients were taking NSAID; however, no difference could be seen in the percentages of B cell subpopulations for those two patients compared with the other pSS patients not receiving NSAID.

A disturbance in B cell trafficking or an increased activation of B cells might explain the altered proportions of PB B cell subpopulations in pSS patients. In addition, there could be an alteration in peripheral B cell differentiation, leading to a bias toward plasma cell differentiation and thus fewer memory B cells in the PB of pSS. Further analysis of both the trafficking and the activation state of the PB B cells in pSS would clarify the role of B cells in the pathology of pSS. The identification of high percentage of activated B cells might be of diagnostic significance in pSS and would be of interest to analyze in other autoimmune disorders.

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