

CpG Drives Human Transitional B Cells to Terminal Differentiation and Production of Natural Antibodies

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The receptor TLR9, recognizing unmethylated bacterial DNA (CpG), is expressed by B cells and plays a role in the maintenance of serological memory. Little is known about the response of B cells stimulated with CpG alone, without additional cytokines. In this study, we show for the first time the phenotypic modification, changes in gene expression, and functional events downstream to TLR9 stimulation in human B cell subsets. In addition, we demonstrate that upon CpG stimulation, IgM memory B cells differentiate into plasma cells producing IgM Abs directed against the capsular polysaccharides of *Streptococcus pneumoniae*. This novel finding proves that IgM memory is the B cell compartment responsible for the defense against encapsulated bacteria. We also show that cord blood transitional B cells, corresponding to new bone marrow emigrants, respond to CpG. Upon TLR9 engagement, they de novo express AID and Blimp-1, genes necessary for hypersomatic mutation, class-switch recombination, and plasma cell differentiation and produce Abs with anti-pneumococcal specificity. Transitional B cells, isolated from cord blood, have not been exposed to pneumococcus in vivo. In addition, it is known that Ag binding through the BCR causes apoptotic cell death at this stage of development. Therefore, the ability of transitional B cells to sense bacterial DNA through TLR9 represents a tool to rapidly build up the repertoire of natural Abs necessary for our first-line defense at birth. *The Journal of Immunology*, 2008, 180: 800–808.

The function of B cells is to secrete Abs. Serum Igs include low- and high-affinity Abs of different isotypes, either produced without intentional immunization (natural Abs) or resulting from antigenic challenge (adaptive Abs). Natural Abs are mostly of IgM isotype and, thanks to the combination of low-affinity and high-avidity, represent an efficient first-line defense against invading pathogens (1). Adaptive Abs, instead, are the final products of the complex response to Ag occurring in the germinal centers, through somatic mutations, affinity selection, and class-switch recombination. They specifically and exclusively recognize their target pathogen and have the function to clear the infection and to prevent its recurrence. Serum Abs represent our serological memory. A constant level of Abs is indispensable to ensure protection against infections. It has been proposed that serological memory is maintained by two independent mechanisms: specific and aspecific recall (2). Antigenic stimulation through recurrent infections or booster immunization is responsible for the specific recall and results in the increase of Abs with a single specificity. Continuous stimulation of TLR9 by small concentration of bacterial DNA is thought to be one of the mechanisms of aspecific

recall, inducing the production of Abs of all isotypes and all specificities. TLRs were first identified in *Drosophila*, where they play a determinant role in embryogenesis and host defense against invading fungal and bacterial pathogens (3). In mammals there is no evidence that TLRs are involved in body organization, but they are extremely important for the recognition of pathogen-associated molecular patterns, such as components of bacterial outer membrane (4, 5). Activation of TLRs initiates an intracellular signaling cascade resulting in the nuclear translocation of NF- κ B transcription factor, known to induce the production of a variety of inflammatory mediators and cytokines and in the phagocytosis of the invading pathogen (6). TLRs are expressed by the cells of the innate immune system and are indispensable for pathogen recognition and clearance. TLRs, however, also play an important role in the adaptive immunity: they mediate the maturation of dendritic cells (DC)² and are also expressed by T and B cells (7). Altered TLRs expression or function may play a role in human disease, affecting the susceptibility to infection or sepsis (8, 9).

TLR9 recognizes CpG motif in unmethylated bacterial DNA (10), although another ligand was recently suggested for this receptor (11). In humans, expression of TLR9 is restricted to B cells (12) and plasmacytoid DC (13). Both in vivo and in vitro CpG oligodeoxynucleotides can be used to stimulate TLR9 (14). CpG has been demonstrated to stimulate human B cells in several experimental conditions, that were based on the use of different combinations of ILs (IL-2, IL-10, IL-15) (15, 16), vitamins (vitamin A) (17), growth factors (BAFF) (16), ligands (CD40L) (16, 17), or anti-Ig in the presence of plasmacytoid DCs (18). Also, mouse B

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² Abbreviations used in this paper: DC, dendritic cell; PnPS, pneumococcal polysaccharide; AID, activation-induced cytidine deaminase; Blimp, B lymphocyte-induced maturation protein.

cells, especially marginal zone B cells, express activation markers and produce Abs in response to CpG (19).

In the human peripheral blood, about half of total B cells, expressing the memory marker CD27 and carrying somatic mutations, are thought to be memory B cells (20, 21). It is assumed that memory B cells are the final product of germinal center reactions, produce high-affinity Abs, and have the function to protect from reinfection. It has been, however, demonstrated that a subset of memory B cells, the IgM memory, can be detected in the absence of germinal centers, require the spleen for their generation, and function and produce natural Abs, including those against *Streptococcus pneumoniae*. This lead to the hypothesis that IgM memory B cells may represent a separate lineage of development, specialized for the first-line protection against infections (22–25).

We have shown that the appearance of IgM memory B cells is developmentally regulated: they are absent in the cord blood and become detectable in the peripheral blood during the first year of life. In infants the absence of IgM memory B cells is associated to the lack of anticapsular pneumococcal polysaccharides (anti-PnPS) IgM in the serum, inability to mount a protective Ab response to polysaccharide vaccines, and increased susceptibility to *S. pneumoniae* infection (22, 26).

It has been shown that human memory B cells proliferate and produce Abs in response to CpG in vitro (27). In this study, we demonstrate that IgM memory B cells produce Abs directed against *S. pneumoniae* upon TLR9 engagement, thereby explaining the correlation between IgM memory B cells and protection against pneumococcal infection (22, 23, 26) at the molecular and cellular level.

We also demonstrate that transitional B cells, corresponding to the most immature B cell type in the peripheral blood (23), respond to TLR9 stimulation, first acquiring the phenotype of IgM memory B cells and then terminally differentiating into plasma cells that produce antibacterial Abs.

Stimulation of innate receptor, therefore, may regulate the final differentiation and function of human transitional B and ensure the first-line protection against infection.

Materials and Methods

Cell isolation

Blood samples were collected from the peripheral vein of adult volunteers and from placental cord of normal, full-term neonates from uncomplicated pregnancies following normal vaginal delivery or caesarian section. Informed consensus was obtained from adult donors. Blood from healthy donors and asplenic patient was obtained from the Bambino Gesù Children Hospital (Rome, Italy) and placental cord blood from the Casa di Cura Santa Famiglia (Rome, Italy). Studies have been reviewed and approved by the ethics committee of these hospitals. Human PBMCs were isolated by Ficoll-Paque Plus (Amersham Biosciences) density-gradient centrifugation.

B cells were purified by negative selection with anti-CD2, anti-CD3, anti-CD14 mAbs (BD Biosciences), using Dynabeads M-450 goat anti-Mouse IgG (DynaL Biotech). To identify B cell subsets, cells were stained with the appropriate combinations of fluorochrome-conjugated Abs to CD10, CD22, CD24, CD27, CD38, CD138 (BD Biosciences), IgA, IgG, IgM (Jackson ImmunoResearch Laboratories) and then separated by cell sorting (FACS Vantage; BD Biosciences). All analysis were performed on a FACSCalibur (BD Biosciences) interfaced to a Macintosh CellQuest computer program. Gated events (50,000) on living cells were analyzed for each sample.

Proliferation assay

Sorted cells were labeled with at the final concentration of 0.1 μ g/ml CMFDA (5-chloromethylfluorescein diacetate, CellTracker; Molecular Probes) and cultured at $2\text{--}3 \times 10^5$ cells per well in 96-well plates in complete RPMI 1640 (InvivoGen) supplemented with 10% FBS (HyClone Laboratories). Human CpG oligodeoxynucleotides (Hycult Biotechnology) was used at the optimal concentration of 2.5 μ g/ml. Control GpC (Invivo-

Gen) was used at the same concentration. Cell proliferation was measured on day 5 by FACSCalibur flow cytometer (BD Biosciences).

Immunofluorescence analysis

Cells were spotted on poly-L-lysine-coated glass slides and fixed at room temperature for 10 min in 2% paraformaldehyde. Cells were permeabilized for 10 min in 0.1% Triton X-100 and blocked for 1 h in PBS with 0.5% BSA. After three washes in PBS, cells were incubated 1 h at with anti-IgM FITC at 1/10 dilution (BD Biosciences). After incubation the cells were washed five times with PBS. To stain DNA, Hoechst dye (Sigma-Aldrich) was added for the last 2 min at a final concentration of 10 μ g/ml. Cells were washed extensively in PBS and slides were mounted in 50% glycerol in PBS.

ELISA immunoassays

Secreted Igs were detected at day 7 by ELISA. Briefly, 96-well plates (Corning) were coated overnight with purified goat anti-human IgA plus IgG plus IgM (Jackson ImmunoResearch Laboratories). After washing with PBS/0.05% Tween and blocking with PBS/gelatin 1%, plates were incubated for 1 h with the supernatants of the cultured cells. After washing, plates were incubated for 1 h with peroxidase-conjugated goat anti-human either IgA or IgG or IgM Abs (Jackson ImmunoResearch Laboratories). The assay was developed with *o*-phenylenediamine tablets (Sigma-Aldrich) as a chromogenic substrate.

ELISA for the quantization of the serotype-specific anti-PnPS IgM, as previously described (26), was performed according to the protocol established by the Vaccines, Immunization, and Biologicals Department of the World Health Organization (Geneva, Switzerland).

Real-time PCR and TaqMan analysis

Total RNA was extracted using RNeasy Mini kit (Qiagen). cDNA was synthesized with High Capacity cDNA Archive kit (Applied Biosystems). Quantitative real-time PCR was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems) and TaqMan reagents (Applied Biosystems), according to the manufacturer's instructions. The sequences of probe and primers used for TLR9 were (probe) ACGATGCCTTCGTG GTCTTCGACAAA, (forward) GGACCTCTGGTACTGCTTCCA, (reverse) AAGCTCGTTGTACACCCAGTCT. Probes and primers for TLR9 were purchased from Applied Biosystems. For quantitation of TLR9 expression, GAPDH was used as endogenous control. Relative quantitation data were performed using the comparative method. Results are expressed relative to PBMC with the threshold cycle $\Delta\Delta$ Ct method as described by the manufacturer's instructions.

Real-time PCR analysis for activation-induced cytidine deaminase (AID) and B lymphocyte-induced maturation protein 1 (Blimp-1) in 7-day cultured cells was performed running triplicates of equal amount of serially diluted cDNA (dilution 1/3) for each sample. Quantitative data were obtained normalizing the threshold cycle value of the target genes in the CD27^{bright} plasma cells population to the threshold cycle value of the CD27⁺ pool (1 = lowest dilution).

Real-time PCR analysis for AID and Blimp-1 in 48-h cultured cells was performed with iCycler iQ detection system (Bio-Rad). TaqMan Gene Expression assays for *AICDA* (AID) (Assay ID Hs00221068_m1) and for *PRDM1* (Blimp-1) (Assay ID Hs00153357_m1) were purchased from Applied Biosystems. *AICDA* or *PRDM1* gene expression levels were quantified using β -actin as endogenous control. β -actin primers and probe were purchased from Applied Biosystems. Results are expressed relative to untreated cells using the $\Delta\Delta$ Ct comparative method.

Results

TLR9 stimulation generates plasma cells from memory B cells and prolongs the survival of mature-naïve B cells

The two largest populations of B cells in the peripheral blood, mature-naïve and memory B cells, can be identified by the expression of CD24 and CD27 (Fig. 1A). CD24⁺, CD27[−], IgM⁺, IgD⁺ mature-naïve B cells (Fig. 1A, ma) correspond to the circulating fraction of the B cells found in the lymphoid follicle, which together with T cells and DC, are the major actors of the adaptive immune response. In healthy adults 30–50% of the B cells are CD24^{bright}CD27⁺ memory B cells (Fig. 1A, me). This population includes IgM memory B cells, expressing IgM and IgD, and switched memory B cells, carrying Igs of other isotypes (Fig. 1A).

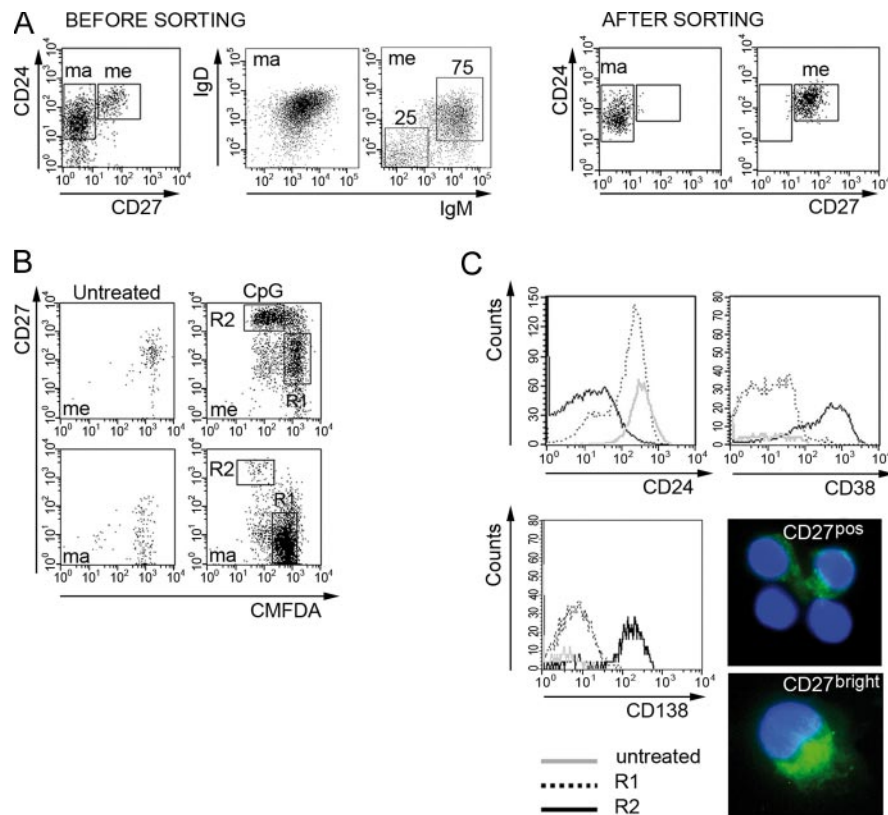


FIGURE 1. TLR9 drives memory B cells to terminal differentiation. *A*, Dot plots show PBMCs from a healthy donor depleted of $CD27^+$, $CD3^+$, and $CD14^+$ cells. Cells were stained before sorting with anti- $CD27$, anti- $CD24$, anti-IgM, and anti-IgD to identify B cell subsets. Mature-naive B cells (ma) gated in $CD24^+CD27^-$ cells are IgM^+ and IgD^+ . Memory B cells (me) are gated in $CD24^+CD27^+$ cells. The staining with anti-IgM and anti-IgD identifies switched memory B cells as IgM^+ and IgD^- and IgM memory B cells as IgM^+ and IgD^{dull} . Number at gated region indicates the percentage of switched memory (25%) and IgM memory (75%) in me. For sorting we used a strategy that left the BCR untouched. Mature-naive B cells (ma) were identified as $CD24^+CD27^-$ and memory B cells (me) as $CD24^+CD27^+$. Purity of the sorted populations was 99%. *B*, Proliferation assay. Sorted memory (*top*) and mature-naive (*bottom*) B cells stained with CMFDA were cultured for 5 days with or without CpG. Region 2 (R2) includes $CD27^{bright}$ proliferating cells and Region 1 (R1) $CD27^+$ nonproliferating cells in stimulated cells. Data shown are representative of seven independent experiments. *C*, Histogram plots show the expression of $CD24$, $CD38$ (*top*), and $CD138$ (*bottom*) in untreated and CpG-stimulated memory B cells. Untreated cells (gray line histogram), CpG-stimulated cells (dotted line histogram) gated in R1, and stimulated cells (black line histogram) contained in R2 are represented. By immunofluorescence we show sorted $CD27^+$ (R1) and $CD27^{bright}$ (R2) cells. IgM is shown in green and the DNA staining (Hoechst dye) in blue. All images were acquired at a magnification $\times 100$.

To study the functional effect of TLR9 signaling, we cultured sorted mature-naive and memory B cells with CpG. Cells were labeled with the CellTracker reagent CMFDA and analyzed after 5 days. At this time most memory B cells in the control wells had undergone apoptosis (data not shown) and few had survived (Fig. 1*B*, untreated). In contrast, in the cultures containing CpG, 50% of the memory B cells up-regulated $CD27$ expression and proliferated (Fig. 1*B*, in R2). In seven independent experiments with sorted memory B cells from different donors, we found that the frequency of $CD27^{bright}$ cells after CpG was 42.9% with SD 8.9%. The rest of the memory B cells showed a prolonged survival (Fig. 1*B*, in R1). In mature-naive B cells the stimulation of TLR9 also improved survival, with a 10-fold higher cell recovery after culture with CpG. Only 3% of mature-naive B cells proliferated (Fig. 1*B*, in R2). Experiment performed in seven different donors confirmed the low frequency of proliferating cells (mean 2.16%, SD 1.3%). These results indicate that CpG alone directly stimulates both memory and mature-naive B cells. The response, however, is different in the two populations and is not identical for each cell in a given subset: half of the memory pool proliferated ($CD27^{bright}$) and the other half was rescued from death ($CD27^+$). In the mature-naive subset the exposure to CpG prolonged the lifespan of all cells and induced proliferation in a minute fraction. Control GpC

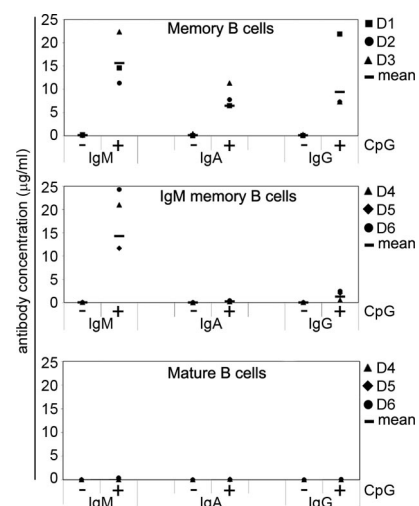
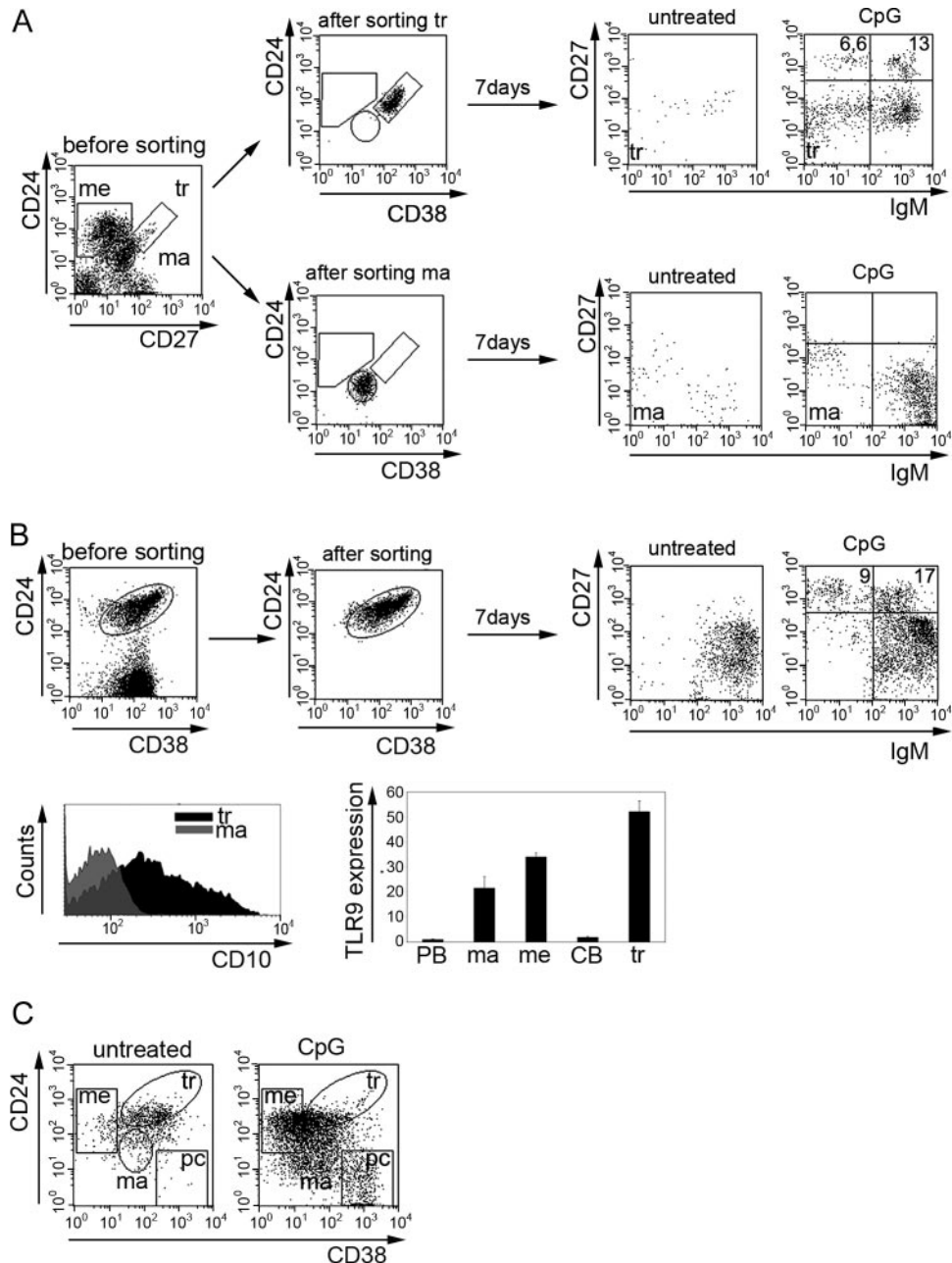


FIGURE 2. Abs secretion in CpG stimulated B cell subsets. ELISA shows histogram for IgM, IgA, and IgG Ab concentrations in the supernatants of 7 days cultures with or without CpG, containing the indicated subpopulations. Symbols represent the response of memory B cells of three donors (numbered as D1, D2, and D3) and IgM memory and mature B cells of three different donors (D4, D5, and D6). Black bar represents the average cell numbers.

FIGURE 3. TLR9 stimulation promotes the differentiation of transitional B cells. **A**, The dot plot on the left shows the CD24 and CD38 staining of PBMCs from adult peripheral blood. Memory B cells (me), mature-naïve B cells (ma), and transitional B cells (tr) are indicated. In the sorted panels, mature-naïve B cells and transitional B cells are shown. Plots on the right demonstrate results after 7 days of culture with CpG-sorted populations stained with anti-CD27 and anti-IgM Abs. The number indicates the percentage of IgM⁺ and IgM⁻ CD27^{bright} plasma cells in this representative donor. **B**, Cord blood cells stained with anti-CD24 and anti-CD38 Abs are shown before and after sorting (top left). Comparison of the expression of CD10 in transitional B cells (filled histogram) and mature B cells (gray-filled histogram) is shown (bottom left). TLR9 expression relative to PBMCs in different cell populations is shown (bottom right), using GAPDH as endogenous control. PBMCs from adult donors (PB), sorted mature-naïve (ma) and IgM memory B cells (me), cord blood PBMCs (CB), and transitional B cells sorted from cord blood are presented. Data represent the mean $\pm$ SD ($n = 3$). The dot plot (top right) shows the expression of CD27 and IgM of cord blood transitional B cells after 7 days in culture with or without CpG. The number indicates the percentages of CD27^{bright} plasma cells expressing either IgM or switched isotypes in this donor. Data shown are representative of three independent experiments. **C**, Expression of CD24 and CD38 in untreated and CpG-treated transitional B cells. Memory-like B cells, mature-naïve B cells, transitional B cells, and plasma cells (pc) are shown.



did not have any significant effect in memory and mature-naïve B cells (data not shown).

As the high expression of CD27 has been correlated with the commitment to the plasma cell lineage (28), we decided to analyze the expression of CD24, CD38, and CD138 in untreated or CpG-stimulated memory B cells (Fig. 1C). The high expression of CD24, typical of circulating memory B cells, remained stable in untreated cultures (Fig. 1C, gray line histogram), was lost in the proliferating CD27^{bright} population (Fig. 1C, black line histogram corresponding to the cells in R2), and decreased in the rest of the memory B cells exposed to CpG (dotted line histogram corresponding to the cells in R1). In contrast, CD38 was absent in R1 for cells both exposed and unexposed to CpG and was high in CD27^{bright} cells (R2). Moreover, CD27^{bright} cells (R2) specifically acquired the expression of the plasma cell marker CD138 (syndecan-1). We next sorted CD27⁺ cells and CD27^{bright} cells from stimulated memory to check intracellular Igs and morphology by immunofluorescence (Fig. 1C). CD27^{bright} cells were larger and

showed high amounts of intracellular IgM compared with CD27⁺ cells. According to the presence of switched memory B cells in the memory pool, some of the CD27⁺ cells were IgM⁻. Thus, CD27^{bright}CD138⁺, derived from memory B cells after TLR9 stimulation, acquired the phenotype of plasma cells, whereas CD27⁺CD138⁻ cells retained the phenotype and morphology of memory B cells. To assess the function of plasma cells generated through TLR9 stimulation, we measured Ab secretion in the culture supernatants (Fig. 2). After 7 days in culture, memory B cells produced IgM, IgG, and IgA. Sorted IgM memory B cells consistently produced IgM and small amounts of IgG. IgM was almost undetectable in the supernatant of mature-naïve B cells.

TLR9 stimulation induces the differentiation of transitional B cells

As mentioned, most of the B cells in the peripheral blood belong to the mature-naïve and memory pools. In the adult, however,

1–2% of peripheral B cells are transitional B cells, recently generated in the bone marrow (23). Transitional B cells cannot be distinguished from mature-naïve B cells using the CD24 and CD27 markers. Transitional B cells can be identified by adding the CD38 marker. Transitional B cells are CD24^{bright}CD38^{bright}CD27[−], mature-naïve B cells are CD24⁺CD38⁺CD27[−], and memory B cells can be identified by the lack of CD38 and the expression of CD24 and CD27 (CD24^{bright}CD38[−]CD27⁺) (23). We sorted transitional B cells and a pure mature-naïve population from the peripheral blood of the same healthy donor (Fig. 3A). Both populations co-expressed IgM and IgD and lacked switched isotypes (data not shown). After 7 days in culture with CpG, mature-naïve B cells, depleted of transitional B cells, did not proliferate (data not shown) and did not generate plasma cells (Fig. 3A, ma). In contrast, upon CpG stimulation, 19.6% of the transitional B cells differentiated into CD27^{bright} plasma cells, expressing not only IgM (13%) but also other isotypes (6.6%) (Fig. 3A, tr). The presence of switched isotypes was unexpected because all transitional B cells co-expressed IgM and IgD at the beginning of the culture. This experiment was reproduced with sorted transitional B cells from two additional donors. The frequency of plasma cells was 27.1% (IgM⁺ (16%), IgM[−] (11.1%)) and 20% (IgM⁺ (13%), IgM[−] (7%)) in these cases.

To confirm these data, we used cord blood as a richer source of transitional B cells (29, 30). In this tissue all B cells have the CD24^{bright}CD38^{bright} transitional phenotype and the majority of them expressed CD10 (Fig. 3B). To determine whether TLR9 is expressed at this stage of B cell development we conducted real-time PCR on isolated B cell subsets. Transitional B cells expressed higher levels of TLR9 compared with IgM memory and mature B cells (Fig. 3B). Upon TLR9 stimulation, similarly to adult transitional B cells, 26% of cord blood transitional B cells proliferated (data not shown) and differentiated into CD27^{bright} plasma cells ($25.5 \pm 2.3\%$, with three different donors, $n = 3$) expressing IgM ($19.2 \pm 2.9\%$, $n = 3$) or switched isotypes ($6.3 \pm 2.3\%$, $n = 3$) (Fig. 3B). The relative expression of CD24 and CD38 demonstrated that the phenotype of most transitional B cells had changed after CpG stimulation: a large part ($41.3 \pm 4.2\%$, $n = 3$) of the cells acquired the phenotype of memory B cells CD24^{bright}CD38[−]CD27⁺ (Fig. 3, B and C, me). A small population of transitional B cells ($21.9 \pm 5.2\%$, $n = 3$) became phenotypically identical with mature-naïve B cells (Fig. 3C, ma). Similarly to those generated from adult memory B cells, cord blood-derived plasma cells expressed high levels of CD38 and lacked CD24 (Fig. 3C, pc). IgM⁺ and IgM[−] plasma cells developed from cord blood only by day 7–9 of CpG stimulation, whereas IgM memory-like CD27⁺ B cells were detectable by day 5 (data not shown).

Cord blood-derived plasma cells were functional. In the supernatants of CpG-stimulated cultures we detected both IgM ($15.2 \pm 5 \mu\text{g/ml}$) and IgG ($2.5 \pm 1.7 \mu\text{g/ml}$) Abs, but never IgA (Fig. 4A).

IgM memory and transitional B cells produce anti-polysaccharide IgM Abs

We have recently shown a correlation between the presence of IgM memory B cells and the protection from lower respiratory tract infections caused by encapsulated bacteria (22, 26). To provide the direct evidence of the functional role of IgM memory B cells against *S. pneumoniae*, we measured the levels of anti-PnPS serotype 14 IgM Abs in CpG-stimulated cultures of sorted IgM memory cells. We choose PnPS serotype 14 because this type is a common in Europe and specific Abs can be detected in the serum of most healthy donors (26).

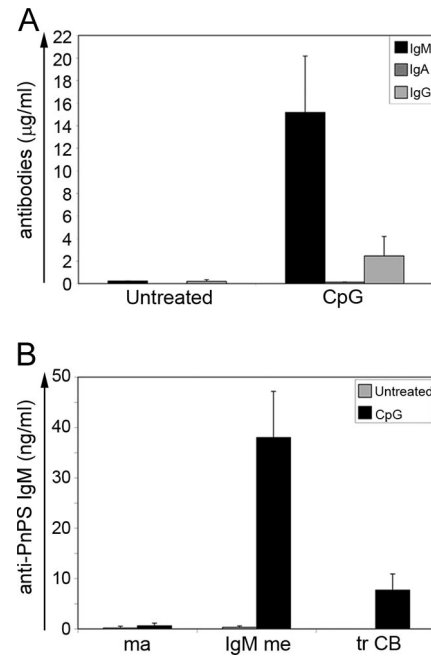


FIGURE 4. Cord blood-derived transitional B cells secrete Abs after TLR9 stimulation. **A**, ELISA shows the concentrations of IgM, IgA, and IgG Abs produced at day 7 of culture by untreated or CpG-stimulated cord blood transitional B cells. Data are the mean $\pm$ SD ($n = 3$). **B**, We show the concentrations of anti-PnPS Abs (serotype 14) produced at day 7 by mature-naïve B cells (ma), peripheral IgM memory B cells (IgM me), and cord blood transitional B cells (tr CB). Data are the mean $\pm$ SD ($n = 3$).

We found that 300,000 IgM memory B cells stimulated with CpG secreted $38 \pm 9 \text{ ng/ml}$ anti-PnPS IgM Abs. Mature-naïve B cells did not secrete anti-PnPS IgM Abs (Fig. 4B). Therefore, the repertoire of IgM memory B cells, isolated from adult peripheral blood contains the anti-PnPS 14 specificity. We calculated that anti-PnPS serotype 14 IgM produced after polyclonal CpG stimulation corresponds to $\sim 0.2\%$ of total IgM detected in the supernatant of IgM memory B cells.

Anti-PnPS IgM were also detectable, at a lower concentration ($7.7 \pm 3.2 \text{ ng/ml}$), in the supernatants of cord blood transitional B cells (Fig. 4B). In this case they were equivalent to 0.08% of total IgM produced by cord blood B cells. Thus, Abs with the same specificity are produced after CpG stimulation not only by naturally occurring IgM memory B cells, but also by transitional B cells.

TLR9 stimulation induces the expression of AID

In humans, anti-polysaccharide Abs generated through a thymus-independent mechanism carry somatic mutations (31). This paradox occurs because somatic hypermutation is thought to happen only in the germinal center in response to thymus-dependent Ags (32). Recently the identification of the AID has shed light on the mechanism of somatic hypermutation and class-switch recombination. AID has been demonstrated to be absolutely required for the induction of somatic hypermutation and class-switch recombination at the Ig loci (33, 34). Both these events are indispensable prerequisites for the production of high-affinity Abs of different isotypes.

The finding that anti-polysaccharide IgM and Abs of different isotypes were always detectable in the supernatants of IgM memory and transitional B cells lead us to hypothesize that the stimulation with CpG may induce AID expression.

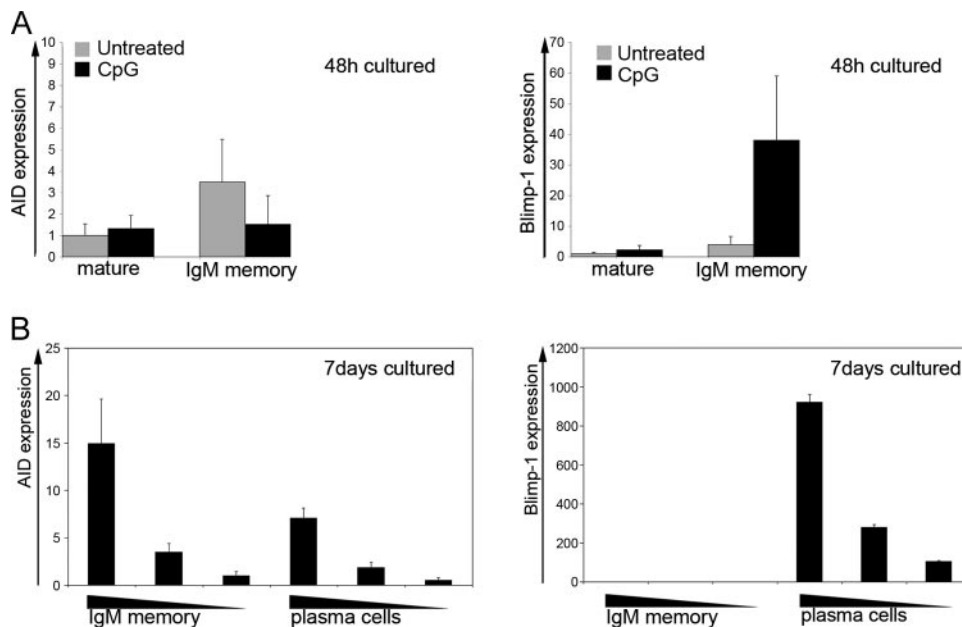


FIGURE 5. Differential gene regulation in the memory pool after CpG stimulation. *A*, AID and Blimp-1 expression was measured by real-time PCR. Sorted mature-naïve and IgM memory B cells were cultured for 48 h with or without CpG. Results are expressed relative to AID or Blimp-1 expression in untreated mature-naïve B cells; β -actin was used as endogenous control. Results are representative of three or more independent experiments. Data show mean $\pm$ SD from triplicate values. *B*, IgM memory B cells were stimulated for an additional 5 days with CpG to allow the differentiation into CD27^{bright} plasma cells and then sorted for the expression of CD27. AID and Blimp-1 mRNA levels were analyzed comparing CD27^{bright} plasma cells to CD27⁺ IgM memory B cells. Histograms show the expression levels for each gene in serially diluted samples (1/3 dilutions). Results are expressed relative to lowest dilution of memory B cells (lowest = 1). Results represent three or more independent experiments. Data show mean $\pm$ SD from triplicate values.

We extracted RNA from mature-naïve and memory B cells stimulated with CpG for 48 h, and we analyzed AID expression by real-time PCR. Results were normalized using β -actin as endogenous control and were plotted as relative values to AID expression in untreated mature-naïve B cells (Fig. 5*A*, left). We found that the basal level of AID mRNA was higher in memory B cells than in mature-naïve B cells. After stimulation with CpG, AID mRNA slightly increased in mature-naïve, but was down-regulated in memory B cells. To further explore the effect of TLR9 stimulation on B cell development, we analyzed the expression of Blimp-1, a transcription factor expressed in plasma cell-committed B lymphocytes, functioning as a master regulator of terminal B cell differentiation. After 48 h of treatment, we found a stronger in-

duction of Blimp-1 mRNA in CpG-treated memory compared with mature-naïve B cells (Fig. 5*A*, right).

After 5–7 days of culture with CpG, half of the memory pool differentiates into plasma cells and the other half maintains the memory phenotype (Fig. 1). These two populations are supposed to express inversely proportional levels of AID and Blimp-1 (35). We sorted, based on the expression of CD27, IgM memory B cells and plasma cells after 7 days of culture with CpG. Real-time PCR was performed on isolated CD27⁺ and CD27^{bright} cells (Fig. 5*B*, respectively, IgM memory and plasma cells). Because we were comparing a proliferating population (terminally developing into plasma cells) to a nonproliferating pool, we could not use any housekeeping gene as endogenous control (36–39). For this reason

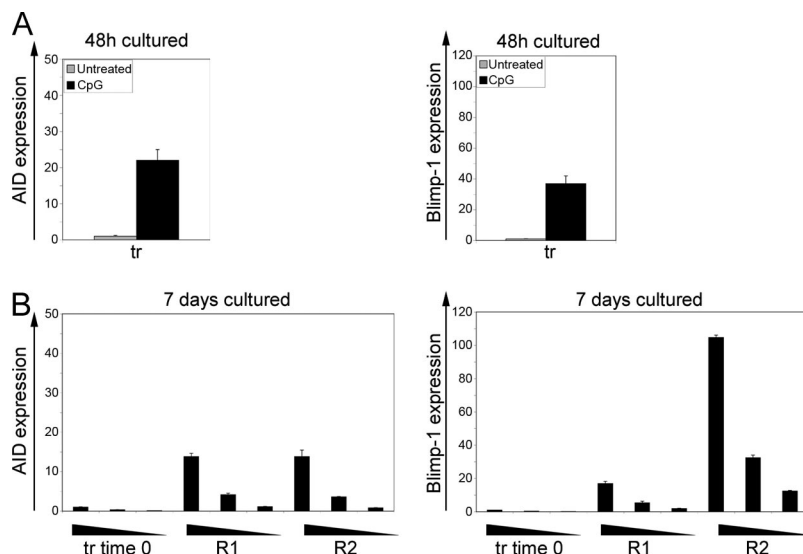
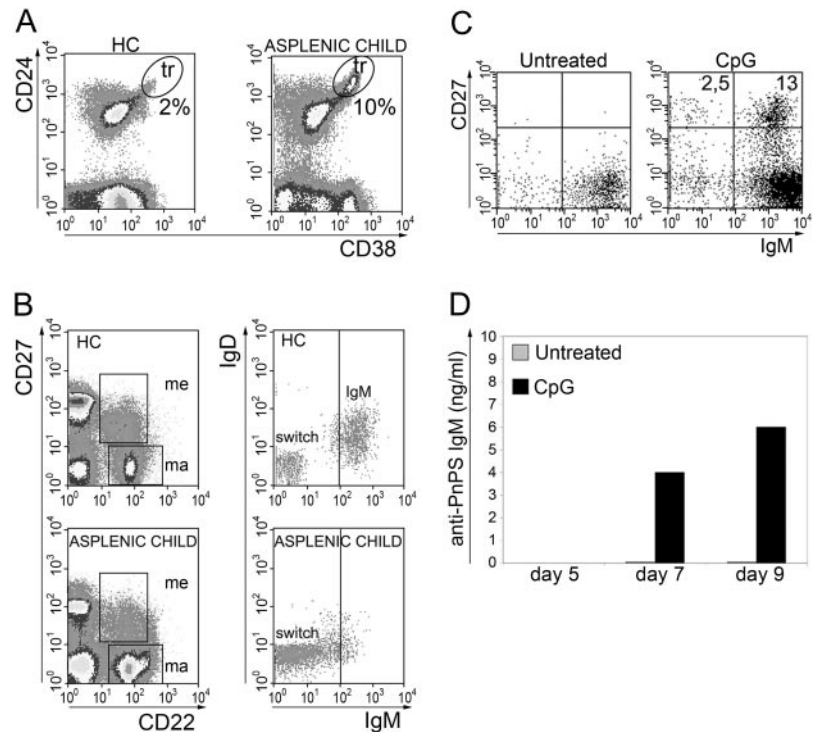


FIGURE 6. AID and Blimp-1 are induced upon TLR9 engagement in transitional B cells. AID and Blimp-1 expression in transitional B cells was measured by real-time PCR. *A*, Transitional B cells (tr) sorted from cord blood, were cultured for 48 h with or without CpG. *B*, After an additional 5 days in culture with CpG, transitional B cells were sorted based on the expression of CD27. AID and Blimp-1 mRNA levels were analyzed comparing CD27^{bright} cells (R2) and CD27⁺ cells (R1) to the transitional pool at time 0 (tr time 0). Results show the expression levels for each gene in serially diluted samples and were expressed relative to the transitional pool at time 0. Results represent three or more independent experiments. Data show mean $\pm$ SD from triplicate values.

FIGURE 7. Transitional B cells give rise to IgM plasma cells producing anti-PnPS IgM in the absence of the spleen. **A**, PBMCs isolated from a 7-year-old asplenic child and from an age-matched healthy control (HC) were analyzed for the expression of CD24 and CD38. Transitional B cells (tr) were identified as CD24^{bright}CD38^{bright} (oval gate). Percentage represents transitional B cells vs total B cells. **B**, PBMCs stained with anti-CD27 and anti-CD22 (left column). Mature-naïve (ma) and memory B cells (me) were gated, respectively, on CD22⁺CD27⁻ and CD22⁺CD27⁺ cells. Gated memory B cells stained for IgM and IgD (right column) are also shown. IgM memory B cells (IgM) are absent in the asplenic child, whereas switched memory compartment B cells (switch) are still present. **C**, Phenotypic analysis of sorted transitional B cells from a 2-mo-old asplenic infant after 9 days of culture with or without CpG. The number indicates the percentages of CD27^{bright} IgM⁻ and CD27^{bright} IgM⁺ plasma cells shown. **D**, ELISA presents anti-PnPS serotype 14 secretion in the supernatants cultured for 5, 7, and 9 days.



we run in parallel the same amount of total cDNA for each sorted populations (see *Materials and Methods*). We observed that AID expression was higher in IgM memory B cells than in plasma cells. On the contrary, Blimp-1 was exclusively expressed in plasma cells (Fig. 5B). Similar experiments were performed on transitional B cells. After 48 h of stimulation AID mRNA was induced in transitional B cells sorted from both adult peripheral blood (data not shown) and cord blood (Fig. 6A). De novo expression of Blimp-1 was detectable at this time (Fig. 6A), thus indicating that commitment to plasma cells terminal differentiation had initiated in culture. To distinguish the expression profile of CD27^{bright} (plasma cells) and CD27⁺ (IgM memory-like) cells, we sorted these two populations developed in vitro after 7 days of culture with CpG. AID mRNA was present in both populations, whereas Blimp-1 was dramatically increased only in the proliferating pool containing the plasma cells (Fig. 6B).

Transitional B cells of asplenic individuals produce anti-PnPS IgM

Asplenic individuals lack IgM memory B cells in the peripheral blood, but have a normal frequency of switched memory B cells (22). We show that upon TLR9 engagement transitional B cells develop into a cell type functionally equivalent to IgM memory B cells. Based on these observations, we questioned whether the spleen plays a role in regulating the TLR9-dependent differentiation of transitional B cells. We found that in the absence of the spleen, the frequency of circulating transitional B cells is higher than in healthy controls (Fig. 7A). This finding is very clear in childhood when the bone marrow output is higher, and less evident in the adult.

We stimulated with CpG transitional B cells isolated from a 2-mo-old asplenic child. After 7 days of culture, transitional B cells differentiated into CD27^{bright} plasma cells and produced anti-PnPS IgM (Fig. 7, C and D).

Discussion

Over 5 years ago, Bernasconi et al. (2) formulated the hypothesis that serological memory, representing the individual own antigenic

experience, may be preserved not only by antigenic recall, but also through aspecific recall. They demonstrated that the combination of CpG and IL-15 was sufficient to induce Ab secretion by memory B cells and suggested that this mechanism may contribute to maintain the diversity and concentration of serum Igs.

Our aim was to measure the intrinsic ability of B cells to sense and react to TLR signals, excluding the modulating and steering action of exogenous cytokines. Therefore, we performed our experiments on sorted B cell populations and never added soluble growth or differentiation factors. In this experimental setting, the measured effects depend on the response of B cells to the initial CpG signal. This response may include the production of cytokines, such as IL-10 and IL-6, generating and maintaining a B cell-intrinsic cytokine-loop.

In this study, we demonstrate that TLR9 stimulation not only induces the differentiation of plasma cells from memory B cells, but also has important effects at the mature-naïve and transitional stage. The stimulation with the CpG prolongs the lifespan of mature-naïve B cells in vitro, suggesting that the size of the human B cell compartment may be regulated by pathogen exposure. Another TLR ligand, LPS, has a similar function in the mouse (40). The addition of a second signal, derived by the BCR and/or IL-10, changes the response of mature B cells.

In humans, the development of transitional B cells strictly depends on the signaling function of the BCR. Genetic mutations of components of the BCR signaling machinery arrest development at the pro- or pre-B cell stage in the bone marrow and result in the ablation of all peripheral B cell pools. The comparison of human genetic diseases with the murine models often demonstrates a more permissive regulation of mouse B cell development (41).

Human transitional B cells are thought to be the precursors of mature-naïve B cells, which in turn differentiate into switched and IgM memory B cells. Switched memory B cells originate from the germinal center reaction, after somatic hypermutation, affinity selection, and class switching. This result has been definitely proven by the study of patients with primary immunodeficiency caused by genetic mutations of CD40L and CD40, AID, and uracil N-glycosylase (42, 43).

The origin of IgM memory B cells is less clear and less studied. It was originally assumed that they also derive from the germinal centers but they can be detected also in patients unable to built germinal centers (19). In addition, two other observations support the hypothesis that IgM memory B cells belong to a lineage of development different from switched memory B cells. First, IgM memory B cells are generated in the spleen during the first 2 years of life, but not in the lymph nodes (22). Secondly, they are responsible for the first-line protection against encapsulated bacterial infection, a task that cannot be fulfilled by switched memory B cells (22, 26).

In this study, we demonstrate that IgM memory-like B cells can be generated in vitro from transitional B cells after TLR9 stimulation. Transitional B cells, exposed to CpG, change their phenotype and function. After 5 days of culture about half of them become phenotypically identical with IgM memory B cells and differentiate into plasma cells secreting IgM and a small amount of IgG in the following 2–4 days (Fig. 4).

In the mouse model it has been recently demonstrated that TLR stimulation induces plasma cell differentiation, Ab production, and AID expression by splenic transitional B cells (44). The induction of AID (Fig. 6) by TLR9 triggering in human transitional B cells is a novel finding. The function of AID in somatic mutation may explain the production of IgM Abs with anti-polysaccharide specificity (32). In contrast, the role of AID in class-switch recombination may justify the production of IgG in our experimental system (Fig. 4). Abs of IgA or IgE isotypes were never found in cultures of IgM memory and transitional B cells. Whereas it has been reported before that CpG inhibits class switching to IgE (45), it was surprising to find no IgA. This observation could be explained either by the requirement of mucosal factors favoring switching to IgA or by the different origin of IgA plasma cells.

In our in vitro model, the stimulus driving the differentiation of transitional B cells is initiated by the interaction of TLR9 with its ligand CpG and, therefore, does not involve a defined Ag or a BCR of selected specificity. It is known that Ag encounter through the BCR causes the apoptotic cell death of transitional B cells, representing an important mechanism of tolerance, with the function of removing unwanted and dangerous specificities from the pool of more mature naive B cells (46–48). Moreover, cord blood transitional B cells are generated and circulate in a sterile environment because bacterial colonization occurs hours after birth (49). Thus, the Abs produced by transitional B cells are natural Abs, generated without intentional immunization (1). Natural Abs constitute an innate repertoire able to recognize the bacteria, which always colonize humans and can therefore be encountered immediately after birth. It remains to be demonstrated whether this repertoire comprises not only germline but also mutated Igs, as suggested by the fact that anti-PnPS IgM always contains somatic mutations (31, 32). The composition of the Ig repertoire may reflect the selective force of pathogens on the host ready-to-use pool of Abs during the co-evolution of host and infectious agents (50).

In vivo transitional B cells are collected in the spleen. This organ is absolutely required for the presence of IgM memory B cells. Patients with congenital asplenia lack IgM memory B cell pool (22), do not produce anti-PnPS IgM, and have a high risk of invasive pneumococcal infection (51). As transitional B cells from an asplenic infant produce anti-PnPS IgM upon TLR9 stimulation, they have the potential to give rise to IgM memory B cells and plasma cells. The absence of IgM memory B cells in asplenic individuals may be explained by the function of the spleen as unique site for their development. Here the slow blood flow and the filtering function of macrophages (52) may favor the encounter of transitional B cells with bacterial DNA and possibly other bac-

terial products initiating the further maturation to IgM memory and plasma cells. In the spleen IgM memory B cells are localized in the marginal zone, admixed with canonical memory B cells (53).

Our findings demonstrate a novel role for TLR9 in human B cells intertwining the innate and adaptive immunity and suggesting once more that the so-called IgM memory B cells are different from canonical memory B cells. We propose that they could be better defined as “natural memory,” taking into consideration their already demonstrated independence from the germinal centers, their production of natural Abs and the role of innate TLR9 in their generation, survival, differentiation, and function.

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Disclosures

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