The Majority of Human Memory B Cells Recognizing RhD and Tetanus Resides in IgM⁺ B Cells

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The Majority of Human Memory B Cells Recognizing RhD and Tetanus Resides in IgM+ B Cells

Luciana Della Valle,* Serge E. Dohmen,† Onno J. H. M. Verhagen,* Magdalena A. Berkowska,* Gestur Vidarsson,* and C. Ellen van der Schoot*

B cell memory to T cell–dependent (TD) Ags is considered to largely reside in class-switched CD27+ cells. However, we previously observed that anti-RhD (D) Igs cloned from two donors, hyperimmunized with D+ erythrocytes, were predominantly of the IgM isotype. We therefore analyzed in this study the phenotype and frequency of D- and tetanus toxoid–specific B cells by culturing B cells in limiting dilution upon irradiated CD40L-expressing EL4.B5 cells and testing the culture supernatant. Most Ag-specific B cells for both TD Ags were found to reside in the IgM-expressing B cells, including CD27+ B cells, in both hyperimmunized donors and nonhyperimmunized volunteers. Only shortly after immunization a sharp increase in Ag-specific CD27+IgG+ B cells was observed. Next, B cells were enriched with D+ erythrocyte ghosts and sorted as single cells. Sequencing of donors and nonhyperimmunized volunteers. Only shortly after immunization a sharp increase in Ag-specific CD27+IgG+ B cells was observed. Next, B cells were enriched with D+ erythrocyte ghosts and sorted as single cells. Sequencing of

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Materials and Methods

Donors

Leukapheresis products or EDTA blood samples were collected from eight hyperimmunized anti-D and four anti-TT donors with high titers at Sanquin with informed consent, and the study was approved by the Ethics Advisory Board of our institute. Donors were hyperimmunized for at least 2 y, except for the youngest donor (RhD1), who was hyperimmunized for 10 mo. The proportions of the different B cell subsets of the hyperimmunized donors and their titers at time of collection are listed in Table I. Moreover, four nonhyperimmunized donors (controls) were included in the study. They all were immunized against TT at least four times in the early period since birth. Three of these donors received their last TT booster at least 10 y before this study and one 5 y earlier. Two of them were also examined 14 d after an additional TT booster.

Production of fluorescent erythrocyte ghosts

Erythrocyte ghosts were produced from 25 ml 3% homozygous for the D Ag and negative for the C Ag of the Rh system, because those RBCs express the highest number of D Ag sites per RBC. The erythrocytes were diluted in PBS and centrifuged for 5 min at 2400 × g at room temperature. After decanting the supernatant, the pelleted was resuspended in 25 ml ice-cold 5 mM sodium biphosphate solution (pH 8.2) and then centrifuged again for 30 min at 21,000 × g at 4˚C. After washing, the purified ghosts were resuspended in the same sodium biphosphate solution and incubated with either a PKH26 (Sigma-Aldrich, St. Louis, MO) probe or with a CFSE (Life Technologies, Carlsbad, CA) probe on ice for 5 min or with a CFSE (Life Technologies, Carlsbad, CA) probe at 37˚C for 15 min. After adding 1% of human serum albumin (200 g/l; Sanquin, Amsterdam, The Netherlands) the ghosts were washed and resuspended in 500 μl IMDM (Lonza, Basel, Switzerland) with 10% FCS (Bodinco, Alkmaar, The Netherlands).

Labeling and sorting of B cells

B cells were purified from mononuclear cells by magnetic separation with CD19 beads (Miltenyi Biotec, Leiden, The Netherlands). B cells were labeled with CD27-allophycocyanin (BD Biosciences, Franklin Lakes, NJ), goat anti-human IgM and IgG (SouthernBiotech, Birmingham, AL) coupled with Alexa Fluor 405 and Alexa Fluor 700 dyes, respectively (Life Technologies). For Ag-specific B cell frequency studies, B cell subsets were sorted by FACSaria II cell sorter (BD Biosciences, San Jose, CA) in 1, 10, 33, 100, 330, 1000, and 3300 cells/well (for gate settings, see Supplemental Fig. 1). To enrich for anti-D–specific B cells, 300 μl PKH26-labeled ghosts and 300 μl CFSE-labeled ghosts were added to a suspension of 60 × 10⁶ CD19⁺ B cells and incubated for 60 min at 4˚C. Anti-D–specific B cells were sorted as single double-positive (PKH26⁺CFSE⁺) B cells per well (Supplemental Fig. 2).

EL4.B5 culture system

The B cells were plated in 96-well flat-bottom plates (Nunc, Roskilde, Denmark). These plates had been preseeded with CD40L-expressing EL4.B5 cells (50 Gy irradiated, 1 × 10⁶/ml) were harvested on day 9 or 10 and 50 μl was added to 50 μl 1% bromelain-treated D⁺ erythrocytes (ccDDEE) in 96-well round-bottom plates. Agglutination was scored after incubation at room temperature and at 37˚C. Agglutinating supernatants were also tested for the lack of agglutination with D⁻ erythrocytes (ccdde).

ELISA plates (Nunc-Immuno MaxiSorp from Sigma-Aldrich) were coated overnight with goat anti-human IgM (1.8 μg/ml), goat anti-human IgG (1.3 μg/ml) (both from Jackson ImmunoResearch Laboratories, West Grove, PA) or with TT (2.3 μg/ml) (Statens Serum Institute, Copenhagen, Denmark) diluted in coating buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.6). Culture supernatant was diluted in 2% milk (Cappima/PBS/0.025% Tween and incubated for 1 h at room temperature. Ab binding was detected with biotinylated goat Fab’ (anti-human IgM or goat Fab’ (anti-human IgG (0.5 μg/ml) (both from BioSource International, Camarillo, CA) and streptavidin-HRP (Thermo Scientific, Waltham, MA).

Calculation of B cell outgrowth

Wells producing Ag-specific Ig were scored and frequencies were calculated with L-Calc software (StemCell Technologies, Grenoble, France). To calculate Ag-specific B cell frequencies per subset, those frequencies were corrected for the number of viable B cells that were found to actually produce IgM or IgG, as determined from 24 wells sorted with single B cells of the different subsets. To estimate the distribution of the Ag-specific B cells among the subsets, we used the Ag-specific B cell frequencies (in relation to Ig production) and the size of each subset (Table I).

BCL6 mutation and Ig analysis

The progeny of single-cell–sorted B cells that produced anti-D or for the control non–anti-D Ig were collected after 8 d and digested in 25 μl lysis buffer (1 × GoTag Flexi buffer; Promega, Madison, WI) and 0.1 mg/ml proteinase K (Roche). A 10th of the lysate was used for BCL6 mutation analysis by nested PCR (see Supplemental Table I) using a GenAmp PCR system 9700 (Applied Biosystems). PCR products were cleaned up (ExoSAP-IT; USB, Cleveland, OH) and sequenced with both nested primers and an extra reverse primer (BCL6-S) (ABI Prism 377 sequencer from Applied Biosystems, Foster City, CA). From the remaining RNA was extracted by TRizol (Invitrogen, Carlsbad, CA) and reverse-transcribed using SMARTer RACE cDNA amplification kit (Clontech, Palo Alto, CA) with the 5’-RACE CDS primer A oligonucleotide. cDNA was amplified using universal primer A mix and four reverse primers (R1 primers in Supplemental Table I) specific to the IGHG,IGHM, IGLK, or IGLL-C region, using the Advantage 2 PCR enzyme system (Clontech). The first PCR product (1.5 μl) was amplified by nested universal primer A and four reverse primers (R2 primers in Supplemental Table I). Both PCR products were cleaned up and sequenced with the appropriate nested R2-C primers. Sequences were

FIGURE 1. Frequency of Ag-specific B cells is similar in IgM⁺ and class-switched IgM⁺ B cells, but IgM⁺ exceeds class-switched B cells in absolute numbers. B cells were sorted based on their IgM⁺ or IgG⁺ surface phenotype. The frequency of Ag-specific Abs was assessed after 10 d of culture with irradiated EL4.B5 cells by either erythrocyte agglutination (rhesus D Ag) or ELISA (TT) in three anti-D and two anti-TT hyperimmunized donors. (A) The frequency of Ag-specific B cells was estimated by limiting dilution analysis and is expressed as a number of Ag-specific B cells per million of total PB B cells of that given subset. Each symbol represents a donor. (B) The relative number of IgM⁺ and IgM⁺ B cells within the Ag-specific B cells was calculated for each donor taking the frequency and subset size of each B cell population into account.
aligned to their closest germline gene segment using IMGT/V-QUEST (http://imgt.org/IMGT_vquest).

Statistical analysis
Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA).

Results
Most Ag-specific B cells are found in the IgM⁺ B cell subset
Because we previously found that most anti-D Ig clones from two hyperimmunized donors were IgM (12), we now sorted IgM⁺ and IgM⁻ B cells in limiting dilution from three other anti-D and two anti-TT hyperimmunized donors. After culture for 10 d, Ag-specific IgGs were identified by agglutination or TT-specific ELISA. The frequency of Ag-specific B cells was highest within the IgM⁻ B cell subset in three donors (two anti-D and one anti-TT) and within the IgM⁺ B cell subset in the other two donors (Fig. 1A). To determine absolute numbers of IgM⁺ and IgM⁻ Ag-specific B cells, the actual size of these B cell subsets was taken into account (Table I). The percentages found in this small group of donors are comparable to data observed in the literature (14–17), although the percentage of CD27⁺IgG⁺ B cells is somewhat lower. Because the IgM⁺ B cell subset was always the largest, the number of IgM⁺ Ag-specific B cells exceeded that of class-switched IgM⁻ cells in all five analyzed hyperimmunized donors (Fig. 1B; mean, 77% of Ag-specific B cells are IgM-expressing B cells).

Ag-specific IgM⁺ B cells reside in both CD27⁺ and CD27⁻ B cell populations
Further frequency analyses were performed on five other anti-D and two anti-TT hyperimmunized donors, but now also distinguishing IgM⁺ B cell subsets by CD27. The frequency of Ag-specific B cells was higher within the CD27⁺ populations (mean of 1 Ag-specific B cell of 4000 cells was found) than in the CD27⁻ B cell population (mean of 1 Ag-specific B cell of 185 and 161 sorted cells in CD27⁺IgM⁺ and CD27⁺IgG⁺ B cells, respectively), whereas in the CD27⁻IgM⁻ subset a mean of 1 Ag-specific B cell of 4000 cells was found. The comparable outgrowth of the three B cell subsets shows that our observation that most Ag-specific B cells express IgM is not due to preferential stimulation of IgM⁺ cells.

RhD- and TT-specific B cells in nonimmunized donors and cord blood
To exclude that these CD27⁺ IgM⁺ B cells were naive B cells, we tested the presence of D-specific B cells in two D⁻ nonimmunized donors, one D⁺ donor, and the presence of TT-specific cells in three cord blood samples. Neither IgM nor IgG Ag-specific B cells were found in any of these cases (data not shown), whereas the outgrowth of B cells was normal. To determine whether IgM⁺ Ag-specific B cells were related to hyperimmunization, TT-specific B cells were characterized in four nonhyperimmunized donors. All showed the highest frequency of TT-specific B cells in the CD27⁺IgM⁺ B cell subset (Fig. 4A), and again most Ag-specific B cells resided in the IgM⁺ B cell subset (88 ± 19%, mean ± SD). In one donor no TT-specific IgM⁺ expressing B cells were found, but unfortunately only 10,000 IgG⁺ B cells were sorted in this donor. Two donors were also analyzed 14 d after a TT booster.

Table I. B cell characteristics and immunization data for all donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (y)</th>
<th>Proportion of CD19⁺ Cells (%)</th>
<th>B Cell Subset Sizes (%) of Total Peripheral CD19⁺ B Cells</th>
<th>Last Booster (d)</th>
<th>Titer*</th>
<th>Hyperimmunization (mo)</th>
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<tbody>
<tr>
<td>Anti-D Rh1</td>
<td>51</td>
<td>5.5</td>
<td>CD27⁺IgM⁺ 72.8⁺nCD27⁺IgM⁻ 19.7⁺nCD27⁺IgG⁺ 43.4⁺n</td>
<td>131</td>
<td>512</td>
<td>10</td>
</tr>
<tr>
<td>Anti-D Rh2</td>
<td>58</td>
<td>7.8</td>
<td>CD27⁺IgM⁺ 80.1⁺nCD27⁺IgM⁻ 17.9⁺nCD27⁺IgG⁺ 43.4⁺n</td>
<td>131</td>
<td>512</td>
<td>154</td>
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<tr>
<td>Anti-D Rh3</td>
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<td>CD27⁺IgM⁺ 56.5⁺nCD27⁺IgM⁻ 37.7⁺nCD27⁺IgG⁺ 43.8⁺n</td>
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<td>225</td>
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<td>Anti-D Rh5</td>
<td>49</td>
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<td>CD27⁺IgM⁺ 66.9⁺nCD27⁺IgM⁻ 20.0⁺nCD27⁺IgG⁺ 28.0⁺n</td>
<td>44</td>
<td>1000</td>
<td>45</td>
</tr>
<tr>
<td>Anti-D Rh6</td>
<td>49</td>
<td>10.5</td>
<td>CD27⁺IgM⁺ 84.9⁺nCD27⁺IgM⁻ 11.7⁺nCD27⁺IgG⁺ 5.0⁺n</td>
<td>290</td>
<td>1000</td>
<td>34</td>
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<td>Anti-D Rh7</td>
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<td>5.1</td>
<td>CD27⁺IgM⁺ 87.1⁺nCD27⁺IgM⁻ 10.2⁺nCD27⁺IgG⁺ 5.0⁺n</td>
<td>130</td>
<td>1000</td>
<td>214</td>
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<tr>
<td>Anti-D Rh8</td>
<td>62</td>
<td>4.4</td>
<td>CD27⁺IgM⁺ 56.1⁺nCD27⁺IgM⁻ 4.4⁺nCD27⁺IgG⁺ 43.8⁺n</td>
<td>116</td>
<td>12</td>
<td>46</td>
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<td>Anti-TT 1</td>
<td>55</td>
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<td>CD27⁺IgM⁺ 77.7⁺nCD27⁺IgM⁻ 22.2⁺nCD27⁺IgG⁺ 43.8⁺n</td>
<td>63</td>
<td>11</td>
<td>62</td>
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<tr>
<td>Anti-TT 2</td>
<td>58</td>
<td>5.8</td>
<td>CD27⁺IgM⁺ 84.7⁺nCD27⁺IgM⁻ 5.9⁺nCD27⁺IgG⁺ 4.9⁺n</td>
<td>570</td>
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<td>Anti-TT 3</td>
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<td>9.9</td>
<td>CD27⁺IgM⁺ 87.3⁺nCD27⁺IgM⁻ 4.8⁺nCD27⁺IgG⁺ 4.8⁺n</td>
<td>169</td>
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<td>86</td>
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<tr>
<td>Anti-TT 4</td>
<td>48</td>
<td>12.0</td>
<td>CD27⁺IgM⁺ 72.1⁺nCD27⁺IgM⁻ 27.8⁺nCD27⁺IgG⁺ 27.8⁺n</td>
<td>&gt;10 y</td>
<td>&lt; 1</td>
<td>—</td>
</tr>
<tr>
<td>Ctrl 1 before</td>
<td>35</td>
<td>17.0</td>
<td>CD27⁺IgM⁺ 64.9⁺nCD27⁺IgM⁻ 32.4⁺nCD27⁺IgG⁺ 21.4⁺n</td>
<td>&gt;10 y</td>
<td>&lt; 1</td>
<td>—</td>
</tr>
<tr>
<td>Ctrl 2 before</td>
<td>30</td>
<td>4.2</td>
<td>CD27⁺IgM⁺ 59.6⁺nCD27⁺IgM⁻ 17.9⁺nCD27⁺IgG⁺ 17.9⁺n</td>
<td>&gt;5 y</td>
<td>1.8</td>
<td>—</td>
</tr>
<tr>
<td>Ctrl 3 before</td>
<td>29</td>
<td>8.8</td>
<td>CD27⁺IgM⁺ 65.8⁺nCD27⁺IgM⁻ 12.9⁺nCD27⁺IgG⁺ 12.9⁺n</td>
<td>&gt;10 y</td>
<td>&lt; 1</td>
<td>—</td>
</tr>
<tr>
<td>Ctrl 4 before</td>
<td>24</td>
<td>10.1</td>
<td>CD27⁺IgM⁺ 77.5⁺nCD27⁺IgM⁻ 22.6⁺nCD27⁺IgG⁺ 22.6⁺n</td>
<td>14</td>
<td>17.6</td>
<td>—</td>
</tr>
<tr>
<td>Ctrl 1 after</td>
<td>35</td>
<td>20.0</td>
<td>CD27⁺IgM⁺ 67.4⁺nCD27⁺IgM⁻ 32.5⁺nCD27⁺IgG⁺ 32.5⁺n</td>
<td>14</td>
<td>6.0</td>
<td>—</td>
</tr>
</tbody>
</table>

*For anti-D, titer is measured based on agglutination, for TT by IgG ELISA.

**IgM⁺ B cell subset size as percentage of sorted CD19⁺ B cells.

*IgM⁻ B cell subset size as percentage of sorted CD19⁺ B cells.

*CD27⁺ B cell subset size as percentage of sorted CD19⁺ B cells.

*CD27⁻ B cell subset size as percentage of sorted CD19⁺ B cells.
(Fig. 4B). The frequencies of TT-specific B cells in the CD27+ IgM+ B cell subset did not change after the booster and were ~1 of 5000. After booster a sharp augmentation of the number of TT-specific CD27+IgG+ cells was observed, from none or only 1 of 60,000 total B cells to 1 of 615 and 1 of 3,000. This increase was accompanied in both donors with a slight decline of TT-specific CD27−IgM+ cells (1 of 7,000 in steady-state to 1 of 20,000 after booster) (Fig. 4B). This increase in frequency of TT-specific CD27+IgG+ cells was paralleled with a rise in anti-TT IgG titer (Table I).

Mutational status of the BCL6 and Ig genes in D-specific B cells

To investigate whether the Ag-specific IgM-expressing cells, especially the CD27+ B cells, are bona fide memory B cells, we studied the mutational status of the Ig and BCL6 genes in D-specific B cells of an anti-D hyperimmunized donor (20, 21). CD27+IgM+, CD27−IgM+, and CD27+IgG+ cells that bound D+ ghosts were sorted as single cells. DNA and RNA were isolated from agglutinating wells. As control, randomly selected B cells producing IgM or IgG were analyzed.

FIGURE 2. The largest proportion of Ag-specific B cells resides in IgM-expressing CD27+ and CD27+ B cell populations. The frequency of Ag-specific B cells in five anti-D and two anti-TT hyperimmunized donors was determined by limiting dilution after sorting CD27+IgM+, CD27−IgM+, and CD27+IgG+ B cell subsets onto EL4B5 cells, allowing them to grow for 10 d before testing supernatants for specific Abs as described in Fig. 1. (A) Frequency of Ag-specific B cells given as a number of Ig-producing Ag-specific B cells per million cells within subset. Black lines indicate geometric means. (B) The frequencies reported are per million total B cells or (C) as relative number of cells from the different subsets. Note that no IgG+ anti-TT B cells were identified from the anti-TT3 donor.

FIGURE 3. Ig production from single B cells is equally good in CD27+ and CD27+ B cells. B cells were sorted as one cell per well and cultured as described in the legend to Fig. 1. Supernatants were tested for IgM and IgG production by ELISA, and the number of Ab-producing B cells are given as a mean percentage (bars), together with SDs from 14 different experiments.

Mutational status of the BCL6 and Ig genes in D-specific B cells

To investigate whether the Ag-specific IgM-expressing cells, especially the CD27+ B cells, are bona fide memory B cells, we studied the mutational status of the Ig and BCL6 genes in D-specific B cells of an anti-D hyperimmunized donor (20, 21). CD27+IgM+, CD27−IgM+, and CD27+IgG+ cells that bound D+ ghosts were sorted as single cells. DNA and RNA were isolated from agglutinating wells. As control, randomly selected B cells producing IgM or IgG were analyzed.

FIGURE 4. IgM-expressing TT-specific B cells are present also in nonhyperimmunized donors. (A) The frequency of TT-specific B cells, in four nonhyperimmunized donors who had not received a TT booster for at least 10 y, was estimated by limiting dilution, as in Figs. 1–3. The frequency is given as the number of B cells per million within a subset. (B) Frequency of TT-specific B cells per million peripheral B cells in two control donors before and 14 d after receiving TT booster immunization.
AID exposure of IgM+ D-specific B cells, including CD27 showing the highest number. Collectively, these data clearly prove a one-tailed Student comparing anti-D–specific and control B cells within B cell subsets using found only in anti-D–specific CD27 anti-D–specific CD27 2 2

anti-D–specific B cells from different subsets are clonally related. Some anti-D–specific B cell clones were clonally related to three CD27 +IgM+ clones, or

in anti-D–specific CD27 +IgM+ cells and not in non–anti-D–specific CD27 +IgM+ cells (Fig. 5). As expected, mutations were present in all CD27 + subsets, with CD27 +IgG+ cells showing the highest number. Collectively, these data clearly prove AID exposure of IgM+ D-specific B cells, including CD27 B cells.

Anti-D–specific B cells from different subsets are clonally related

The results of sequence analysis of rearranged Ig genes from 36 D-specific B cell clones of donor RhD4 are summarized in Fig. 6. The lengths of the CDR3 regions were similar for the three B cell subsets. For 20 of 36 sequences, a related clone was found either within the same subset or between subsets. Mutations found in the BCL6 gene were identical in the CD27 +IgM+ clone 4A2 and the CD27 +IgM+ clone 10B6, as well as for the related clones 13B5 and 12E5. In Fig. 7 the clonal relationship is displayed. Six of 13 CD27 +IgM+ cells (46%) and 6 of 15 CD27 +IgG+ cells (40%) showed clonal relatedness, whereas only 2 of 8 CD27 +IgM+ cells (25%) were related. Some anti-D–specific B cell clones were clonally related among the three different B cell subsets. In particular, 4 of 8 CD27 +IgM+ cells were related to three CD27 +IgM+ clones, whereas only 1 of 15 CD27 +IgG+ cells were clonally related with cells of the other two subsets. One clone was even present in all three subsets.

Discussion

In this study, we show that most circulating Ag-specific memory B cells in anti-D and anti-TT hyperimmunized individuals are IgM+ B cells, in some donors even CD27 +IgM+ B cells, and that these IgM+ B cell are bona fide memory cells. We have studied two strong TD Ags, a particulate (RhD) as well as a soluble (TT) Ag, and they showed comparable results. Our findings in humans support previous data observed in mice suggesting the existence of multiple layers of B cell memory with different effector functions (8).

Using a CD40/CD40L culture system, the frequency of Ag-specific B cells in PB was analyzed in anti-D and anti-TT hyperimmunized donors in steady-state. In line with our previous findings (12), we demonstrate that the most in number of either anti-D- or anti-TT-specific B cells expressed IgM, although the highest frequency of Ag-specific B cells was found in most donors within the CD27 +IgM+ subset. It was excluded that this was an artifact of our CD40L culture approach, because both IgM- and IgG-secreting B cells were equally stimulated in this system in contrast to studies showing discrepancies between naive and memory cells using a similar method (18, 19). The different B cell source (tonsils and spleen versus PB) likely containing more activated B cells compared with PB, the absence of a more complex cytokine environment, and a reduced number of CD40L feeder cells per culture could be responsible for these disparities.

D-specific B cells were only found in alloimmunized individuals and TT-specific B cells were absent in cord blood, indicating that they are elicited upon immunization. A key characteristic of memory B cells is the presence of somatic hypermutations within Ig genes. The same mutation machinery affects off-target genes (23, 26, 27) where mutations occur at a lower frequency (27). Although the classical idea was that most IgM+ memory B cells are produced in a GC-independent fashion (11, 29, 30), we found that all anti-D–specific CD27 +IgM+ and CD27 +IgG+ B cells harbored mutations in their Ig genes, whereas in non–anti-D–producing cells, mutations were found only in the CD27 + B cell subsets. Additionally, several anti-D CD27 +IgM+ and CD27 +IgM+ clones contained mutations in BCL6. Somatic mutations in IgM+ cells have been found before (25, 31–33) in CD27 +IgM+ B cells (25) as well as in CD27 +IgG+ B cells (34). However, to our knowledge we are the first to describe these mutations in TD Ag-specific CD27 +IgM+ B cells. The anti-D–specific IgM+ populations (both CD27 + and CD27 −) showed fewer mutations in

<table>
<thead>
<tr>
<th>Donor</th>
<th>B Cell Subset</th>
<th>PCR Efficiency</th>
<th>One Allele/Two Alleles Amplified</th>
<th>Mutated Cells (%)</th>
<th>No. Mutations for Each Sequence</th>
<th>Mutation Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>CD27 +IgM+</td>
<td>8/8</td>
<td>0/8</td>
<td>3 (37.5)</td>
<td>1 × 2, 1 × 1, 1 × 1</td>
<td>0.0384</td>
</tr>
<tr>
<td></td>
<td>CD27 +IgG+</td>
<td>13/14</td>
<td>0/13</td>
<td>2 (15.3)</td>
<td>1 × 1, 1 × 1</td>
<td>0.0118</td>
</tr>
<tr>
<td></td>
<td>CD27 +IgG+</td>
<td>11/15</td>
<td>1/10</td>
<td>4 (36.4)</td>
<td>1 × 1, 1 × 1, 1 × 1, 1 × 1</td>
<td>0.0293</td>
</tr>
<tr>
<td>Controls</td>
<td>CD27 +IgM+</td>
<td>8/8</td>
<td>0/8</td>
<td>2 (25.0)</td>
<td>1 × 1, 1 × 2</td>
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<td>CD27 +IgM+</td>
<td>8/8</td>
<td>0/8</td>
<td>1 (12.5)</td>
<td>1 × 3</td>
<td>0.028846</td>
</tr>
</tbody>
</table>

*Numbers of positive PCRs versus single clones analyzed.

*Number of PCR products containing one allele could be confirmed in one case only (one sequence of the anti-D hyperimmunized donor harbored a mutation that made clear that only one allele was amplified). For all the other unmutated PCR products, we assumed that both alleles were amplified.

*The derived percentage of mutations in a total of 650 bp of the Bcl6-MMC considered from each sequence as described by Seifert and Kükpers (25).
**FIGURE 6.** Analysis of anti-D–specific B cell IGVH and IGVL sequences. The schematic diagram illustrates the clonal relationship in the H (A) and L (B) chains of anti-D–specific B cells isolated from the RhD4 hyperimmunized donor. Each small vertical stick represents a point mutation, and each horizontal line indicates a unique B cell sequence, whereas the clonally related B cells and shared mutations are grouped in different colors. Each cell ID and the B cell subset are given. Additionally, the V(D)J region usage and the CDR3 amino acidic length are also displayed to point out the closeness of the different B cell clones. VH and VK/VL genes were sequenced for all 36 cells, except for four VH sequences and one VK/VL sequence that could not be amplified. VH gene usage was in 15 cases restricted to use VH4–34, with 13 residing in both IgM-expressing B cell subsets and of which 11 showed clonal relatedness. These 11 sequences also restrictedly used the same VL1–39 gene. Two B cell clones were found to be clonally related by their VL3–19 gene sequence only, because in one clone the Ig VH sequence was not amplified.
and Lanzavecchia (45) identified a minor population of CD27detected after a prolonged time from the last booster. Also, Wirths effector cells (IgM +IgD+) or IgM-only cells (35)
et al. (46) described CD27FcRH4, but these CD27 cells undergo less affinity maturation. Because we did not stain for responding to T cell–independent Ags (36–38) and developing inresent the first line of defense against bacterial infections and blood-borne Ags and pathogens. For this reason they might rep-
in a specialized area of the spleen where they have unique access to these memory cells are thought to be marginal zone B cells residing(47). CD27 tonsils and were found to be absent from PB (data not shown)
= 15) subsets derived from the (8, 41, 42). We now definitively show that IgM+ B cells are of activation markers, and replication history, although limited in healthy control donors with a very low TT titer. Although most
in the same or a different number of somatic point mutations as indicated by the numbers within the circle. Colors match the scheme used in Fig. 6.

another general assumption is that memory B cells express CD27 (1). In all donors we found Ag-specific B cells in the CD27+IgM+ population, and in three of seven hyperimmunized donors this population even represented most Ag-specific B cells. We found these cells years after the last booster and in individuals without a measurable anti-TT titer. This suggests that CD27 expression is not expressed on all IgM+ memory B cells, Dogan et al. (8) observed that peanut agglutinin, the classical marker for GC B cells in mice, also did not stain most Ag-specific IgM+ B cells detected after a prolonged time from the last booster. Also, Wirths and Lanzavecchia (45) identified a minor population of CD27–PB B cells that behaved similar to memory cells and were identi-
tified based on their inability to extrude rhodamine. However, this CD27– memory population consisted mainly of IgG+ and IgA+ cells, supporting the findings of another group that showed the presence of PB memory CD27+ IgG+ (14, 35). Ehrhardt et al. (46) described CD27– memory B cells in tonsils expressing FcRH4, but these CD27– memory cells seem to be confined to the tonsils and were found to be absent from PB (data not shown) (47). CD27– memory B cells have been described in patients with chronic immune activation such as systemic lupus erythe-
ma-tosus (48) and HIV-1 (49). Because hyperimmunization might cause chronic immune activation, we analyzed TT-specific B cells in healthy control donors with a very low TT titer. Although most
TT-specific B cells were found in the CD27+IgM+ subset in steady-state, a considerable percentage of the TT-specific B cells were CD27–IgM+ in all four donors, and in two donors the frequency of TT-specific CD27+ IgG+ B cells exceeded the frequency of TT-specific CD27+IgM+ B cells. Therefore, we conclude that the presence of CD27– IgM+ and CD27+IgM+ Ag-specific cells is not linked to chronic immune activation, although the loss of CD27 might be more pronounced after repeated immunizations, as CD27 can be shed from activated B and T lymphocytes (50). Alterna-
tively, the lack of CD27 might represent the inability to gain sustained support from CD70-expressing activated T cells to com-
plete the GC reaction in an early CD40L-mediated TD manner. This may thereby explain why CD27– B cells harbor a lower rate of mutations than do their CD27+ counterpart (51–54).

Because upon booster immunizations mainly IgG is produced and Ag-specific IgM is predominately seen only during primary immune response, we applied our culture system also after recent booster. Indeed, a 25-fold rise in frequency of TT-specific CD27+ IgG+ cells and a 2.5-fold decrease in TT-specific CD27+ IgM+ cells were observed 14 d after immunization. Probably CD27+ IgG+ B cells do not really recirculate in the PB but preferentially reside in clusters near the contracted GCs from where they can rapidly proliferate upon secondary challenge (55).

Although the role of IgM+ memory cells in a secondary re-
response remains unclear, two studies described possible roles for IgM+ B cells in mice (8, 9). Dogan et al. (8) suggested that there is a memory compartment of long-lived and mutated IgM+ cells, which can reinitiate a GC round upon further antigenic challenge and partly switch to replenish the IgG+ memory pool. In their model, the IgG+ cells have an immediate effector and protective function by rapidly becoming IgG-secreting plasmocytes but display little ability to re-enter the GC. Pape et al. (9) also pro-
posed a role for the Ag-specific Ig. IgG+ B cells were activated in the presence of high-affinity neutralizing serum whereas IgM+ memory B cells poorly respond until the IgG titers wane. They observed that the number of IgM+ memory B cells remained stable, up to 500 d after priming, whereas the IgG+ B cells de-
clined with a half-life of ~50 d (9). This discrepancy between the two murine studies may be related to the persistence of GC reac-
tions in the response to particulate Ags: SRBCs in the study of Dogan et al. (8), and soluble Ags in the study of Pape et al. (9, 56).

It is relevant that we obtained similar results using a soluble Ag (TT) and a particulate Ag (D+ RBCs), and that the kinetics of the
memory subsets also resembled the observations of Pape et al. More recently, another group studied the response to a TD Ag infection in mice and defined a subset of IgM memory B cells expressing CD11c, CD73, and PD-L2 (57). The absence of this IgM memory subset was found to abrogate the onset of IgG recall responses following a specific Ag challenge, thereby (again) supporting the idea that IgM memory plays an important role in maintaining long-term immunity.

Our findings in humans give support to the above-mentioned results obtained in mice (8, 9, 57), suggesting that IgM memory represents a pool of long-lasting memory cells continuously replenishing the IgG memory pool after repeated exposure. This may be beneficial upon second encounter with a mutated form of the original pathogen, triggering their ability to re-enter the GC, switch, and acquire additional mutations, resulting in higher affinity to the mutated Ag, as also suggested in an elegant murine study describing GC-independent unmutated IgG+ memory B cells (58), which, similar to the IgM memory B cells characterized in this study, are implied to confer a great flexibility to the immune system by retaining germline sequences. Meanwhile, IgG memory B cells originating from the first encounter mainly form a rapid burst of plasma cells that boost the level of serum Ab, but with little capacity of re-initiating a GC. Our findings on the clonal relationship between IgG and BCL6 mutations among the d-specific B cells isolated from all B cell subsets support this model of multiple layers of memory responses. Because anti-d–specific IgM IgG memory populations (both CD27+ and CD27−) harbored fewer mutations compared with their CD27+ IgG counterparts, this suggests that they underwent fewer rounds of affinity maturation.

In conclusion, our findings show that both in hyperimmunized donors and control donors who received booster vaccinations in the distant past, most RhD- and TT Ag–specific B cells circulating in PB reside in the IgM-expressing B cell subset. These IgM+ B cells seem to provide a long-lasting memory. Their lower level of mutations make these cells more flexible in responding to variants of the same pathogen compared with IgG+ B cells carrying a highly specific BCR (59).

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Representative CD27^−IgM^+, CD27^+IgM^+ and CD27^+IgG^+ B cells sorting. A) Gating strategy for the flow cytometric isolation of CD27^− and CD27^+ PB B cells. B) Dot plots indicate the sizes of CD27^−IgM^+ B cells and C) CD27^+IgM^+ and CD27^+IgG^+ B-cell subsets sorted for the limiting dilution studies. Gates show percentages of CD27^−IgM^+ (B), CD27^+IgM^+ and CD27^+IgG^+ B cells (C).
Supplemental Figure 2: Gating strategy for anti-D-specific PB B cells. R$_2$R$_2$ RhD$^+$ red blood cells (RBCs) were stained with either red-fluorescent PKH26 or green-fluorescent CFSE membrane dyes and then incubated with PB B cells. Gates show percentages of double positive dots representing anti-D-specific CD27$^-$IgM$^+$ (A), CD27$^+$IgM$^+$ (B) and CD27$^+$IgG$^+$ (C) B cells isolated from the hyperimmunized RhD-4 anti-D donor.
Supplemental Table 1: **Reverse primer sequences for nested Ig-PCR on RACE products and Forward (F), Reverse (R) and extra reverse (S) primer sequences for nested BCL6-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>R1-Cγ1-4</td>
<td>5’-TGTCCACCTTGGTTGCTGGGCT-3’</td>
</tr>
<tr>
<td>R1-Cμ</td>
<td>5’-CACTGGAAGAGGCACGTGTTTTCT-3’</td>
</tr>
<tr>
<td>R1-Cκ</td>
<td>5’-ACACTCTCCCCTGTTGAAGCTCTTTG-3’</td>
</tr>
<tr>
<td>R1-Cλ</td>
<td>5’-TGAACATTCTGTAGGGGCCACTGTCTT-3’</td>
</tr>
<tr>
<td>R2-Cγ1-4</td>
<td>5’-TTCCGGGGAAGTAGTTCCTTGACCAG-3’</td>
</tr>
<tr>
<td>R2-Cμ</td>
<td>5’-GGAATTCTCAGGAGACGGAGG-3’</td>
</tr>
<tr>
<td>R2-Cκ</td>
<td>5’-CAGATTTCAACTGCTCATCAGATGGC-3’</td>
</tr>
<tr>
<td>R2-Cλ</td>
<td>5’-CACCAGTGTGGCCTTGTTGGCTTG-3’</td>
</tr>
<tr>
<td>BCL6-1F</td>
<td>5’-GCCGGACACCAGGTGATTATT-3’</td>
</tr>
<tr>
<td>BCL6-1R</td>
<td>5’-CTCTCATTAGGAAGATCACGGC-3’</td>
</tr>
<tr>
<td>BCL6-2F</td>
<td>5’-AATGCTTTGGCTCAAAGTTTTC-3’</td>
</tr>
<tr>
<td>BCL6-2R</td>
<td>5’-TAGACACGATACTTTCATCTCATCTG-3’</td>
</tr>
<tr>
<td>BCL6-S</td>
<td>5’-CAAGGAAAGCAGTTTGCAAGC-3’</td>
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