T cell–independent development and induction of somatic hypermutation in human IgM$^+$IgD$^+$CD27$^+$ B cells

Ferenc A. Scheeren,[1] Maho Nagasawa,[1] Kees Weijer,[1,2] Tom Cupedo,[3] Jörg Kirberg,[4] Nicolas Legrand,[1] and Hergen Spits[1]

[1]Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, Netherlands
[2]Netherlands Cancer Institute–Antoni van Leeuwenhoek Ziekenhuis, 1066 CX Amsterdam, Netherlands
[3]Erasmus University Medical Center, 3000 CA Rotterdam, Netherlands
[4]University of Lausanne, Department of Biochemistry, 1066 Epalinges, Switzerland

IgM$^+$IgD$^+$CD27$^+$ B cells from peripheral blood have been described as circulating marginal zone B cells. It is still unknown when and where these cells develop. These IgM$^+$IgD$^+$CD27$^+$ B cells exhibit somatic hypermutations (SHMs) in their B cell receptors, but the exact nature of the signals leading to induction of these SHMs remains elusive. Here, we show that IgM$^+$IgD$^+$CD27$^+$ B cells carrying SHMs are observed during human fetal development. To examine the role of T cells in human IgM$^+$IgD$^+$CD27$^+$ B cell development we used an in vivo model in which Rag2$^{-/-}\gamma_c^{-/-}$ mice were repopulated with human hematopoietic stem cells. Using Rag2$^{-/-}\gamma_c^{-/-}$ mice on a *Nude* background, we demonstrated that development and induction of SHMs of human IgM$^+$IgD$^+$CD27$^+$ B cells can occur in a T cell–independent manner.

CORRESPONDENCE
Hergen Spits:
spits.hergen@gene.com

Abbreviations used: AID, activation-induced cytidine deaminase; HIS, human immune system; HSC, hematopoietic stem cell; MLN, mesenteric lymph node; MZ, marginal zone; SHM, somatic hypermutation; shRNA, short hairpin RNA.

Human memory B cells can be separated into four different CD27$^+$ B cell populations that carry somatic hypermutations (SHMs), namely classical isotype-switched B cells, rare IgD-only and IgM-only B cells, and an IgM$^+$IgD$^+$ subpopulation (1–4). The IgM$^+$IgD$^+$CD27$^+$ B cell population is also referred to as the IgM memory pool. Recently, these IgM$^+$IgD$^+$CD27$^+$ cells in the peripheral blood were shown to be recirculating marginal zone (MZ) B cells based on phenotype and gene expression profiling (5, 6). Unlike MZ B cells in rodents, human MZ B cells are recirculating through the peripheral blood and do contain SHMs (7). However, the notion that mouse MZ B cells are sessile is challenged by a recent finding that mouse MZ B cells shuttle between MZ and follicles, clearly showing that these cells recirculate similar as in human (8). MZ B cells participate in T cell–independent responses to polysaccharide antigens and in the initial defense against blood-borne pathogens (5, 9–11).

It is unknown when and where IgM$^+$IgD$^+$CD27$^+$ B cells develop. In children under the age of 2 yr no response can be detected against T-independent infections (12, 13). However, IgM$^+$IgD$^+$CD27$^+$ B cells are already present at birth, albeit at low numbers (13). Whether or not IgM$^+$IgD$^+$CD27$^+$ B cells are present in the fetus is also still unknown. Human MZ B cells in the spleen and lymph nodes, as well as circulating IgM$^+$IgD$^+$CD27$^+$ B cells in the peripheral blood and neonatal cord blood, have been shown to carry SHMs (1, 13–15). Because no active immune responses are thought to happen in the fetus, these data suggest that development and induction of SHMs of IgM$^+$IgD$^+$CD27$^+$ B cells are not prompted by an active immune response. After the age of 2 yr, the frequency of IgM$^+$IgD$^+$CD27$^+$ B cells in the blood is increased as is the frequency of SHMs in these cells (13). This observation correlates with the appearance of the anatomical structure of the MZ in the spleen and

F.A. Scheeren's present address is Stanford University School of Medicine, Stanford, CA 94305.
H. Spits' present address is Genentech, South San Francisco, CA 94080.
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effective humoral immunity against T cell–independent infections (16). Thus, $IgM^+IgD^+CD27^+$ B cells in young children are formed well before the anatomical structure of the MZ is present. The percentage of $IgM^+IgD^+CD27^+$ B cells in the blood is reduced in the elderly, correlating with a decreased humoral immunity against T cell–independent infections (17). The spleen has been suggested to be the primary organ for $IgM^+IgD^+CD27^+$ B cell development because adult asplenic patients have severely decreased $IgM^+IgD^+CD27^+$ B cell numbers and exhibit poor B cell responses against T cell–independent infections (6). However, it is unknown whether the spleen is the site of $IgM^+IgD^+CD27^+$ B cell development, or whether the spleen supports the survival to this cell subset in a particularly efficient manner. It is also unknown how SHMs are induced in $IgM^+IgD^+CD27^+$ B cells. SHMs are strictly dependent on activation-induced cytidine deaminase (AID) (18, 19). MZ B cells in the spleen do not express the AID protein, as determined by immunohistochemistry (20), strongly suggesting that the SHM process does not occur in the spleen but at a different location.

Hyper-IgM patients with either CD40 or CD40L deficiency have $IgM^+IgD^+CD27^+$ B cells but lack classical switched memory B cells (21). These patients lack germinal centers, in which SHMs are induced in a T cell–dependent way. In both types of patients, the SHM frequency in the $IgM^+IgD^+CD27^+$ B cell population was similar to what is seen in healthy donors. Because T cells predominantly activate CD40 via CD40L expression during a T cell–dependent germinal center reaction, it has been suggested that the development of MZ B cells and the induction of SHMs are T cell independent. However, it is conceivable that T cells are in fact involved in the development and induction of SHMs in a CD40-independent manner. It is important to note that the formation of germinal centers can occur in mice in the absence of CD40L and CD40, although at a much lower level than in wild-type animals (22). Furthermore a new CD40 ligand has recently been described in humans, C4b binding protein, which binds an activated complement component (23, 24).

Here, we show that $IgM^+IgD^+CD27^+$ B cells are present in the human fetus between weeks 14–18 of gestation. These cells strongly resemble MZ B cells based on phenotype and the presence of SHMs. Furthermore, AID expression could be detected in mature B cells from the fetal liver and mesenteric lymph nodes (MLNs) but was absent in B cells from the spleen and BM, suggesting that SHMs occur in the liver and/or MLNs. Using $Rag2^{-/-}\ \gamma_C{}^{-/-}$ mice repopulated with human fetal liver $CD34^+CD38^-$ hematopoietic stem cells (HSCs), we show that the generation of $IgM^+IgD^+CD27^+$ B cells is NOTCH2 dependent, supporting the notion that these cells are MZ-like B cells. Further experiments in $Rag2^{-/-}\ \gamma_C{}^{-/-}$ mice on a *Nude* background, in which T cell development cannot occur due to defective thymus development, revealed that the generation of human $IgM^+IgD^+CD27^+$ B cells is T cell independent. In the absence of T cells, the average number of mutations in $IgM^+IgD^+CD27^+$ B cells that had undergone SHMs was similar to control mice. We conclude that both the development of $IgM^+IgD^+CD27^+$ B cells as well as the induction of SHMs in this cell subset can occur in a T cell–independent manner.

RESULTS

IgD^+CD27^+ B cells are present during human fetal development

$IgM^+IgD^+CD27^+$ B cells can be detected in neonatal cord blood, suggesting that these cells developed before birth. To understand when and where these cells developed during fetal life, we performed an extensive flow cytometry analysis for IgD^+CD27^+ B cells in the human fetus. The fetal tissue used in these experiments ranged from week 14 to 18 of gestation. Adult spleen and neonatal cord blood were used as a positive control. IgD^+CD27^+ B cells were found in fetal cord blood, liver, MLNs, spleen, and BM (Fig. 1 A). Additional B cell stainings for surface IgD and CD27 expression in fetal tissue are shown in Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070447/DC1. The percentages of the IgD^+CD27^+ B cells in the different organs were on the average between 3 and 4.8% of IgD^+ B cells (Fig. 1 B). We did not find significant differences between the different fetal organs analyzed. In addition, we did not observe significant differences in the frequencies of IgD^+CD27^+ B cells between fetuses from gestational week 14 to 18 (Fig. S2). The IgD^-CD27^+ B cells found in the fetal liver, MLNs, and BM were pro–/pre–B cells based on the expression of CD34 and the lack of BCR expression (Fig. S3). This confirms the findings of Vaskova et al. (25) who described transient expression of CD27 on early B cells that lack light chain rearrangements in human BM.

We next determined whether the observed phenotype of fetal IgD^+CD27^+ B cells corresponded to the phenotype of MZ B cells using flow cytometry analysis of different fetal organs and fetal cord blood (Fig. 1 C). In neonatal cord blood, the IgD^+CD27^+ B cells are $sIgM^+CD21^{high}CD1c^{high}$. In fetal cord blood and MLNs, the expression of CD21 was comparable to that in control neonatal cord blood. However, expression of CD21 was lower in the fetal liver, spleen, and BM. CD1c expression in neonatal cord blood was similar to that of fetal cord blood, MLNs, and spleen. In fetal liver and BM, IgD^+CD27^+ B cells expressed low levels of CD1c. This suggests that the fetal spleen, liver, and BM contain IgD^+CD27^+ B cells with a more immature phenotype as compared with fetal cord blood and MLNs. The IgD^+CD27^+ B cell population in the fetal spleen appears to be in between these two stages. Collectively, our data indicate that the phenotype of IgD^+CD27^+ B cells in fetal cord blood and fetal MLNs is similar to that in neonatal cord blood. Although sIgM, CD21, and CD1c are expressed on IgD^+CD27^+ B cells in both the fetal and adult spleen, the expression levels are different (Fig. 1 C). The biological significance of the difference in protein expression is presently unknown.

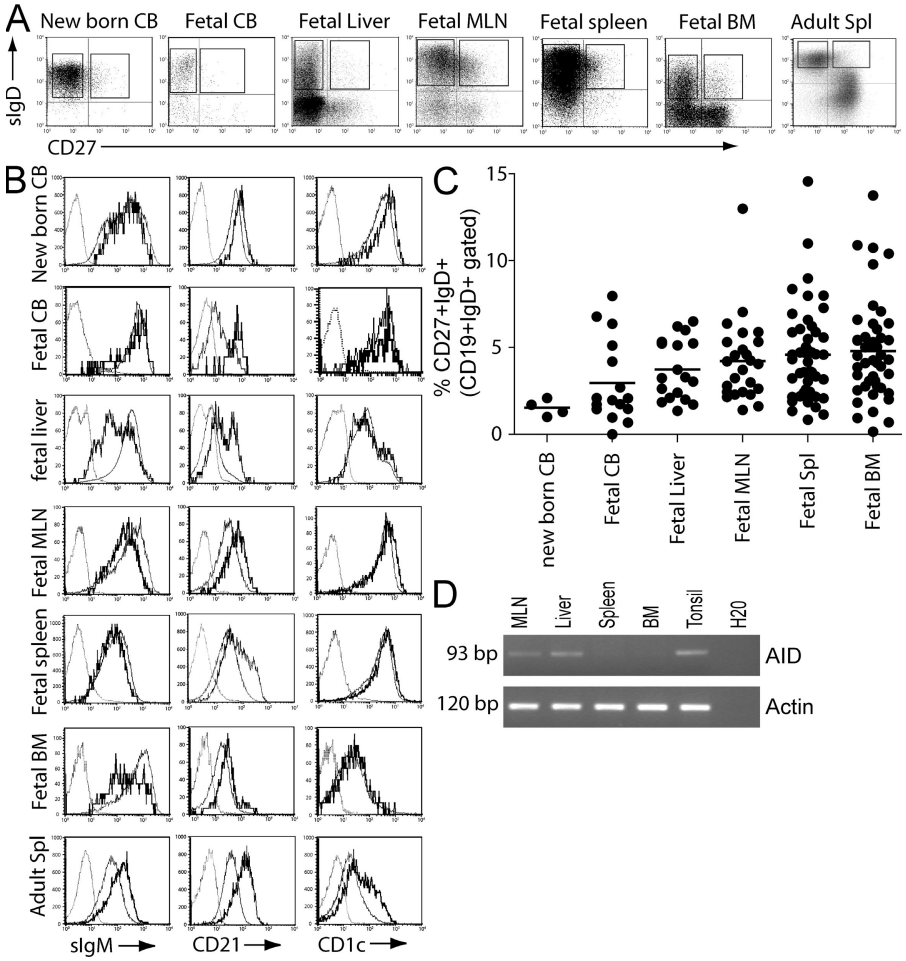

Figure 1. Mature B cells with an MZ B cell phenotype are present in the human fetus. (A) Flow cytometry analysis for CD27 and sIgD of neonatal cord blood (CB), fetal cord blood, liver, MLNs, spleen, and BM. Dot plots presented are gated on $CD19^+$ cells. (B) Percentages of $CD27^+$ cells within the $CD19^+sIgD^+$ gate (fetal cord blood, $n = 16$; fetal liver, $n = 18$; fetal MLNs, $n = 25$; fetal spleen, $n = 47$; fetal BM, $n = 45$). (C) Flow cytometry analysis for sIgM, CD12, and CD1c of the $CD19^+sIgD^+CD27^-$ cells (thin line) and $CD19^+sIgD^+CD27^+$ cells (bold line) using gates indicated in A. Dotted lines represent the staining with the matched isotype control. (D) RT-PCR for AID expression in fetal MLNs, liver, spleen, and BM. Adult tonsil was used as a positive control. Fetal liver B cells were enriched for B cells by CD19 MACS isolation because after Ficoll isolation of mononuclear cells, still <1% of the cells were B cells. The other samples had ≥20% $CD19^+$ cells and thus were not further purified. Data are from one donor representative of three.

AID expression is detected in the human fetal liver and MLNs, and fetal IgD^+CD27^+ B cells have SHMs

A proportion of the $CD19^+IgD^+CD27^+$ B cells derived from adult and neonatal cord blood contains AID-driven SHMs (1, 13). We examined whether AID was expressed in the different fetal organs. AID expression was detected by RT-PCR in mRNA from the fetal liver and MLNs but not from the spleen and BM (Fig. 1 D). As expected, AID was detected in tonsil mRNA. We extended these findings by performing a semiquantitative RT-PCR for AID on sorted IgD^+ B cells from the fetal liver, spleen, and BM (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070447/DC1). Both fetal spleen and fetal BM mature B cells were negative for AID expression, whereas fetal liver B cells were positive, suggesting that the fetal liver and MLNs are the organs where SHMs take place.

The presence of AID in the fetal liver and MLNs may imply that the Ig-encoding genes in $CD19^+IgD^+CD27^+$ cells contain SHMs. Therefore, $CD19^+IgD^+CD27^+$ and $CD19^+IgD^+CD27^-$ cells derived from the fetal spleen were sorted, mRNA was isolated, and an RT-PCR specific for V_H3-Cμ was performed. Products were cloned into a vector and sequenced. Approximately 20% of the sequences contained SHMs (Table I). Collectively, these data show that SHMs are present on fetal IgD^+CD27^+ B cells.

Human $IgM^+IgD^+CD27^+$ B cells develop in humanized mice and exhibit SHMs

To determine whether development of $IgD^+IgM^+CD27^+$ B cells and induction of SHMs are dependent or independent of T cells, we used an in vivo model for human lymphocyte development. We and others have set up a human mouse

Table I. IgH SHM analysis of fetal spleen B cells

B cells	Total unique sequences	Mutated sequences[a]	% of mutation frequency[b]	Mutation range
Donor 1 IgD^+CD27^-	11	0	–	–
Donor 1 IgD^+CD27^+	18	3 (17%)	2.3	5–7
Donor 2 IgD^+CD27^+	19	3 (16%)	2.7	5–11
Donor 3 IgD^+CD27^+	29	6 (21%)	2.4	3–12

[a]Only unique rearrangements were considered for analysis. Sequences scored as mutated contained three or more mutations (≥1% mutation frequency). In brackets is the frequency of mutated sequences among all sequences.
[b]The percentage of point mutations among the total number of sequenced nucleotides of mutated sequences.

model in which development of the human immune system (HIS) can be studied (26, 27). We used alymphoid $Rag2^{-/-}$ $\gamma c^{-/-}$ mice (28) to monitor the development of human $IgD^+IgM^+CD27^+$ B cells. To this end, we injected sorted $CD34^+CD38^-lineage^-$ fetal liver HSCs into sublethally irradiated newborn BALB/c $Rag2^{-/-}$ $\gamma c^{-/-}$ mice. After 6–8 wk, the resulting HIS (BALB-Rag/γ) mice showed a robust reconstitution by human cells, including T cells, B cells, and plasmacytoid DCs in the different lymphoid organs (27, 29). The level of human reconstitution, as indicated by the frequency of $huCD45^+$ cells, ranged from 30 to 90% in peripheral blood of HIS (BALB-Rag/γ) mice, with a large majority of human $CD19^+$ B cells (Fig. S5, A and B, available at http://www.jem.org/cgi/content/full/jem.20070447/DC1). Most reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice (>10% $huCD45^+$) contained both human IgM and IgG in their serum (Fig. S5 C), with titers similar to previously published observations (26, 27). IgD^+CD27^+ B cells could be detected in the spleen and the blood of HIS (BALB-Rag/γ) mice (Fig. 2 A). A more detailed phenotype of splenic IgD^+CD27^+ B cells showed that they were $sIgM^+CD21^{high}CD1c^+$ (Fig. 2 B), i.e., they were similar to MZ B cells found in the MZ and peripheral blood of human beings. The frequency of $CD27^+$ cells among IgD^+CD19^+ B cells was around 2.5 and 1% in the spleen and blood, respectively (Fig. 2 C). The percentages found in the reconstituted mice are similar to the percentages found in young children (13).

A detailed analysis of the anatomical structure of the spleen of reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice (n = 12) failed to reveal MZ structures (not depicted) despite the presence of $IgM^+IgD^+CD27^+$ B cells in the circulation and peripheral organs. This suggests that there is a disconnection between the anatomical MZ structure in the spleen and the presence of IgD^+CD27^+ B cells. Similarly, in children under the age of 2 yr, $IgM^+IgD^+CD27^+$ B cells can be found in the peripheral blood despite the absence of anatomical MZ structure (13, 16).

To determine whether the $IgD^+IgM^+CD27^+$ B cells, which developed in the repopulated HIS (BALB-Rag/γ) mice, also had undergone SHMs we sequenced V_H3-Cμ heavy chain regions derived from these cells. Splenic $CD19^+IgD^+CD27^+$ and $CD19^+IgD^+CD27^-$ cells were sorted from three independent HIS (BALB-Rag/γ) mice, mRNA was isolated, and an RT-PCR specific for V_H3-Cμ was performed. Sequencing of the cloned PCR products revealed that approximately half of the sequences from the $CD19^+IgD^+CD27^+$ cells contained SHMs (Table II). These data demonstrate that the human IgD^+CD27^+ B cells in the HIS (BALB-Rag/γ) mice have undergone SHMs.

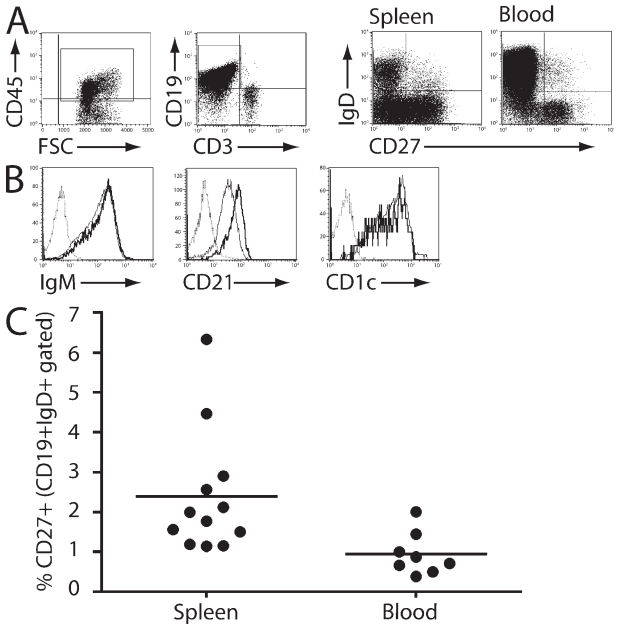

Figure 2. Human B cells with MZ B cell phenotype are present in HIS (BALB-Rag/γ) mice. (A) Flow cytometry analysis of blood and spleen lymphocytes of the human HSC-reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice. The sIgD-CD27 dot plots were obtained after gating on $CD45^+$ and $CD19^+CD3^-$ cells, as indicated by the outlined areas. (B) Expression of sIgM, CD21, and CD1c by $CD19^+sIgD^+CD27^-$ cells (thin line) and $CD19^+sIgD^+CD27^+$ cells (bold line) from the spleens of HIS (BALB-Rag/γ) mice. Dotted lines represent staining with the matched isotype control. (C) Percentage of $CD27^+$ cells within the $CD19^+sIgD^+$ population in the spleen and the blood of HIS (BALB-Rag/γ) mice. Data were obtained from 12 reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice with human HSC s isolated from five independent donors.

Table II. IgH somatic hyper mutation analysis of spleen B cells of HIS (BALB- Rag/γ) mice

B cells	Total unique sequences	Mutated sequences[a]	% of mutation frequency[b]	Mutation range
Donor 1 IgD$^+$CD27$^-$	20	0	–	0–2
Donor 1 IgD$^+$CD27$^+$	15	10 (67%)	2.6	3–14
Donor 2 IgD$^+$CD27$^+$	18	8 (44%)	3.1	3–16
Donor 3 IgD$^+$CD27$^+$	10	4 (40%)	4.5	4–18

[a]Only unique rearrangements were considered for analysis. Sequences scored as mutated contained three or more mutations (≥1% mutation frequency). In brackets is the frequency of mutated sequences among all sequences.
[b]The percentage of point mutations among the total number of sequenced nucleotides of mutated sequences.

IgD$^+$IgM$^+$CD27$^+$ B cells resemble MZ B cells

To obtain further evidence that IgD$^+$CD27$^+$ B cells that developed in the HIS (BALB-Rag/γ) mice were MZ-like B cells, we made use of a genetic approach. In normal mice, MZ B cell development is dependent on NOTCH2, as NOTCH2$^{-/-}$ mice lack this B cell subset (30). Therefore, we investigated whether NOTCH2 was required for human IgD$^+$CD27$^+$ B cell development in HIS (BALB-Rag/γ). We made use of a lentiviral vector to inhibit NOTCH2 expression through short hairpin RNA (shRNA) interference. The NOTCH2-specific shRNA lentiviral vector induced a 60% reduction of NOTCH2 mRNA levels as determined by RT-PCR in the B cell line Raji (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20070447/DC1). We compared the effects of the NOTCH2-specific shRNA lentiviral-transduced cells with the control shRNA lentiviral-transduced cells within the same HIS (BALB-Rag/γ) mouse. We used two different fluorescent markers, YFP and GFP, to distinguish between the two vectors. Human FL CD34$^+$CD38$^-$ HSCs were transduced with either a YFP control vector or with a GFP vector expressing the shRNA cassette to knock down NOTCH2. After lentiviral transduction, control and NOTCH2 knockdown-transduced cells were mixed and injected in neonatal Rag2$^{-/-}$ γc$^{-/-}$ mice. 8 wk later, repopulated mice were killed and the percentage of CD19$^+$IgD$^+$CD27$^+$ cells was determined in the GFP and the YFP population of the spleen. The ratio of YFP versus GFP was equal 8 wk after reconstitution in the HIS (BALB-Rag/γ) mice as compared with the original inoculum, demonstrating that there was no selection due the shRNA-mediated knockdown of NOTCH2. The shRNA-mediated knockdown of NOTCH2 resulted in a 50% reduction of CD19$^+$IgD$^+$CD27$^+$ cells as compared with the control-transduced cells (Fig. 3 A) and the non-transduced cells (not depicted). Importantly the shRNA-mediated NOTCH2 knockdown did not result in decreased frequency of other immune cell subsets, e.g., CD3$^+$ T cells (Fig. 3 B), showing the specificity of the developmental block. These data strongly suggest that in humans, NOTCH2 is important for the development of IgM$^+$IgD$^+$CD27$^+$ B cells.

Development of IgD$^+$CD27$^+$ B cells and induction of SHMs can occur in a T cell–independent manner

To determine whether development of IgD$^+$CD27$^+$ B cells is strictly dependent on the presence of T cells, we made use of Rag2$^{-/-}$ γc$^{-/-}$ mice on a *Nude* background. These mice have no functional thymus, and therefore T cell development cannot take place (31, 32). HIS (BALB-*nude*-Rag/γ) mice showed a similar level of human engraftment in peripheral blood than control HIS (BALB-Rag/γ) mice (Fig. S5, A and B). In control HIS (BALB-Rag/γ) mice, a functional mouse thymus was present and fully supported human T cell development, leading to colonization of peripheral lymphoid organs by mature human T cells (Fig. 4 A). In contrast, no T cells could de detected in the different lymphoid organs of HIS (BALB-*nude*-Rag/γ) mice, indicating that no thymus-independent T cell development occurs in this humanized mouse model. Nonetheless, IgD$^+$CD27$^+$ B cells were present in the blood and spleen of HIS (BALB-*nude*-Rag/γ) mice (Fig. 4 B). A more detailed phenotypic analysis of the splenic IgD$^+$CD27$^+$ B cells revealed

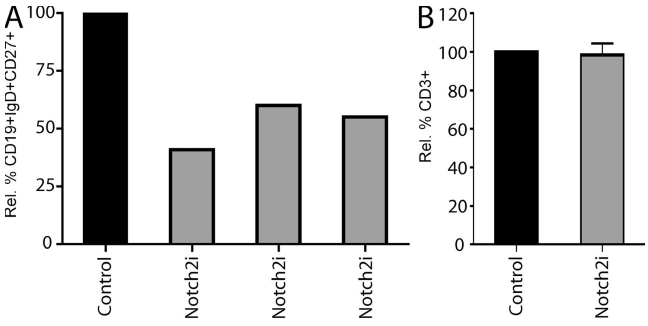

Figure 3. NOTCH2 is essential for development of CD19$^+$sIgD$^+$ CD27$^-$ cells. (A) The relative percentage of CD19$^+$sIgD$^+$CD27$^+$ cells in the spleen of HIS (BALB-Rag/γ) mice reconstituted with human HSCs transduced either with the NOTCH2 shRNA-mediated knockdown construct (GFP$^+$ cells) or a control knockdown construct (YFP$^+$ cells). (B) Relative percentage of the CD3$^+$ cells with the NOTCH2 shRNA-mediated knockdown construct (GFP$^+$ cells) compared with the CD3$^+$ cells with a control knockdown construct (YFP$^+$ cells). Three HIS (BALB-Rag/γ) mice from three different experiments using different donors for HSC transductions were analyzed. The shRNA-mediated NOTCH2 knockdown is indicated as siNOTCH2.

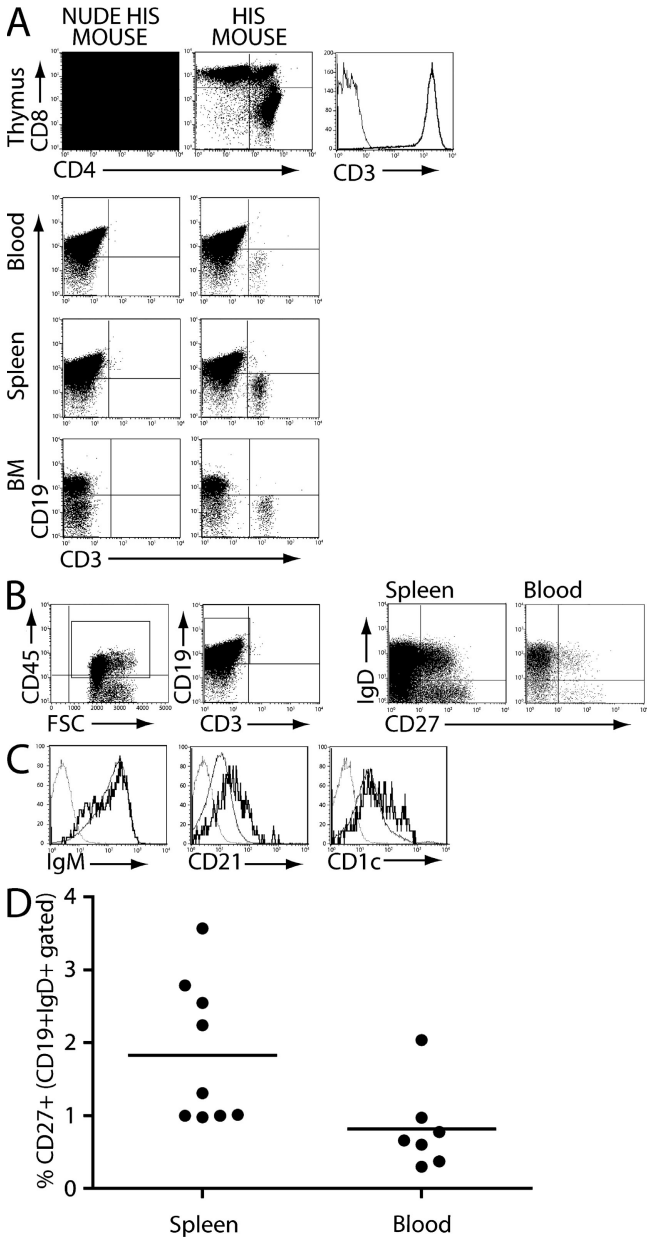

Figure 4. MZ B cells develop in the absence of T cells. (A) Rag2$^{-/-}$ $\gamma c^{-/-}$ mice on *Nude* background were reconstituted with human HSCs, and flow cytometry analysis was performed 9 wk later to detect T cells (CD4, CD8, and CD3) and B cells (CD19). (B) Flow cytometry analysis of blood and spleen lymphocytes of HIS (BALB-*nude*-Rag/γ). sIgD-CD27 dot plots were obtained after gating on CD45$^+$ and CD19$^+$CD3$^-$ cells, as indicated by the outlined areas. (C) Expression of sIgM, CD21, and CD1c of CD19$^+$sIgD$^+$CD27$^-$ cells (thin line) and CD19$^+$sIgD$^+$CD27$^+$ cells (bold line) on splenocytes of HIS (BALB-*nude*-Rag/γ) mice. Dotted lines represent the staining with a matched isotype control. (D) Percentage of CD27$^+$ cells within the CD19$^+$sIgD$^+$ population in the spleen and the blood lymphocytes of HIS (BALB-*nude*-Rag/γ) mice. In total, nine HIS (BALB-*nude*-Rag/γ) mice, which were reconstituted with HCS from three different donors, were analyzed.

that the cells were sIgM$^+$CD21^{high}CD1c$^+$ (Fig. 4 C). The percentages of IgD$^+$CD27$^+$ B cells in the spleen and blood were similar between HIS (BALB-*nude*-Rag/γ) mice (Fig. 4 D) and control HIS (BALB-Rag/γ) mice (Fig. 2 C). From these data, we conclude that the development of human IgD$^+$CD27$^+$ B cells can occur in a T cell–independent manner in vivo.

To determine the impact of the absence of T cells on the induction of SHMs, we determined the frequency of clones carrying SHMs in the VH3-Cμ region in three repopulated HIS (BALB-*nude*-Rag/γ) mice. Sequence analysis of the RT-PCR product showed that ~25% of these cells had undergone SHMs as compared with the 50% of IgD$^+$CD27$^+$ B cells derived from the control HIS (BALB-Rag/γ) mice (Table III). Importantly, the IgD$^+$CD27$^+$ B cells from the HIS (BALB-*nude*-Rag/γ) mice that had undergone SHMs had similar numbers of mutations, indicating that the induction of SHMs can occur in a T cell–independent manner.

DISCUSSION

We report here that postrearrangement Ig diversification occurs in utero in human B cells via induction of SHM. We describe that in the human fetus during weeks 14–18 of gestation, IgM$^+$IgD$^+$CD27$^+$ B cells that have undergone SHM are present. AID expression, which is a prerequisite for the induction of SHM, could be detected only in the fetal liver and MLNs, suggesting that the induction of SHM occurs in these organs. In both organs, IgM$^+$IgD$^+$CD27$^+$ B cells are detected that are CD21^{high} and CD1c^{high}. One could speculate that these are more mature IgM$^+$IgD$^+$CD27$^+$ B cells as compared with fetal BM IgM$^+$IgD$^+$CD27$^+$ B cells, which express low levels of surface CD21 and/or CD1c. However, there are obvious limitations when working with human material, making it difficult to address the maturation status of human IgM$^+$IgD$^+$CD27$^+$ B cells. To address the question of whether development and induction of SHM of IgM$^+$IgD$^+$CD27$^+$ B cells is a T cell–independent process, we made use of a recently developed in vivo–humanized mouse model. We observed IgM$^+$IgD$^+$CD27$^+$CD21^{high}CD1c^{high} B cells in such HIS (BALB-Rag/γ) mice. These cells contained SHMs and strongly resembled human MZ B cells phenotypically.

It is well documented that there are differences between rodent and human MZ B cells because human MZ B cells recirculate and contain SHMs, unlike rodent MZ B cells (5, 6, 13–15). Moreover, on an anatomical level, the spleens of humans and rodents are slightly different (7). In contrast to mice, humans have an inner and outer MZ surrounded by a perifollicular zone, which can be directly irrigated by blood vessels. However, such MZ structures were not found in HIS (BALB-Rag/γ) mice (not depicted). This observation is in line with findings that in children under the age of 2 yr, no CD27$^+$ MZ B cells are present in histological sections of the spleen, despite the fact that IgM$^+$IgD$^+$CD27$^+$ B cells can already be found in the blood of newborns (15) and, as shown here, in the fetus. This clearly indicates that the development of IgM$^+$IgD$^+$CD27$^+$ B cells does not depend on the presence of anatomical MZ structure.

Table III. IgH SHM analysis of spleen B cells of HIS (BALB-*nude*-Rag/γ) mice

B cells	Total unique sequences	Mutated sequences[a]	% of mutation frequency[b]	Mutation range
Donor 1 IgD$^+$CD27$^+$	32	7 (22%)	2.6	3–14
Donor 2 IgD$^+$CD27$^+$	34	9 (26%)	2.4	3–10

[a]Only unique rearrangements were considered for analysis. Sequences scored as mutated contained three or more mutations (≥1% mutation frequency). In brackets is the frequency of mutated sequences among all sequences.
[b]The percentage of point mutations among the total number of sequenced nucleotides of mutated sequences.

In mice it is established that NOTCH2 is essential for MZ B cell development, as NOTCH2 knockout mice do not have MZ B cells (30). Combining the in vivo human–mouse model with shRNA-mediated knockdown for NOTCH2, we established that development of IgM$^+$IgD$^+$CD27$^+$ B cells in HIS (BALB-Rag/γ) mice is NOTCH2 dependent.

Using Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice on a *Nude* background, in which no T cell development is possible due to the lack of a functional thymus, we demonstrated that development and induction of SHMs of human IgM$^+$IgD$^+$CD27$^+$ B cells is a T cell–independent process. However, the frequency of IgM$^+$IgD$^+$CD27$^+$ B cells that had undergone SHM decreased from 40–67% in control HIS (BALB-Rag/γ) mice to 22–26% in HIS (BALB-*nude*-Rag/γ) mice. Importantly, IgM$^+$IgD$^+$CD27$^+$ B cells in HIS (BALB-*nude*-Rag/γ) mice that had undergone SHM showed a similar level of mutation frequency per cell as compared with control HIS (BALB-Rag/γ) mice. Collectively, this indicates that T cells are probably involved in increasing the numbers of these cells, but they are not essential to induce SHM in IgM$^+$IgD$^+$CD27$^+$ B cells.

The fact that IgM$^+$IgD$^+$CD27$^+$ B cells develop during fetal life raises the question whether IgM$^+$IgD$^+$CD27$^+$ B cells still develop and undergo induction of SHM during adult life. An answer to this question might come from adult patients with rheumatoid arthritis, who have been depleted of B cells using an anti-CD20 antibody (33). Even 2 yr after the anti-CD20 treatment, the percentages of the IgD$^+$CD27$^+$ B cells in these patients are still severely reduced as compared with healthy controls, suggesting that the development of IgD$^+$CD27$^+$ B cells is not efficient in adults. On the other hand, numbers of CD27$^+$ class-switched B cells are also maintained at low levels after anti-CD20 treatment, suggesting a general developmental defect in CD27$^+$ memory B cells not necessarily restricted to the IgD$^+$CD27$^+$ B cell population.

We hypothesize that the development and the induction of SHM in IgD$^+$CD27$^+$ B cells occurs mainly during fetal development and in young children. It has been shown that upon vaccination, IgD$^+$CD27$^+$ B cells from adults can undergo additional SHM (5). It will be of interest to determine whether the mechanism of vaccination-induced SHM is different from the mechanism involved in utero.

Postrearrangement Ig diversification has been reported in several species including birds and mammals, resulting in B cells that are able to recognize T-independent antigens and mount a high affinity immune response (34, 35). This process takes place in utero and early in life in the gut-associated lymphoid tissue by SHM and/or gene conversion. Our data indicate that a similar process occurs in humans via induction of SHM during fetal life. This process most likely takes place in the fetal liver and fetal MLNs, as AID expression could be detected in these organs. The MLN is considered to be part of the gut-associated lymphoid tissue (36). In this respect, it is interesting to note that in mice with a highly restricted BCR repertoire, SHM can be detected in B cells with an immature phenotype. In the absence of T cells a similar mutation frequency was observed (37). These data are in line with our findings that induction of SHM can occur in a T cell–independent manner; however, in this mouse model SHM only occurred when the BCR repertoire was severely restricted.

Igs that recognize autoantigen are removed from the repertoire at two checkpoints during B cell development in the BM and the periphery (38, 39). Recently, a third checkpoint against self-reactive IgM during IgM$^+$CD27$^+$ B cell development in humans was described (40). This novel checkpoint for B cell tolerance is active between the naive and IgM$^+$CD27$^+$ B cells that have undergone SHM. Interestingly, the data suggest that self-reactive IgM antibodies are removed before the onset of SHM.

In conclusion, here we show that in utero postrearrangement Ig diversification via SHM occurs in human IgM$^+$IgD$^+$CD27$^+$ B cells. Using a humanized mouse model, we furthermore show that the IgM$^+$IgD$^+$CD27$^+$ B cells strongly resembled MZ B cells and that development and induction of SHM can occur in the absence of T cells. The T cell–independent IgM$^+$IgD$^+$CD27$^+$ B cell development and induction of SHM strongly suggest that classical switched memory B cells and IgM$^+$IgD$^+$CD27$^+$ B cells develop along separate pathways.

MATERIALS AND METHODS

Mice. BALB/c Rag2$^{-/-}$ γc$^{-/-}$ mice and BALB/c Rag2$^{-/-}$ γc$^{-/-}$ mice on a *Nude* background were bred and maintained in isolators and fed autoclaved food and water under a protocol approved by the AMC-UVA. Repopulated Rag2$^{-/-}$ γc$^{-/-}$ mice with cells of the HIS-(BALB-Rag/γ) mice were generated as described previously with minor changes (27). In brief, newborn (<1-wk-old) Rag2$^{-/-}$ γc$^{-/-}$ mice received sublethal (3.5 Gy) total body irradiation with an x-ray source and were injected intra-hepatically with 0.5–10^6 CD34$^+$ human fetal liver cells or 0.5 to 2 × 10^6 CD34$^+$CD38$^-$lineage$^-$ human fetal liver cells. For the lineage depletion, we used antibodies against CD3,

CD19, CD56, and BDCA2. All manipulations with the mice were performed under a laminar flow. Cell suspensions were prepared in RPMI medium with 2% fetal calf serum.

Cell purification. After the preparation of single cell suspension and isolation of mononuclear cells by density gradient centrifugation over Lymphoprep Ficoll-Hypaque (Nycomed Pharma), a magnetic enrichment of fetal liver $CD34^+$ cells or B cells (>98% pure) was performed by using the CD34 Progenitor Cell Isolation kit or the CD19 MACS kit, respectively (Miltenyi Biotec). This was followed by cell sorting on a FACS Aria (Becton Dickinson). Human fetal liver was obtained from elective abortions, with gestational age ranging from 14 to 20 wk. The use of this tissue was approved by the Medical Ethical Committee of the AMC-UVA and was contingent on informed consent.

Flow cytometric analysis. Cell suspensions were labeled with anti–human mAb targeting the following cell surface markers: CD1a (T6-RD1), CD21 (BL13; Beckman Coulter), CD3 (SK7), CD4 (SK3), CD8 (SK1), CD19 (HIB19), CD45 (2D1), IgM (G20-127), IgD (IA6-2; BD Biosciences), CD27 (LT27; AbD Serotec), CD27 (LG.7F9; eBioscience), BDCA1/CD1c, (AD5-8E7), and BDCA2 (AC144; Miltenyi Biotec). Stained cells were analyzed with an LSR II (Becton Dickinson), and flow cytometric data were processed with CELLQuest software (Becton Dickinson).

RT-PCR. Total mRNA was isolated from cells using the RNeasy mini kit (QIAGEN). mRNA was reverse-transcribed in a volume of 20 µl containing 5× first-strand buffer, 500 µM dNTPs, 25 µg/liter oligo(dT), and 200 U superscript II RT (Invitrogen). A portion of the cDNA solution (1 µl) was amplified by PCR in a 50-µl solution containing 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM dNTPs, 2.5 U *Taq* DNA polymerase (Invitrogen), and 30 pmol of each primer. PCR conditions were as follows: a 7-min denaturing step at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, and a final 7-min extension at 72°C. Actin and AID primers were similar to those described previously (41).

The PCR conditions for the *VH3*-Cμ genes were similar to those described above, including a 7-min denaturing step at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, and a final 10-min extension at 72°C. The oligonucleotide used for RT-PCR was as follows: *VH3* forward, 5′-GGGGGTCCCTGAGACTCTC-3′.

Quantitative real-time PCR analysis was performed with an iCycler PCR (Bio-Rad Laboratories). The iQ SYBR Green Supermix (Bio-Rad Laboratories) was used for amplification. After incubation at 95°C for 6 min, 40 cycles of amplification were performed. Each cycle consisted of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The oligonucleotides used for real-time RT-PCR were as follows: *Notch2* forward, 5′-TGTGAATGCAGTGGATGACC-3′ and *Notch2* reverse, 5′-GGGCAGCAAGAAACAGAGG-3′; *Actin* forward, 5′-GGATGCAGAAGGAGATCACTG-3′ and *Actin* reverse, 5′-CGATCCACACGGAGTACTTG-3′.

Cloning and sequencing of PCR products. PCR products were cloned into Topo TA cloning vectors (Invitrogen). Subsequently, mini-preps were made of each individual clone and both strands of the inserts were sequenced to obtain the sequence. Sequencing was performed with an ABI sequencer (PerkinElmer) using the dye-terminator cycle-sequencing kit (PerkinElmer).

Assignment of mutations. The sequences found were compared with published germline sequences using the National Center for Biotechnology Information Immunoglobulin blast on the Internet (http://www.ncbi.nlm.nih.gov/igblast/) to identify mutations. The last nucleotide position of the V gene was excluded from the mutational analysis in view of possible nucleotide deletions at the joining sites.

Lentivirus production. Replication-defective self-inactivating HIV vectors were produced by transient transfection of 293T cells using FUGENE (Roche) and three different plasmids. The plasmids used were the VSV-G envelope coding plasmid pMD.G; the packaging plasmid pCMVDR8.91, designed to provide the Gag, Pol, Tat, and Rev proteins to produce the virus particle; and the transfer vector pTRIPU3-E1 (42). pTRIPU3-E1 has been modified to include the siRNA cassette from the pSUPER vector containing the human NOTCH2-targeting sequence 5′ (43, 44), and it carries the enhanced *GFP* or *YFP* gene driven by the elongation factor-1α promoter. 48 h after transfection, medium was replaced and two samples of virus were collected at 24 and 48 h. The virus-containing supernatants were centrifuged for 4 min at 1,800 rpm to remove cells and then were passed through a 0.22-mm filter and kept at −80°C until use.

Transduction protocol. Transduction of $CD34^+$ human cells was performed by one cycle of overnight exposure to viral supernatant on retronectin (Takara Shuzo, Co.) -coated 24-well plates in the absence of cytokines. The next day, cells were washed and injected intra-hepatically in the newborn mice.

IgM and IgG ELISA. To measure Ig levels in the serum of the reconstituted mice, we performed an ELISA for IgM and IgG using the serum of the mice. Microtiter plates (9018; Costar) were coated with 100 µl/well of anti-IgM (10 µg/ml goat anti–human polyclonal; Jackson ImmunoResearch Laboratories) or anti-IgG (10 µg/ml goat anti–human polyclonal; Jackson ImmunoResearch Laboratories) in PBS. ELISA was developed with horseradish peroxidase–labeled goat anti–human IgM and IgG antibodies (Dako) and horseradish peroxidase substrates (Sigma-Aldrich). OD_{405} was measured with a microplate reader (MDS Analytical Technologies).

Online supplemental material. Fig. S1 shows four representative IgD/CD27 dot plots of analyzed fetal tissue. Fig. S2 shows the gestational age of the analyzed fetal tissue and the percentage of IgD^+CD27^+ B cells within the IgD^+ B cell population. Fig. S3 shows that $CD19^+sIgD^-CD27^+$ B cells express CD34 and lack BCR expression. Fig. S4 shows that AID is expressed in the IgD^+ B cells of fetal liver but not in fetal BM and fetal spleen IgD^+ B cells. Fig. S5 shows the reconstitution efficiency of repopulated HIS mice, the percentage of $CD45^+$, $CD3^+$, and $CD19^+$ cells, and the IgG and IgM levels in the serum. Fig. S6 shows the knockdown efficiency for the NOTCH2 shRNA-mediated knockdown construct. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070447/DC1.

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REFERENCES

1. Klein, U., K. Rajewsky, and R. Kuppers. 1998. Human immunoglobulin (Ig)M^+IgD^+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J. Exp. Med.* 188:1679–1689.
2. Klein, U., R. Kuppers, and K. Rajewsky. 1997. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood.* 89:1288–1298.

3. van Es, J.H., F.H. Meyling, and T. Logtenberg. 1992. High frequency of somatically mutated IgM molecules in the human adult blood B cell repertoire. *Eur. J. Immunol.* 22:2761–2764.
4. Paramithiotis, E., and M.D. Cooper. 1997. Memory B lymphocytes migrate to bone marrow in humans. *Proc. Natl. Acad. Sci. USA.* 94:208–212.
5. Weller, S., M.C. Braun, B.K. Tan, A. Rosenwald, C. Cordier, M.E. Conley, A. Plebani, D.S. Kumararatne, D. Bonnet, O. Tournilhac, et al. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood.* 104:3647–3654.
6. Kruetzmann, S., M.M. Rosado, H. Weber, U. Germing, O. Tournilhac, H.H. Peter, R. Berner, A. Peters, T. Boehm, A. Plebani, et al. 2003. Human immunoglobulin M memory B cells controlling *Streptococcus pneumoniae* infections are generated in the spleen. *J. Exp. Med.* 197:939–945.
7. Mebius, R.E., and G. Kraal. 2005. Structure and function of the spleen. *Nat. Rev. Immunol.* 5:606–616.
8. Cinamon, G., M.A. Zachariah, O.M. Lam, F.W. Foss Jr., and J.G. Cyster. 2008. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat. Immunol.* 9:54–62.
9. Guinamard, R., M. Okigaki, J. Schlessinger, and J.V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol.* 1:31–36.
10. Martin, F., A.M. Oliver, and J.F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity.* 14:617–629.
11. Zandvoort, A., and W. Timens. 2002. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. *Clin. Exp. Immunol.* 130:4–11.
12. Cowan, M.J., A.J. Ammann, D.W. Wara, V.M. Howie, L. Schultz, N. Doyle, and M. Kaplan. 1978. Pneumococcal polysaccharide immunization in infants and children. *Pediatrics.* 62:721–727.
13. Weller, S., A. Faili, S. Aoufouchi, Q. Gueranger, M. Braun, C.A. Reynaud, and J.C. Weill. 2003. Hypermutation in human B cells in vivo and in vitro. *Ann. NY Acad. Sci.* 987:158–165.
14. Dunn-Walters, D.K., P.G. Isaacson, and J. Spencer. 1995. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. *J. Exp. Med.* 182:559–566.
15. Tierens, A., J. Delabie, L. Michiels, P. Vandenberghe, and C. De Wolf-Peeters. 1999. Marginal-zone B cells in the human lymph node and spleen show somatic hypermutations and display clonal expansion. *Blood.* 93:226–234.
16. Zandvoort, A., M.E. Lodewijk, N.K. de Boer, P.M. Dammers, F.G. Kroese, and W. Timens. 2001. CD27 expression in the human splenic marginal zone: the infant marginal zone is populated by naive B cells. *Tissue Antigens.* 58:234–242.
17. Shi, Y., T. Yamazaki, Y. Okubo, Y. Uehara, K. Sugane, and K. Agematsu. 2005. Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. *J. Immunol.* 175: 3262–3267.
18. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell.* 102:553–563.
19. Revy, P., T. Muto, Y. Levy, F. Geissmann, A. Plebani, O. Sanal, N. Catalan, M. Forveille, R. Dufourcq-Labelouse, A. Gennery, et al. 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell.* 102:565–575.
20. Willenbrock, K., B. Jungnickel, M.L. Hansmann, and R. Kuppers. 2005. Human splenic marginal zone B cells lack expression of activation-induced cytidine deaminase. *Eur. J. Immunol.* 35:3002–3007.
21. Weller, S., A. Faili, C. Garcia, M.C. Braun, F.F. Le Deist, G.G. de Saint Basile, O. Hermine, A. Fischer, C.A. Reynaud, and J.C. Weill. 2001. CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc. Natl. Acad. Sci. USA.* 98:1166–1170.
22. Gaspal, F.M., F.M. McConnell, M.Y. Kim, D. Gray, M.H. Kosco-Vilbois, C.R. Raykundalia, M. Botto, and P.J. Lane. 2006. The generation of thymus-independent germinal centers depends on CD40 but not on CD154, the T cell-derived CD40-ligand. *Eur. J. Immunol.* 36:1665–1673.
23. Brodeur, S.R., F. Angelini, L.B. Bacharier, A.M. Blom, E. Mizoguchi, H. Fujiwara, A. Plebani, L.D. Notarangelo, B. Dahlback, E. Tsitsikov, and R.S. Geha. 2003. C4b-binding protein (C4BP) activates B cells through the CD40 receptor. *Immunity.* 18:837–848.
24. Lazarevic, V., A.J. Myers, C.A. Scanga, and J.L. Flynn. 2003. CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol M. tuberculosis infection. *Immunity.* 19:823–835.
25. Vaskova, M., E. Fronkova, J. Starkova, T. Kalina, E. Mejstrikova, and O. Hrusak. 2008. CD44 and CD27 delineate B-precursor stages with different recombination status and with an uneven distribution in non-malignant and malignant hematopoiesis. *Tissue Antigens.* 71:57–66.
26. Traggiai, E., L. Chicha, L. Mazzucchelli, L. Bronz, J.C. Piffaretti, A. Lanzavecchia, and M.G. Manz. 2004. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science.* 304:104–107.
27. Gimeno, R., K. Weijer, A. Voordouw, C.H. Uittenbogaart, N. Legrand, N.L. Alves, E. Wijnands, B. Blom, and H. Spits. 2004. Monitoring the effect of gene silencing by RNA interference in human CD34+ cells injected into newborn RAG2−/− gammac−/− mice: functional inactivation of p53 in developing T cells. *Blood.* 104:3886–3893.
28. Weijer, K., C.H. Uittenbogaart, A. Voordouw, F. Couwenberg, J. Seppen, B. Blom, F.A. Vyth-Dreese, and H. Spits. 2002. Intrathymic and extrathymic development of human plasmacytoid dendritic cell precursors in vivo. *Blood.* 99:2752–2759.
29. Schotte, R., M. Nagasawa, K. Weijer, H. Spits, and B. Blom. 2004. The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J. Exp. Med.* 200:1503–1509.
30. Saito, T., S. Chiba, M. Ichikawa, A. Kunisato, T. Asai, K. Shimizu, T. Yamaguchi, G. Yamamoto, S. Seo, K. Kumano, et al. 2003. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity.* 18:675–685.
31. Nehls, M., B. Kyewski, M. Messerle, R. Waldschutz, K. Schuddekopf, A.J. Smith, and T. Boehm. 1996. Two genetically separable steps in the differentiation of thymic epithelium. *Science.* 272:886–889.
32. Blackburn, C.C., C.L. Augustine, R. Li, R.P. Harvey, M.A. Malin, R.L. Boyd, J.F. Miller, and G. Morahan. 1996. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc. Natl. Acad. Sci. USA.* 93:5742–5746.
33. Roll, P., A. Palanichamy, C. Kneitz, T. Dorner, and H.P. Tony. 2006. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Arthritis Rheum.* 54: 2377–2386.
34. Lanning, D., B.A. Osborne, and K.L. Knight. 2004. Immunoglobulin genes and generation of antibody repertoires in higher vertebrates: a key role for GALT. *In* Molecular Biology of B cells. Elsevier, New York. 433–448.
35. Reynaud, C.A., C. Garcia, W.R. Hein, and J.C. Weill. 1995. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. *Cell.* 80:115–125.
36. Newberry, R.D., and R.G. Lorenz. 2005. Organizing a mucosal defense. *Immunol. Rev.* 206:6–21.
37. Mao, C., L. Jiang, M. Melo-Jorge, M. Puthenveetil, X. Zhang, M.C. Carroll, and T. Imanishi-Kari. 2004. T cell-independent somatic hypermutation in murine B cells with an immature phenotype. *Immunity.* 20:133–144.
38. Meffre, E., R. Casellas, and M.C. Nussenzweig. 2000. Antibody regulation of B cell development. *Nat. Immunol.* 1:379–385.
39. Wardemann, H., S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, and M.C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science.* 301:1374–1377.
40. Tsuiji, M., S. Yurasov, K. Velinzon, S. Thomas, M.C. Nussenzweig, and H. Wardemann. 2006. A checkpoint for autoreactivity in human IgM^+ memory B cell development. *J. Exp. Med.* 203:393–400.

41. Smit, L.A., R.J. Bende, J. Aten, J.E. Guikema, W.M. Aarts, and C.J. van Noesel. 2003. Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin's lymphomas of germinal-center phenotype. *Cancer Res.* 63:3894–3898.
42. Sirven, A., E. Ravet, P. Charneau, V. Zennou, L. Coulombel, D. Guetard, F. Pflumio, and A. Dubart-Kupperschmitt. 2001. Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. *Mol. Ther.* 3:438–448.
43. Fan, X., I. Mikolaenko, I. Elhassan, X. Ni, Y. Wang, D. Ball, D.J. Brat, A. Perry, and C.G. Eberhart. 2004. Notch1 and notch2 have opposite effects on embryonal brain tumor growth. *Cancer Res.* 64:7787–7793.
44. Brummelkamp, T.R., R. Bernards, and R. Agami. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science.* 296:550–553.