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

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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

Janne Ø. Bohnhorst,* Marie B. Bjørgan,* Jørn E. Thøen,† Jacob B. Natvig,* and Keith M. Thompson*

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 Bm36–46), 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+/H11546 phenotype, previously known as naive Bm1 cells, expressed CD27. The CD38+ IgD+ population therefore includes both naive Bm1 cells and IgD+ 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) 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 defining 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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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 Lo-

visken 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 × 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 × g. The cells were diluted in PBS and layered on Isopaque-Ficoll separation medium (Lymphoprep; Nycomed, Oslo, Norway) and cen-

trifuged for 25 min at 450 × g. T cells were depleted from purified mononuclear cells (MNC) by rosetting with anti-IgM/IgG3/biotinylated sheep anti-human IgM (Upstate Biotechnology, Lake Placid, NY) and a second round of anti-IgG3/PE-treated SRBC. The cell suspension was layered on Isopaque-Ficoll separation medium (Lymphoprep) and centrifuged for 25 min at 450 × g. T cells were depleted from the separated 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’2)), all from Immunotech (Luminy, France); CD3 (T cell specific: FITC, IgG2a, 33-2A3), IgM (PE, IgG2a, J112), 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, G137), all from BD Pharmingen (San Diego, CA); IgD (FITC, F(ab’2)2, 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 I. Clinical, laboratory, and medication data for the pSS patients

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*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 (×10^9/liter).

### Table II. Clinical, laboratory, and medication data for the RA patients

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*Antinuclear Ab 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 (×10^9/liter).

*Also receiving Salazopyrin (sulfasalazine).
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 µl) 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://facs.scripps.edu/).

Sorting of CD27+ and CD27− PB B cells and of the Bm1 (CD38+ IgD+) and Bm2 (CD38+ IgD−) 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− B cells were sorted into CD27+ and CD27− subpopulations and collected into two tubes on a FACSVantage (BD Biosciences).

Molecular analysis of V_H region from CD27+, CD27−, Bm1 (CD38+ IgD+), and Bm2 (CD38+ IgD−) PB B cells

The V_H region sequences were obtained by extracting mRNA from the sorted B cells with oligo(dT)12–14-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_H1, V_H3, V_H4, and V_H6 regions were done by PCR using 2 µl of the single-stranded cDNA product in a final volume of 50 µl containing 200 µM (0.2 mM) concentrations of each dNTP, 40 pmol of the C primer, 40 pmol of one of the V_H primers, 5 µl of the 10× 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_H families were used (V_H1, 5′-CCATGGAAC GGACCTGGGGG-3′; V_H3, 5′-CCATGGAGTTGGGCTAGG-3′; V_H4, 5′-ATGAAAAACCTCGTGCTTT-3′; V_H6, 5′-ATGTGCTGTCCTCCCTCAT-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 (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_H genes (2 µl) were cloned by using a Zero blunt TOPO PCR cloning kit for sequencing with chemically competent XL1-blue (Stratagene). An aliquot (4 µl) 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, CD34, CD27, 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...
(GC) B cell subpopulation (CD38⁺IgD⁻) is undetectable or absent in PB. However, surprisingly, a small proportion of GC founder cells (CD38⁺IgD⁺) 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⁺ + Bm348). Tonsils consisted of ~30% resting B cells (Bm1, early Bm5, and Bm5) and 70% activated B cells (Bm2, Bm2⁺ + Bm348, Bm3, and Bm4) (Fig. 2).

The CD38⁺IgD⁺ B cell subpopulation contains a significant IgD⁺ 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⁺IgD⁺ 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⁺ 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⁺ B cells were clearly somatically mutated by analyzing the sequences from expressed V_H region genes of seven IgM⁺CD27⁺ PB clones and eight IgM⁺CD27⁻ PB clones. All CD27⁺ V_H sequences showed somatic mutations (Table IV). The mean somatic mutation frequency of the IgM⁺CD27⁺ B cells was 3.1% (range 0.7–7.4%). Only one of the eight CD27⁻ B cell clones showed evidence of somatic mutation. In addition, three sequences obtained from the CD38⁺IgD⁺ B cell subpopulation (IgM⁺, CD27 unknown) were all mutated (mean 5.6%, range 1.0–12.9%; Table V).

![FIGURE 1.](image1.png) CD19⁺ 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.

![FIGURE 2.](image2.png) A, Percentages of CD19⁺ B cell subpopulations expressing the cell surface markers CD10, CD44, CD77, CD23, IgM, CD27, and CD95. B, Percentage of each subpopulation of CD19⁺ 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.
The activated naive Bm2 population characterized by the phenotype CD38+/IgD+ was the largest subpopulation in both PB and tonsils (PB 59%; tonsil 46%). In comparison with the CD38+/IgD− 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+/IgD+/Bm2 or GC founder cells (Bm2+/Bm3+Bm4) in 9 of the 12 PB samples. A high percentage of the CD38+/IgD+ cells expressed CD10 (80% in PB; 83% in tonsil; p = 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, the previously described sequencing analysis of CD27+ and CD27− 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+/IgD+ B cells in PB, confirms that GC cells do not circulate.

Two IgD− 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− memory B cells could be divided into two subpopulations, one subpopulation still expressing CD38 (CD38−IgD−, early Bm5) and one that had down-regulated the CD38 expression (CD38+IgD+, Bm5). The percentages of CD27+ 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− memory B cells are class switched than the tonsillar IgD− memory B cells, in that IgM was expressed on a significantly lower percentage of both IgD− memory B cell subpopulations from PB. The early Bm5 sub population contained 37% IgM+ 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).
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 cell subpopulations (Bm2 and Bm2'+ Bm34δ). 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'+ Bm34δ 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'+ Bm34δ 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 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'+ Bm34δ (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'+ Bm34δ) 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 co-staining 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 IV. Somatic mutation in VH region genes from IgM+CD27+ and IgM+CD27− PB (CD19+) B cells from healthy donors

<table>
<thead>
<tr>
<th>Clones</th>
<th>VH Segment</th>
<th>D Segment</th>
<th>J Segment</th>
<th>Mutations (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC1+6</td>
<td>1-8</td>
<td>4-17</td>
<td>JH5b</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td>HC1+2</td>
<td>1-8</td>
<td>5-12</td>
<td>JH4b</td>
<td>21 (7.4%)</td>
</tr>
<tr>
<td>HC3+6</td>
<td>1-34</td>
<td>2-2</td>
<td>JH4b</td>
<td>3 (1.1%)</td>
</tr>
<tr>
<td>HC4+5</td>
<td>4-59</td>
<td>NF</td>
<td>JH4b</td>
<td>9 (3.3%)</td>
</tr>
<tr>
<td>HC4+8</td>
<td>4-34</td>
<td>NF</td>
<td>JH4b</td>
<td>6 (2.1%)</td>
</tr>
<tr>
<td>HC4+2</td>
<td>4-4</td>
<td>NF</td>
<td>JH4a</td>
<td>16 (5.6%)</td>
</tr>
<tr>
<td>Total mean (Range)</td>
<td>3.1% (0.7–7.4%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The numbers of base pairs sequenced from each VH 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.

Table V. Somatic mutation in VH region genes from IgM+IgD+CD38− (Bm2') and IgM+IgD+CD38− (Bm1 cells) PB (CD19+) B cells from healthy donors

<table>
<thead>
<tr>
<th>Clones</th>
<th>VH Segment</th>
<th>D Segment</th>
<th>J Segment</th>
<th>Mutations (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JO3.3</td>
<td>6-1</td>
<td>5-24</td>
<td>JH4b</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>JO3.6</td>
<td>6-1</td>
<td>3-10</td>
<td>JH5</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Total mean (Range)</td>
<td>0.0% (1.0–12.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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 VH 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.
RA. There were no significant differences between RA and healthy donors.

identified and compared circulating B cell differentiation stages in PB

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++/IgD−) was missing in PB. In 1994, Pascau 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++.). 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++/IgD+ subpopulation

The lack of a specific memory B cell marker has been a limitation of the Bm1–Bm5 classification system. CD27 has recently been 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++/IgD+ PB B cells, previously defined as unmutated naive Bm1 cells. Sequencing analysis of VH gene segments from CD27+ and CD27− PB B cells of healthy donors confirmed that the CD27 marker discriminates between memory and naive B cells. This indicates that CD38++/IgD+ B cells include memory/somatically mutated IgD+ B cells in addition to naive B cells. Moreover, three VH gene sequences obtained from the sorted CD38++/IgD+ PB B cells were all mutated. We should also have obtained unmutated sequences from the VH genes analyzed as this population also contains the naive Bm1 cells. However, because CD27+ B cells are over-represented in the PB CD38++/IgD+ subpopulation, the sequences are likely to have been obtained only from the CD27+ B cells. Previously, the CD38++/IgD+ (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++/IgD+ subpopulation examined in the previous study probably included both the CD38++/IgD+ and the CD38++/IgD− (Bm2) subpopulations found in the present analysis. This combined population would therefore have a very low percentage of CD27+ cells. Thus the sequences obtained might only come from CD27− cells, due the low percentage of CD27+ cells. IgD+ memory or somatically mutated B cells have previously been described in both PB (19) and tonsils (21, 29), and these IgD+ memory B cells have possibly been “hidden” in the CD38/IgD classification system.

Some GC founder cells circulate

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

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

<table>
<thead>
<tr>
<th>Differentiation Step</th>
<th>PB</th>
<th>Secondary Lymphoid Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive B cells</td>
<td>CD38+IgD*</td>
<td>Bm1*</td>
</tr>
<tr>
<td>CD38+IgD*</td>
<td>Bm2</td>
<td></td>
</tr>
<tr>
<td>GC founder cells</td>
<td>CD38++IgD−</td>
<td>Bm2′ + Bm3δ45</td>
</tr>
<tr>
<td>CD38++IgDweak</td>
<td>Bm2′ + Bm3δ45</td>
<td></td>
</tr>
<tr>
<td>CD38++IgD+</td>
<td>Early Bm3</td>
<td></td>
</tr>
<tr>
<td>Memory B cells</td>
<td>CD38+IgD+</td>
<td>Early Bm5</td>
</tr>
<tr>
<td>CD38–IgD−</td>
<td>Bm5</td>
<td></td>
</tr>
</tbody>
</table>

* This subpopulation also contains some CD27+IgD+ memory B cells.

Table VI. Expression of selected surface markers on tonsillar CD19+ B cells and Bm1–Bm5 subpopulations

<table>
<thead>
<tr>
<th></th>
<th>CD38+IgD+</th>
<th>CD38+IgD+</th>
<th>CD38++IgD+</th>
<th>CD38++IgD+</th>
<th>CD38++IgD+</th>
<th>CD38–IgD+</th>
<th>CD38–IgD+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD10</td>
<td>−</td>
<td>− (+)</td>
<td>+ (−)</td>
<td>+ (−)</td>
<td>− (−)</td>
<td>− (+)</td>
<td>+ (−)</td>
</tr>
<tr>
<td>CD44</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD77</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CD23</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IgM</td>
<td>+</td>
<td>+</td>
<td>+ (−)</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Subpopulation</td>
<td>Bm1</td>
<td>Bm2</td>
<td>Bm2′ + Bm3δ45</td>
<td>Bm3 + Bm4</td>
<td>Early Bm5</td>
<td>Bm5</td>
<td>Bm5</td>
</tr>
</tbody>
</table>

a −, 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+/IgD+ B+ PB 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_H gene sequences obtained from Bm2-sorted cells were found to be unmutated. In contrast, many of the tonsillar CD38+/IgD+ B cells express CD27 and CD77, which suggests that these cells are starting to develop into GC cells (CD38+/IgD+/CD27+/CD77-/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 previous believed.

The GC cells are not circulating, and the IgD+ 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+/-) 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′+ Bm3Δ46) 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-CD27+ 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′+ Bm3Δ46) 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.

Acknowledgments

We thank the doctors and nurses at Lovisenberg Hospital for providing tonsils and Dr. Jan Egil Henriksen at Diakonhjemmet Hospital for providing patient material. We also thank Gøril Olsen (Institute of Pathology, National Hospital, Oslo, Norway), for providing help with the FACS sorting of 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.

References


