Loss of Precursor B Cell Expansion but Not Allelic Exclusion in *VpreB1/VpreB2* Double–deficient Mice

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Abstract

The pre–B cell receptor consists of immunoglobulin (Ig) μ heavy chains and surrogate light chain, i.e., the VpreB and λ5 proteins. To analyze the role of the two VpreB proteins, mice lacking the *VpreB1* and *VpreB2* genes were generated. *VpreB1$^{-/-}$VpreB2$^{-/-}$* mice were impaired in their B cell development at the transition from pre–BI to large pre–BII cells. Pre–BII cells did not expand by proliferation, consequently 40–fold less small pre–BII and immature B cells were found in bone marrow, and the generation of immature and mature conventional B cells in spleen appeared reduced. In addition, only low numbers of B–1a cells were detected in the peritoneum. Surprisingly, Ig heavy chain allelic exclusion was still active, apparently ruling out a signaling role of a VpreB1/VpreB2–containing receptor in this process.

Key words: B cell development • surrogate light chain • pre–B cell receptor • B cell deficiency • B1–a B cells

Introduction

Mouse B cell development follows a sequence of cellular stages which are characterized by selective expression of cell surface receptors and stage-specific genes, and which can be ordered in development by their status of rearrangement in the IgH and L chain gene loci (1–6). One selective marker of early B cell development is the surrogate light (SL)[1] chain composed of the VpreB and λ5 proteins (7–11).

Mice have two *VpreB* genes, *VpreB1* and *VpreB2*, separated by an unknown distance on chromosome 16 (8). The two genes show 97% identity at the nucleotide and amino acid level, giving rise to a difference of four amino acids in the protein. The *VpreB1* gene is expressed in all cells that express λ5, whereas *VpreB2* RNA is detected in ∼30% of these same cells as determined by single cell reverse transcription (RT)-PCR (12). Both *VpreB* gene products are functional and can form an SL chain with λ5 (12, 13).

The earliest identifiable B-lineage progenitor already transcribes, and probably also expresses as protein, the SL chain (14, 15). The cell population is in the process of DJ$_H$ rearrangements, and is phenotypically characterized as B220$^+$CD19$^-$c-kit$^+$flk-2$^+$. B220$^+$CD19$^+$c-kit$^+$flk-2$^-$ pre-BI cells follow, which have both *IgH* chain alleles DJ$_H$

rearranged and express the SL chain as protein associated with gp130 (13, 16). The function of this protein complex is unknown, especially because pre-BI cells are generated in λ5-deficient mice, even in elevated numbers (17).

At the transition of pre-BI to pre-BII cells, V to DJ$_H$ rearrangements are initiated. Whenever they occur in-frame, a μH chain can be produced which is then probed for its capacity to associate with SL chain and form a pre-B cell receptor (pre-BCR) on the cell surface (18, 19). The pre-BCR expressing pre-BII cells are stimulated to enter the cell cycle and become large pre-BII cells. They downregulate the expression of c-kit, SL-chain, and of the rearrangement machinery, i.e., recombination activating gene (*RAG*)-*1*, *RAG-2*, and terminal deoxynucleotidyl transferase (TdT), and upregulate CD25 expression (2, 20). Allelic exclusion is established at the IgH chain alleles, either because the first allele was nonproductively VDJ$_H$ rearranged, or because the cell is inhibited from recombining the second DJ$_H$ rearranged allele. The latter is thought to be effected both by the immediate downregulation of the rearrangement machinery, as well as by a closing of the DJ$_H$ rearranged allele, both signaled by the deposition of pre-BCR in the surface membrane of large pre-BII cells (21–24). Hence, cells with productive VDJ$_H$ rearrangements resulting in a μH chain which is incapable of forming a pre-BCR are not allelically excluded in wild-type B-lineage cells (25). Large pre-BII cells proliferate for two to five divisions, then come to rest as small pre-BII cells which continue to express CD25 (26).

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[1]*Abbreviations used in this paper:* BCR, B cell receptor; ES, embryonic stem; RAG, recombination activating gene; RT, reverse transcription; SL, surrogate light.

At the transition of large to small pre-BII cells, rearrangements at the *IgL* chain gene loci are initiated. Whenever they are productive and lead to the synthesis of a μH chain pairing IgL chain, immature sIgM+sIgD−AA4.1+ B cells are formed which become subject to selection by autoantigen (21). Negative selection deletes autoreactive B cells, whereas B-1 cells may be generated by positive selection (27). Finally, mature, conventional B cells appear to be generated in two steps, first by migration of immature cells from bone marrow to spleen, second by maturation of immature to mature, sIgM+sIgD+AA4.1− cells in spleen (28). SL chain becomes undetectable in small pre-BII cells, as large pre-BII cells loose pre-BCR surface expression. This appears to confine all functional influences of the SL chain to cells before the small pre-BII cell stage.

In mice lacking the transmembrane portion of μH chain (μmT) the development of B cells appears impeded at the transition from the pre-BI into large pre-BII stage, so that the cells can V to DJ$_H$ rearrange the *IgH* locus but cannot express a membrane-bound pre-BCR and are unable to enter the cell cycle (29). In the μmT mice, the μH chains are allelically included, i.e., cells expressing two μH chain proteins from the two IgH chain alleles are found in 9% of all cells at that stage (30). Mice lacking λ5 (λ5−/−) are also impaired in B cell development at the transition from pre-BI into the large pre-BII stage, again evident in a lack of proliferation of pre-BII cells (17, 31). However, λ5 deficiency does not result in a complete block in B cell development, as low numbers of small pre-BII, immature, and mature B lymphocytes are produced. Surprisingly, IgH allelic exclusion is functioning in λ5−/− mice (25). One hypothesis to explain this finding is that a pre-BII cell can make a modified pre-BCR consisting of μH chain and the VpreB polypeptide(s), unable to signal proliferation, but able to signal allelic exclusion.

We have recently described the analysis of mice lacking the *VpreB1* (*VpreB1*−/−) gene (32). These mice use a SL chain composed of VpreB2 and λ5 which appears to be sufficient, although not as efficient as VpreB1 (or VpreB1 plus VpreB2) to form a functional SL chain. A partial block at the transition from pre-BI to large pre-BII cells was found, possibly due to lowered efficiency of this SL chain in its capacity to pair with different μH chains. However, the cells that reach the large pre-BII stage are expanded in a normal fashion.

Here we have established mice lacking both *VpreB1* and *VpreB2* in the germline (*VpreB1*−/−*VpreB2*−/−) in order to analyze the combined effects of these two genes on B cell development and IgH allelic exclusion.

Materials and Methods

Targeting Vectors. Conventional DNA techniques were used to construct the targeting vectors (33). The *VpreB1* targeting vector has been described previously (32). In brief, the *VpreB1* vector contained 5.3 kb of genomic sequence from the *VpreB1* locus and the *neomycin* resistance gene. In the *VpreB2* targeting vector (129

DNA), the entire *VpreB2* coding sequence was replaced by the 1.9-kb *hygromycin* resistance gene. The final *VpreB2* targeting vector comprised from 5′ to 3′: 1.2 kb of 5′ *VpreB2* genomic sequence, the *hygromycin* resistance gene (opposite transcriptional orientation), and 7.2 kb of 3′ *VpreB2* genomic sequence (sequence data are available from GenBank/EMBL/DDBJ under accession no. AC005817). Restriction enzyme sites are indicated in Fig. 1 A (SalI site originates from the vector). Homologous integration introduced two new EcoRI restriction enzyme sites.

ES Cells and Transfections. Embryonic stem (ES) cells were grown on irradiated embryonic fibroblasts (resistant to G418 and hygromycin) in IMDM (Life Technologies) supplemented with MEM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, penicillin, and streptomycin, 0.1% LIF, and 15% FCS (Boehringer). The *VpreB1*-targeted Y2 clone (32) was used as the recipient of the *VpreB2* targeting vector. Resistant clones (150 μg/ml hygromycin) were picked 7–9 d after transfection, expanded, and frozen, and genomic DNA was prepared.

Screening of Targeted Clones. ES clones were screened by PCR using primers no. 1 and no. 2 (Fig. 1 A). Homologous recombination results in a product of 1.8 kb. Primer sequences: *VpreB2* no. 1, 5′-ATGCCCACTTGTCTGCCTCTATC-3′; *hygromycin*: no. 2, 5′-ACCGATGGCTGTGTAGAAGTACT-3′. Conditions: 40 cycles of 94°C, 30 s; 60°C, 30 s; 72°C, 2.5 min. A total of 585 hygromycin-resistant clones were analyzed and 6 clones were targeted in the *VpreB2* locus. Positive ES clones were also analyzed by Southern blotting. Several probes located outside the targeting vector were tested but on account of the high degree of repetitive sequences in the *VpreB2* region these did not hybridize specifically. One probe (Fig. 1 A) was better than the others; it also gave rise to a high background, but did confirm proper targeting of the *VpreB2* locus (data not shown).

Establishing Mice Targeted in Both the VpreB1 and VpreB2 Loci. The murine *VpreB1* and *VpreB2* genes are both located on chromosome 16 at an unknown distance from each other (8). As it was not possible to determine if the *VpreB2* locus had been targeted on the same chromosome as the *VpreB1*-targeted locus, five ES clones were injected into blastocysts, chimeric mice established and bred with C57BL/6 females. The agouti offspring (all five clones gave rise to germ line transmission) were screened for recombination at the *VpreB1* and *VpreB2* loci. Targeting on the same chromosome gives rise to 50% of the pups being positive for both targeting events (ES94 and ES163), whereas targeting on separate chromosomes gives rise to 50% of the pups being positive for the *VpreB1* and 50% for the *VpreB2* event (ES47, ES65, and ES223). *VpreB1*+/−*VpreB2*+/− heterozygous mice from ES163 and ES94 were then inter-crossed to establish *VpreB1*−/−*VpreB2*−/− mice. The mice have been bred and investigated under proper Project Licenses (80/1143, 1263) approved by the Home Office, UK.

Screening of VpreB1/VpreB2-targeted Mice. After establishing the ES94 and ES163 lines, offspring were screened by PCR. For the *VpreB1* locus, primer nos. 3, 4, and 5 were used (32). For the *VpreB2* locus, two separate reactions were performed using primer nos. 6 and 7, primer nos. 8 and 9 (Fig. 1): *VpreB1*: (no. 3) 5′-TGCTCATGCTGCTGGCCTAT-3′; *VpreB1*: (no. 4) 5′-CTCCGGAGCCCCACGGCA-3′; *Neomycin*: (no. 5) 5′-TTG-GCTACCCGTGATATTGCTGA-3′; *VpreB2*: (no. 6) 5′-CT-CATGCTGCTGGCCCAC-3′; *VpreB2*: (no. 7) 5′-TCGTG-GCTCCGGAGCCCCACAGCG-3′; *hygromycin*: (no. 8) 5′-GGGTAAATAGCTGCGCCGATGG-3′; *hygromycin*: (no. 9) 5′-GCCGCAGCGATCGCATCCATGG-3′. Conditions: *VpreB1*, 30 cycles of 94°C, 20 s; 55–60°C, 20 s; 72°C, 30 s; *VpreB2*, 4 min

at 94°C, then 30 cycles of 94°C, 30 s; 60°C, 20 s; 72°C, 15 s. *Hygromycin*: same conditions as *VpreB2*.

RT-PCR Analysis. Total RNA was prepared from ∼10^7 cells using RNAzol (Tel-Test Inc.) followed by cDNA reaction using random primers (Life Technologies). cDNAs were analyzed by PCR using primers specific for *HGPRT*, λ5, *VpreB1* (nos. 3 and 4), and *VpreB2* (nos. 6 and 7) as described previously (12).

Analysis of VpreB and λ5 Protein. Bone marrow cells from young mice were cultured on irradiated stromal cells (S17) in the presence of IL-7 (10% supernatant) in IMDM supplemented with 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% FCS. Pre-BI cells, which grow out after a week in culture (34), were analyzed on a FACSCalibur™ (Becton Dickinson) using mAbs VP245 recognizing VpreB1 and VpreB2, and LM34 recognizing λ5 (12, 13).

FACS® Analysis of Lymphoid Organs. Bone marrow cell suspensions were prepared by flushing out femurs using HBSS (Life Technologies) supplemented with 10 mM Hepes and 3% FCS. Spleen and thymus cell suspensions were prepared using conventional techniques. Peritoneal cells were prepared by lavage of the peritoneal cavity with 3–5 ml HBSS supplemented as above. Cells were stained in HBSS (supplemented as above) with either two or three mAbs in combination with propidium iodide (0.25 μg/ml PI, analyzed in FL3) to exclude dead cells and then analyzed on a FACSCalibur™ (Becton Dickinson). The following mAbs were used: FITC- and allophycocyanin (APC)-labeled anti-B220/CD45R (clone RA3-6B2); biotin- and FITC-labeled anti-CD19 (clone 1D3); biotin- and PE-labeled anti–c-kit/CD117 (clone ACK 45); biotin-labeled anti-CD43 (clone S7); biotin-labeled anti-CD25 (clone 7D4); biotin-labeled anti-IgM (clone R6-60.2); PE-labeled anti-IgM^a (clone DS-1); FITC-labeled anti-IgM^b (clone AF6-78); FITC-labeled anti-CD4 (clone RM4-5); biotin-labeled anti-CD8α (clone 53-6.7); biotin-labeled anti-CD5 (clone 53-7.3); biotin-labeled anti-Igκ (clone R5-240); and FITC-labeled anti-Igλ (clone R26-46; all from BD PharMingen). Biotin-labeled anti-IgD (clone 1.19), anti-λ5 (clone LM34), and anti-VpreB (clone VP245) were purified and biotinylated in the laboratory. Polyclonal FITC-conjugated goat anti–mouse-IgM was from BD PharMingen. When biotinylated mAbs were used, positive cells were revealed using PE-labeled streptavidin (Southern Biotechnology Associates, Inc.). To determine IgM allotype, cells from both femurs were stained with APC-labeled anti-B220, biotinylated anti-IgD, and PE-labeled anti-IgM^a in the presence of 5% rat serum for 30 min. The cells were washed twice and incubated with FITC-labeled anti-IgM^b and SA-Tricolor (Caltag) for 30 min in the presence of 5% rat serum, then washed as before. This procedure was crucial to achieve proper staining of the cells.

Serum Titres. 5-mo-old wild-type, heterozygous, and homozygous *VpreB1/VpreB2* double-mutant mice were bled and the sera analyzed for the presence of IgM. ELISA plates were coated with 2 μg/ml of an mAb recognizing mouse IgM (R6-60.2; BD PharMingen). The samples were serially diluted, added to the plates, and bound IgM was detected with a biotinylated goat anti–mouse IgM antiserum (Sigma-Aldrich) and streptavidin-biotinylated horseradish peroxidase (Amersham Pharmacia Biotech) using o-phenylene-diamine (Sigma-Aldrich) as substrate. The ELISA was measured in a Multiskan MCC/340 plate reader (Titertek) at 492 nm. The concentration of IgM was calculated using purified mouse IgM (Sigma-Aldrich) as standard.

Immunizations. Wild-type, heterozygous, and homozygous *VpreB1/VpreB2* double-mutant mice, at the age of ∼4–5 mo, were immunized with 100 μg alumprecipitate of either phenyl-

oxazolone coupled to OVA (Ox-OVA) or OVA, or 5 μg NP-Ficoll. 2 wk after immunization, the mice were bled and the sera analyzed for the presence of antigen-specific Abs (IgM, IgG). ELISA plates were coated with either Ox-BSA, OVA, or NP-OVA at a concentration of 10 μg/ml. Samples were serially diluted and added to the plates. Antigen-specific IgM was detected with a biotinylated goat anti–mouse IgM antiserum (Sigma-Aldrich), and IgG with a biotinylated goat anti–mouse IgG antiserum (Sigma-Aldrich). The detection was as described above.

Results

Establishment of VpreB1$^{-/-}$VpreB2$^{-/-}$ Mice. To analyze how the two *VpreB* genes, on chromosome 16 of the mouse, influence B cell development, the two genes were replaced by homologous integration of antibiotic resistance genes. Earlier we have described the targeting of the *VpreB1* gene (32), which yielded the Y2 ES clone that is neomycin resistant and heterozygous for the targeting event. This ES clone was used as recipient for the *VpreB2* targeting construct. The *VpreB2* targeting vector was made by substituting the *VpreB2* coding sequences for the gene encoding *hygromycin* resistance. It included ∼9 kb of *VpreB2* homologous sequences (Fig. 1 A). As both *VpreB* genes are located on chromosome 16, and if the targeting of the *VpreB2* construct occurs randomly and is not influenced by the first integration, half of all successfully targeted ES clones should have both targeting constructs (*VpreB1* and *VpreB2*) integrated on the same chromosome. Out of five double *VpreB1/VpreB2* targeted ES clones, two were found to have integrated the two vectors on the same chromosome (ES94 and ES163). The others, ES47, ES65, and ES223, were found to be targeted for *VpreB1* on one, and for *VpreB2* on the other chromosome 16.

Targeting of the *VpreB2* locus was determined by PCR, in which homologous recombination gives rise to a 1.8-kb product (Fig. 1 A, primer nos. 1 and 2). Fig. 1 B shows that ES94 and ES163 both gave rise to a 1.8-kb PCR product, whereas the original E14 and the recipient Y2 cells did not. Heterozygous and homozygous *VpreB1/VpreB2* double-mutant mice from ES94 and ES163 also produced the 1.8-kb PCR product whereas the respective wild-type mice did not, demonstrating that homologous recombination had occurred at the *VpreB2* locus. The targeting of the *VpreB2* locus was further confirmed by Southern blotting (see Materials and Methods, and data not shown). Further analysis of ES94 and ES163, using the *hygromycin* resistance gene as a probe, demonstrated that ES94 carried only one copy of the *VpreB2* targeting vector, whereas ES163 had integrated at least one more copy elsewhere in the genome (data not shown). To determine the status of the *VpreB1* locus, we used Southern blotting (32) and, as shown in Fig. 1 D, the *VpreB1* locus remained intact after the second targeting event. The deletion of the two *VpreB* genes was further confirmed by Southern blotting using *VpreB1* cDNA as a probe (data not shown). Thus, ES94 and ES163 gave rise to mice lacking both *VpreB1* and *VpreB2*. As the targeting of *VpreB1* and *VpreB2* was correct in both strains
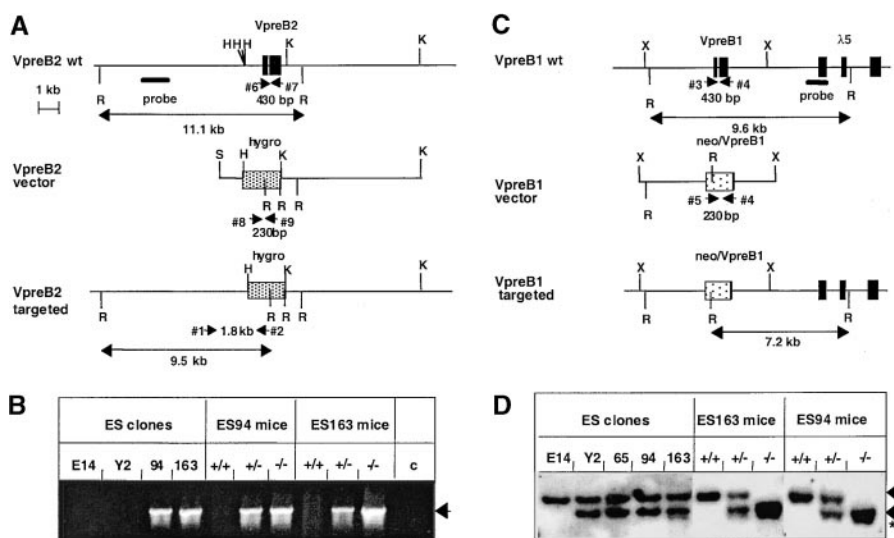
Figure 1. Homologous recombination of *VpreB1* and *VpreB2*. (A) *VpreB2* genomic locus before and after homologous integration. The targeting vector consists of 8.4 kb of *VpreB2* genomic sequences and the *VpreB2* coding sequence replaced by the *hygromycin* (hygro) resistance gene. Shown is the location of primers used for detection of homologous recombination (nos. 1 and 2), giving rise to a 1.8-kb PCR product. Offspring were later screened by PCR with primer nos. 6 and 7 and nos. 8 and 9, and the expected size of the respective product is indicated. The probe for Southern blotting and expected fragments are indicated. Restriction enzymes: R, EcoRI; H, HindIII; K, KpnI; S, SalI. (B) The arrow indicates the 1.8-kb PCR product (primer nos. 1 and 2) upon homologous recombination of the *VpreB2* locus. E14, original nontargeted ES cells; Y2, *VpreB1*-targeted E14 cells; ES94 and ES163, *VpreB2*-targeted Y2 cells; ES94 and ES163 mice, mice established from these ES clones. (C) The *VpreB1* genomic locus before and after homologous integration. The *VpreB1* targeting vector consisted of 5.3 kb of *VpreB1* genomic sequences and the *neomycin* (neo) resistance gene which replaced the *VpreB1* coding sequence. Primer nos. 3, 4, and 5 were used to screen for genotype and the expected size of the respective product is shown. The probe used for Southern blotting is indicated and expected fragments. Restriction enzymes: R, EcoRI; X, XbaI. (D) Homologous recombination of the *VpreB1* locus using the probe in C. Samples are as indicated in B, except ES65. The asterisk (*) shows the extra band detected in ES163 ES cells and in ES163 +/− and −/− mice.

we analyzed mice derived from both ES94 and ES163 (*VpreB1*$^{-/-}$*VpreB2*$^{-/-}$).

Lack of VpreB RNA Expression in VpreB1$^{-/-}$*VpreB2*$^{-/-}$ *Mice.* After establishing that the mice were correctly targeted, we investigated whether any RNA encoding *VpreB* could be detected. For this purpose, we analyzed RNA from bone marrow cells by RT-PCR using primers that were specific for the respective *VpreB* gene (12). RNA encoding *VpreB1* and *VpreB2* was detected in both wild-type and heterozygous mice, whereas there was no message found in *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice, as revealed for both ES163 and ES94 (Fig. 2 A). The λ5 gene, which encodes the other component of the SL chain, is located just downstream of the *VpreB1* gene (Fig. 1 C). To find out if the expression of this gene had been affected by the targeting events, expression of this gene was also analyzed. λ5 RNA was present in all samples, independent of genotype, suggesting that its expression had not been altered. As a control for the RT-PCR assays, RNA representing a house keeping gene (*HGPRT*) was measured and was detected in all bone marrow samples independent of genotype. Thus, *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice lacked *VpreB1* and *VpreB2* RNA but expressed λ5 RNA at apparently normal levels.

Lack of VpreB Protein Expression. The lack of *VpreB* RNA implied that there should also be a lack of VpreB protein. To test this, we analyzed in vitro–cultured pre-BI cells which express the SL chain on the cell surface together with a set of glycoproteins, i.e., the pro-BCR (13, 34, 35). Pre-BI cells from heterozygous ES94 mice expressed VpreB on the cell surface (Fig. 2 B), as demonstrated by staining with an mAb (VP245) recognizing both VpreB1 and VpreB2 (12, 13). In contrast, pre-BI cells from *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice were not recognized by the
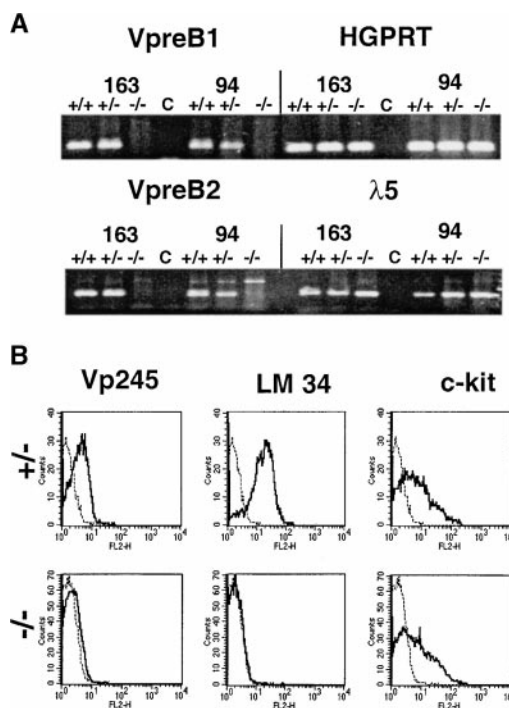

Figure 2. Lack of VpreB expression in *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ pre-B cells. (A) Total RNA from bone marrow cells of the indicated mice. RT-PCR was performed for *HGPRT*, *VpreB1*, *VpreB2*, and λ5. C indicates negative control. (B) In vitro cultured pre-BI cells were stained for B220 in combination with VP245 (anti-VpreB), LM34 (anti-λ5), or c-kit and analyzed by FACS®. The histograms from the VP245, LM34, and CD117 stains are shown.

VP245 Ab and, hence, did not express VpreB on the cell surface. The pre-BI cultures, independent of genotype, expressed both B220 (CD45R) and c-kit (CD117) on the cell surface (Fig. 2 B, and data not shown). Thus, pre-BI cells from the *VpreB1/VpreB2* double-deficient mice lacked surface VpreB expression.

As $\lambda 5$ is also part of the pro-BCR and was expressed as RNA in *VpreB1$^{-/-}$VpreB2$^{-/-}$* bone marrow cells, we analyzed the in vitro–cultured cells for the presence of this protein using the mAb LM34 (13). Pre-BI cells from heterozygous mice expressed $\lambda 5$ on the cell surface. In contrast, the cells from *VpreB1$^{-/-}$VpreB2$^{-/-}$* mice were not recognized by the LM34 Ab and thus did not express $\lambda 5$ on the cell surface (Fig. 2 B). Analysis of cells from ES163 mice demonstrated that both VpreB and $\lambda 5$ could be detected on the surface of pre-BI cells derived from wild-type and heterozygous but not homozygous *VpreB1/VpreB2* double-mutant mice (data not shown). Thus, the targeting of the two *VpreB* genes resulted in a lack of VpreB protein on the surface of pre-BI cells. In addition, although expressed as RNA, $\lambda 5$ protein was also not detected on the cell surface.

B Cell Development Is Impaired in VpreB1$^{-/-}$VpreB2$^{-/-}$ Mice at the Transition from Pre-BI to Pre-BII Cells. After confirming that the targeting of both *VpreB* genes on the same chromosome resulted in a lack of VpreB RNA and protein expression we analyzed whether this had an effect on B cell development. We observed no differences between wild-type and heterozygous *VpreB1/VpreB2* double-mutant mice (data not shown). The data from these mice were therefore pooled and represent control mice.

The number of cells in different bone marrow cell populations of control mice varied between two- and sevenfold depending on age. Therefore, the mice were separated into two age groups. Table I summarizes the data from mice aged 10 and 50–70 d, respectively. As shown in Table I, the lack of both *VpreB1* and *VpreB2* caused a slight decrease in total number of nucleated bone marrow cells in young mice. This difference became more apparent in older mice (~60% of control). Furthermore, the numbers of B220^{+} cells in young and old *VpreB1$^{-/-}$VpreB2$^{-/-}$* mice were also lower, around half that of control mice. The number of CD19^{+} lymphocytes was similarly decreased (Table I). It has been shown by Rolink et al. that most of the B220^{+}CD19^{-} cells in bone marrow not only include pro-B cells but also progenitors of other cell types, e.g., NK cells (36). However, the total number of B220^{+}CD19^{-} cells in *VpreB1$^{-/-}$VpreB2$^{-/-}$* mice was similar to that in control mice. Hence, it appears that the lack of both *VpreB1* and *VpreB2* has either no or very little effect on this cell population.

To determine at which point of development the defect in both *VpreB* genes impedes B-lineage cell generation the sizes of cellular pools of pre-BI, large and small pre-BII, and immature and mature B cells in bone marrow were determined by FACS® analysis using B220, CD19, c-kit, CD25, and cell size as distinguishable markers (Fig. 3 A and Table I). *VpreB1/VpreB2* double-deficient mice showed an about twofold increase in the number of B220^{+}c-kit^{+} pre-BI cells

Table I. *Impaired B Cell Development in VpreB1$^{-/-}$VpreB2$^{-/-}$ Mice*

Bone marrow population	Days[*]	N[‡]	Control (mean ± SEM)	N[‡]	Homozygous (mean ± SEM)
Total	10	3	14.60 ± 3.54	3	11.83 ± 0.44
	50–70	6	33.92 ± 3.19	6	20.70 ± 4.32
B220^{+}	10	3	2.97 ± 0.88	3	1.57 ± 0.13
	50–70	6	10.63 ± 1.03	6	3.95 ± 0.80
CD19^{+}	10	3	2.84 ± 0.88	3	1.38 ± 0.10
	50–70	6	9.98 ± 1.10	6	3.07 ± 0.79
B220^{+}c-kit^{+}	10	3	0.40 ± 0.13	3	1.01 ± 0.11
	50–70	6	0.94 ± 0.30	6	1.71 ± 0.41
B220^{+}CD25^{+}	10	3	1.24 ± 0.29	3	0.03 ± 0.003
	50–70	6	6.33 ± 0.81	6	0.64 ± 0.20
B220^{+}IgM^{+}	10	3	0.48 ± 0.10	3	0.02 ± 0.01
	50–70	6	3.43 ± 0.36	5	0.40 ± 0.15

The mean and SEM of absolute numbers ($\times 10^6$) are shown for the indicated cell populations in the bone marrow of control and homozygous VpreB1/VpreB2 double-mutant mice.
[*]Age of mice.
[‡]Number of mice analyzed.

compared with control mice. The number of CD19^{+}c-kit^{+} cells was also increased about twofold (data not shown). By contrast, the number of B220^{+}CD25^{+} pre-BII cells was severely reduced. In young mice, the difference in cell numbers, compared with control littermates, was ~40-fold, whereas it was ~10-fold in older mice. These results suggest that the lack of *VpreB1* and *VpreB2* expression result in a defect in the generation of pre-BII from pre-BI cells, i.e., at the stage of surface pre-BCR expression.

Lack of Proliferative Expansion at the Pre-BII Cell Stage. Pre-BII cells (B220^{+}CD25^{+}) can be divided into large cycling and small resting cells (2). We determined the ratio between these subsequent stages of B cell development in *VpreB1/VpreB2* double-deficient old mice. The number of B220^{+}CD25^{+} cells in young *VpreB1$^{-/-}$VpreB2$^{-/-}$* mice was too low to give meaningful results (Fig. 3 A; <1%). In control mice, the ratio of large versus small pre-BII cells was 1:4, whereas in *VpreB1/VpreB2* double-mutant mice the ratio was 1:1–1.5. Thus, in *VpreB1/VpreB2* double-deficient mice there was an ~5-fold reduction in large and an ~15-fold reduction in small pre-BII cells. Hence, the lack of both VpreB1 and VpreB2 proteins affected the production of both large and small pre-BII cells, with the greatest impact on small pre-BII cells. It suggests that, as in $\lambda 5^{-/-}$ mice, the proliferative expansion of pre-BII cells does not occur and/or that the progression from large to small cells is impaired.

Reduced Numbers of Immature and Mature B Cells in Bone Marrow of VpreB1/VpreB2 Double-mutant Mice. In *VpreB1$^{-/-}$VpreB2$^{-/-}$* mice, the total numbers of sIgM^{+} immature and mature B cells were severely reduced, ~25-fold in young and 10-fold in old mice. Comparable reductions in num-
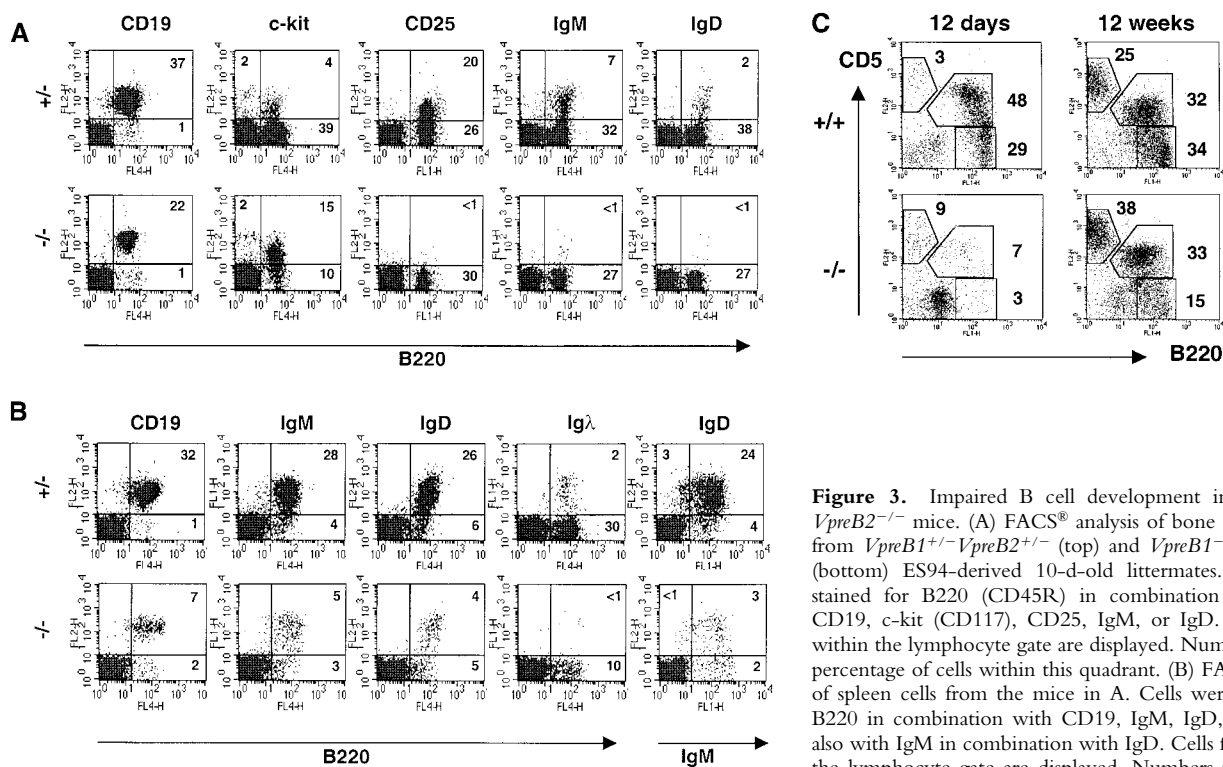
Figure 3. Impaired B cell development in $VpreB1^{-/-}$ $VpreB2^{-/-}$ mice. (A) FACS® analysis of bone marrow cells from $VpreB1^{+/-}VpreB2^{+/-}$ (top) and $VpreB1^{-/-}VpreB2^{-/-}$ (bottom) ES94-derived 10-d-old littermates. Cells were stained for B220 (CD45R) in combination with either CD19, c-kit (CD117), CD25, IgM, or IgD. Cells falling within the lymphocyte gate are displayed. Numbers indicate percentage of cells within this quadrant. (B) FACS® analysis of spleen cells from the mice in A. Cells were stained for B220 in combination with CD19, IgM, IgD, or Igλ, and also with IgM in combination with IgD. Cells falling within the lymphocyte gate are displayed. Numbers indicate percentage of cells within this quadrant. (C) FACS® analysis of peritoneal cells from $VpreB1^{+/+}VpreB2^{+/+}$ and $VpreB1^{-/-}VpreB2^{-/-}$ ES94 mice stained for B220 and CD5 aged 12 d and 12 wk, respectively. Cells falling within the lymphocyte gate are displayed. The numbers indicate the percentage of cells within these regions.

bers of sIgM$^+$κL$^+$ B cells were observed (data not shown). As shown in Fig. 3 A, sIgM$^+$sIgD$^+$ mature B cells in bone marrow were reduced as much as the sIgM$^+$ immature B cells.

As small pre-BII cells are the precursors of immature B cells, we analyzed the sizes of these two populations. Independent of genotype, the proportion of immature B cells was ~50% of the sIgM$^+$ population. In control mice, ~5 × 10^6 small pre-BII cells and ~1.5 × 10^6 immature B cells were found, whereas in the $VpreB1/VpreB2$ double-mutant mice these values were ~0.4 × 10^6 and ~0.2 × 10^6, respectively. From these data it appears likely that the $VpreB1/VpreB2$ double defect does not significantly affect the transition from small pre-BII to immature B cells in the bone marrow.

In conclusion, in the absence of VpreB1 and VpreB2 protein, bone marrow was enriched in pre-BI cells, decreased in large, and decreased even more in small pre-BII cells, whereas the differentiation of the latter population into immature B cells was unaffected. These data suggest a defect at the transition from pre-BI to large pre-BII cells, due to either a lack of proliferative expansion at, or an impairment of the progression through the pre-BII stage, resulting in the production of much lower numbers of pre-BII and immature B cells.

Decreased Numbers of B Cells in the Spleen of $VpreB1^{-/-}$ $VpreB2^{-/-}$ Mice. Bone marrow is a primary lymphoid organ which generates B lymphocytes that migrate as immature B cells into the spleen where they mature. There-

fore, we analyzed the effect of the $VpreB1/VpreB2$ double mutation on the generation of splenic B cells. Data shown in Fig. 3 B and Table II demonstrate that the lack of both $VpreB1$ and $VpreB2$ caused a strong reduction in the number of B220$^+$CD19$^+$ B cells. The number of cells defined as either B220$^+$ or CD19$^+$ cells decreased ~10-fold in young and ~5-fold in older mutant mice. Total nucleated cells were ~70% of normal in young and ~45% in older mice.

Only half of the B220$^+$ cells were also sIgM$^+$ in double-mutant mice, whereas 80–90% were sIgM$^+$ in control littermates. In the sIgM$^+$ B cell pool, an ~15- and 5-fold reduction was seen in $VpreB1^{-/-}VpreB2^{-/-}$ young versus old mice, respectively. Further determinations of the numbers of sIgD-expressing cells showed that 60–70% of the sIgM$^+$ cells were also IgD$^+$ in young double-mutant mice, compared with ~90% in control littermates. However, in old double-mutant mice this percentage had reached normal levels (90–100%). In actual cell numbers, ~5-fold fewer immature (sIgM$^+$sIgD$^-$) and ~20-fold fewer mature (sIgM$^+$sIgD$^+$) B cells were found in young, and ~5-fold fewer mature B cells in old double-mutant mice. Thus, with age, the number of B cells increased in $VpreB1^{-/-}VpreB2^{-/-}$ mice, and most of these cells were of a mature phenotype.

Furthermore, the ratio of Ig κL versus λL chain expression in splenic B cells was found to be similar (15:1 versus 30:1) as determined in old control and double-mutant mice, respectively. This suggests that the lack of *VpreB1*

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and *VpreB2* expression did not affect the rearrangements at the *IgL* chain gene loci, and the repertoire selection of κL⁺ versus λL⁺ sIgM⁺ B cells. Comparable results were obtained with the second strain of *VpreB1⁻/⁻VpreB2⁻/⁻* mice (ES163, data not shown). In addition, no alterations were found in the numbers of thymocytes or in CD4⁺ and CD8⁺ T cells in the periphery of double-mutant mice (data not shown).

In conclusion, in the absence of *VpreB1* and *VpreB2*, the number of B-lineage cells in the spleen of young mice was greatly decreased with a large proportion of these cells expressing an immature phenotype. However, in older mice, most of the B cells showed a mature phenotype although their numbers had not fully recovered at 70 d of age.

Impaired Development of B-1a Cells in the Peritoneum of VpreB1⁻/⁻VpreB2⁻/⁻ Mice. Although a large proportion of splenic B cells are expected to belong to the conventional B cell compartment, B cells in the peritoneum appear enriched for B-1 type B cells (37). B-1a cells are characterized as B220⁺CD5⁺sIgM^high^sIgD^dull^. Fig. 3 C and Table III show that the number of B-1a cells was reduced eightfold in young (10–12-d-old) mice, whereas at 9–13 wk of age the B-1a compartment in the peritoneum of double-mutant mice had reached the size of that in wild-type littermates. Heterozygous *VpreB1/VpreB2* mice were comparable in their B-1a development to wild-type mice. The B220⁺CD5⁺ B-1a cells of double-mutant mice were also analyzed for other markers, such as sIgM^high^ and sIgD^dull^, and were found to be normal (data not shown).

The analyses of peritoneal cells also demonstrated that the percentages and absolute numbers of other B-lineage cells, i.e., of B220⁺CD5⁻ cells were reduced ~15-fold in 10–12-d-old *VpreB1/VpreB2* double-mutant mice (Fig. 3 C and Table III). Independent of genotype, almost all these cells

were IgM⁺ with varying IgD levels, demonstrating that they were B-lineage cells. In contrast to the B220⁺CD5⁺ B-1a cells, the B220⁺CD5⁻ B cells had not reached normal levels by 9–13 wk of age. In contrast, both to B-1a and B220⁺CD5⁻ cells, the B220⁻CD5⁺ non–B-lineage compartment was normal both in young and old double-mutant mice (Fig. 3 C and Table III). We conclude that the generation of the B-1a cell pool in peritoneum is initially affected by the lack of *VpreB1* and *VpreB2*. With age this pool fills to normal size even in *VpreB1⁻/⁻VpreB2⁻/⁻* mice. However, as with B cells in spleen of double-mutant mice, the remaining peritoneal B cell populations, which would be either B-1b or conventional B cells, have not yet fully recovered at 70 d of age.

Normal Levels of IgM in Serum. Sera from 5-mo-old *VpreB1/VpreB2* double-mutant mice were analyzed by ELISA for the amounts of IgM and found to be ~0.8–1 mg/ml in *VpreB1⁻/⁻VpreB2⁻/⁻* mice, compared with ~0.8–0.9 mg/ml in wild-type and heterozygous littermates. Thus, serum IgM levels appeared not to be negatively affected by the lack of *VpreB1* and *VpreB2*.

T Cell–dependent and –Independent Immune Responses. To investigate whether B cells in *VpreB1⁻/⁻VpreB2⁻/⁻* mice were able to mount an immune response, we immunized mice 4–5 mo of age with T cell–dependent antigens (oxazolone coupled to OVA or OVA without hapten) as well as with a T cell–independent antigen (NP-Ficoll). After 2 wk, the mice were bled and the antigen-specific Ab responses analyzed. The results in Fig. 4 demonstrate that *VpreB1⁻/⁻VpreB2⁻/⁻* mice were able to respond to both types of antigens. In terms of IgM and IgG responses against NP-Ficoll, *VpreB1⁻/⁻VpreB2⁻/⁻* mice mounted an as good immune response as wild-type and heterozygous control mice (Fig. 4, and data not shown). In the case of T cell–dependent antigens, *VpreB1⁻/⁻VpreB2⁻/⁻* mice showed slightly lowered antigen-specific IgG responses

Table II. *Decreased Numbers of B Cells in the Spleen of VpreB1⁻/⁻VpreB2⁻/⁻ Mice*

Population	Days*	N‡	Control (mean ± SEM)	N‡	Homozygous (mean ± SEM)
Total	10	3	45.50 ± 9.57	3	30.67 ± 4.06
	50–70	6	136.17 ± 10.80	6	59.33 ± 10.45
B220⁺	10	3	9.63 ± 2.38	3	1.10 ± 0
	50–70	6	44.83 ± 3.53	6	9.88 ± 1.77
CD19⁺	10	3	9.20 ± 2.60	3	0.99 ± 0.07
	50–70	6	43.80 ± 3.65	6	8.90 ± 1.45
B220⁺IgM⁺	10	3	8.48 ± 2.18	3	0.58 ± 0.06
	50–70	6	36.97 ± 3.15	6	8.58 ± 1.33
B220⁺IgD⁺	10	3	7.49 ± 2.02	3	0.39 ± 0.09
	50–70	6	39.82 ± 2.98	6	7.96 ± 0.90

The mean and SEM of absolute numbers ($\times 10^6$) are shown for the indicated cell populations in the spleen of control and homozygous VpreB1/VpreB2 double-mutant mice.
*Age of mice.
‡Number of mice analyzed.

Table III. *Reduction in B-1a B Cells in VpreB1⁻/⁻VpreB2⁻/⁻ Mice*

Age	Genotype	N*	B-1a	B220⁺CD5⁻	B220⁻CD5⁺
10–12 d	+/+	3	0.31 ± 0.04	0.17 ± 0.03	0.04 ± 0.01
	+/−	5	0.24 ± 0.10	0.14 ± 0.07	0.03 ± 0.01
	−/−	3	0.04 ± 0.01	0.01 ± 0	0.03 ± 0.01
16 d	+/+	2	0.58 ± 0.01	0.64 ± 0.05	0.08 ± 0.01
	+/−	6	0.80 ± 0.01	0.77 ± 0.12	0.14 ± 0.02
	−/−	2	0.15 ± 0.04	0.08 ± 0.01	0.13 ± 0.01
9–13 wk	+/+	5	31.25 ± 6.57	49.03 ± 7.90	29.42 ± 7.03
	+/−	6	33.05 ± 8.75	47.65 ± 9.01	29.51 ± 6.89
	−/−	8	21.22 ± 3.38	13.01 ± 1.73	31.08 ± 6.24

The mean ± SEM of absolute numbers (10^4) are shown for B-1a (B220⁺CD5⁺), B220⁺CD5⁻ and B220⁻CD5⁺ lymphocyte populations in the peritoneum of mice of indicated VpreB1/VpreB2 genotype and age.
*Number of mice.

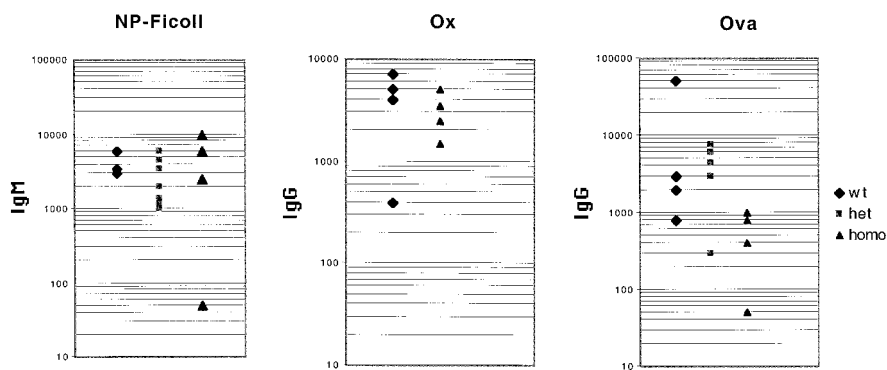**NP-Ficoll**   **Ox**   **Ova**

Figure 4. T cell–dependent and –independent immune responses. 4 to 5-mo-old mice of the indicated genotypes, were immunized with either NP-Ficoll or alum-precipitates of either oxazolone-ovalbumin (Ox) or ovalbumin (Ov). 2 wk later, the mice were bled and the sera analyzed in ELISA. The data are represented as titers. Preimmune sera were <1. wt, wild-type; het, heterozygous; homo, homozygous.

compared with control mice (Fig. 4). Thus, the emerging B cells in *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice are functional in terms of immune responses.

Allelic Exclusion in Immature B Cells. It has been proposed that the pre-BCR signals allelic exclusion of the IgH chain. As λ5$^{-/-}$ mice still display allelic exclusion, it has been hypothesized that VpreB alone, in the absence of λ5, can pair with μH chain and cause IgH allelic exclusion (25). This would imply that the lack of *VpreB1* and *VpreB2* would cause IgH allelic inclusion. We have shown previously that the single *VpreB1* deficiency does not abolish allelic exclusion (32). Hence, VpreB2 could still function as a component of a pre-BCR signaling allelic exclusion. This should no longer be possible in *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice. To test this, IgM^a/IgM^b heterozygous bone marrow cells from *VpreB1*/*VpreB2* double mutant and control ES94 mice were analyzed for expression of IgM^a and IgM^b allotypes on single B cells by FACS®. Upon allelic inclusion, the calculated theoretical value of double producers (IgM^{a}IgM^b) would be 12% (30). Due to the very low number of immature B cells (B220$^+$IgM$^+$IgD$^-$) in the bone marrow of double-mutant mice, all cells from both femurs were collected and analyzed. As shown in Fig. 5, in wild-type mice, ~55% of the IgM$^+$ cells expressed only IgM^a and 44% only IgM^b on their surface, whereas 1.2% of the cells stained positive for both markers. In the *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice, the percentages of cells expressing either IgM^a or IgM^b only were very similar to that of control mice. Most importantly,

the percentage of cells expressing both IgM^a and IgM^b was only 2%. This demonstrates that in the absence of *VpreB1* and *VpreB2*, immature B cells in the bone marrow were still allelically excluded at the IgH locus.

Discussion

The production and surface deposition of the pre-BCR on pre-BII cells has been shown to signal these cells to enter two to five rounds of cell division, expanding the individual μH chain repertoire within the developing B-lineage cells between 2- and 30-fold (26). Whenever the pre-BCR cannot be deposited on the surface, be it that the DJ$_H$/DJ$_H$-rearranged pre-BI cell has not entered an in-frame V$_H$ to DJ$_H$ rearrangement preventing the expression of a μH chain, that the μH chain made from a productive rearrangement cannot pair with SL chain (25), that the μH chain cannot be deposited in the surface membrane because of a lack of its transmembrane portion (30), that no Ig rearrangement can occur in pre-BI cells as is the case in SCID and *RAG*-deficient mice (38–40), or that production of the λ5-component of the pre-BCR is defective as in λ5$^{-/-}$ mice (17, 31), then this proliferation of pre-BII cells does not occur either in vivo or in vitro (26, 41). The analyses of the precursor B cell compartments in the bone marrow of *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice presented here allow us now to add one more important deficiency of the pre-BCR to this list, which abolishes the proliferative expansion of precursor B cells at the transition from pre-BI to pre-BII cells. Although we have shown previously that the single deficiency of one of the *VpreB* genes of the mouse, i.e., the *VpreB1* gene, does not impede in a major way this proliferative expansion of pre-BII cells (32), the double deficiency of both known *VpreB* genes does. This result then establishes that proliferative expansion of large pre-BII cells is controlled by surface deposition of a pre-BCR composed of μH chain, λ5-protein, and either VpreB1 or VpreB2 protein, and that no other VpreB-like protein can assume the role of the two VpreB proteins for this cellular function. Hence, VpreB3, the protein encoded by *8HS20*, also expressed in early B cell development, does not replace VpreB1 and VpreB2 in this signaling function (42).

VpreB1$^{-/-}$*VpreB2*$^{-/-}$ B-lineage cells show strikingly similar impairment in B cell development to λ5-defective

IgM^a +/+   -/-
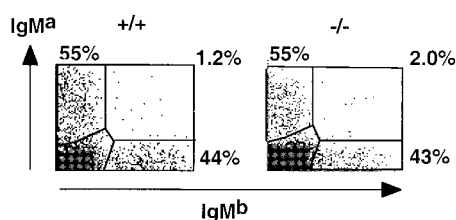55%   1.2%   55%   2.0%
44%   43%
IgM^b

Figure 5. Allelic exclusion of Ig μH chain expression on *IgM^{a}IgM^b* F1 B cells. FACS® analysis of bone marrow cells from ES94-derived *VpreB1/VpreB2* double-mutant littermates of the indicated genotype at the age of 74 d. All cells from both femurs were stained for B220 in combination with IgD, IgM^a, and IgM^b. Cells falling both within the lymphocyte and the B220$^+$IgD$^-$ gate were analyzed for expression of IgM^a and IgM^b. The numbers indicate the percentage of cells within these regions. Only 10% of the gated cells are shown for wild-type.

cells (17, 31). Because proliferative expansion of pre-BII cells is abolished, the generation of B-lineage cells from DJ_H/DJ_H-rearranged pre-BI cells becomes less efficient (31). For $\lambda5^{-/-}$ pre-BI cells it has been shown that the kinetics and rates of formation of sIgM$^+$ and sIg$^-$ B cells is indistinguishable from wild-type pre-BI cells. This argues that it is not efficiency of progression in differentiation through the pre-BI to pre-BII to immature B cell stages, but rather the lack of proliferation of $\lambda5^{-/-}$ pre-BII cells that accounts for the overall B cell developmental defect (31). Thus, in vitro studies of the kinetics and rates of differentiation have yet to be done with $VpreB1^{-/-}VpreB2^{-/-}$ pre-BI cells, in order to further investigate the similarity or possible difference in the differentiation capacities of wild-type, $\lambda5^{-/-}$, and $VpreB1^{-/-}VpreB2^{-/-}$ pre-BI cells.

Beyond the pre-BII stage of B cell development, no major effect was observed in $VpreB1^{-/-}VpreB2^{-/-}$ mice, a situation very similar to that in $\lambda5^{-/-}$ mice. This is illustrated by the B-1a cell compartment and the IgM levels in serum, which become normalized soon after birth, resembling those of wild-type mice, whereas the defect in the accumulation and proper filling of the conventional B cell compartment remains visible for several months after birth. Also, the decreased cellularity of the peripheral, conventional B cell compartment does not impede T cell–independent or T cell–dependent responses of the $VpreB1^{-/-}VpreB2^{-/-}$ mice.

Major changes in V_H-repertoires of μH chain originally expressed in V_H to DJ_H-rearranging precursor B cells are observed in wild-type mice as the pre-BCRs form (18). In fact, approximately half of all originally generated μH chains cannot pair with SL chain to form and deposit a pre-BCR on the cell surface. Hence, these are excluded from the proliferative expansion of large pre-BII cells and consequently are infrequent or absent in the repertoire of small pre-BII, immature, and mature B cells. In $\lambda5^{-/-}$ mice, IgL chains appear to take the functional role of SL chain. Therefore, a very similar change in the V_H repertoire of μH chains produced occurs in $\lambda5^{-/-}$ mice at the transition of precursor B into immature B cells (18). It remains to be investigated whether a similar change in the V_H repertoire of μH chains can also be observed in $VpreB1^{-/-}VpreB2^{-/-}$ mice which, again, would possibly be mediated by IgL chains.

It has been proposed that surface deposition of pre-BCRs on precursor B cells, at the transition from pre-BI to pre-BII cells, also signal the cells to allelically exclude the V_H to DJ_H rearrangement at the DJ_H-rearranged second IgH chain allele. This, in fact should be the case in half of all developing B-lineage cells in which the second allele remains DJ_H rearranged (5). The signal should cause a rapid downregulation of the expression of the rearrangement machinery, i.e., RAG-1, RAG-2, and TdT transcripts and proteins. It should also signal a closure of the DJ_H-rearranged IgH chain allele for further V_H to DJ_H rearrangements, especially in cells at later stages of development, i.e., in small pre-BII cells which reactivate the recombination machinery and V_L to J_L rearrange the κL and λL chain gene loci (20).

It was already surprising that $\lambda5$-deficient mice still showed allelically excluded B cell compartments (25). To explain allelic exclusion in $\lambda5^{-/-}$ mice, it was proposed that a modified pre-BCR consisting of μH chain and VpreB protein might act as signaling receptor (25). In line with this suggestion, it was observed that μH chains could bind to VpreB1 and VpreB2 proteins in the absence of $\lambda5$ protein, and that SL chain pairing μH chains could do so, whereas nonpairing μH chains could not (unpublished observations). In $VpreB1^{-/-}VpreB2^{-/-}$ precursor B cells, such modified pre-BCRs should not be made. Further, it has been shown that $\lambda5$ alone cannot form a $\lambda5$-μH chain modified pre-BCR, as in the absence of VpreB protein, $\lambda5$ appears not to bind to a whole series of SL chain pairing as well as nonpairing μH chains (unpublished observations). All the more surprising, therefore, is our present finding that allelic exclusion of the μH chain does still function in the double-defective $VpreB1^{-/-}VpreB2^{-/-}$ mice.

How, then, can allelic exclusion happen? It appears from studies with μH chain transmembrane region–deficient mice (30) and from studies of double μH chain–producing B cells of wild-type mice, in which only one μH chain can pair with SL chain, that membrane insertion of the μH chain is required for allelic exclusion. In the absence of both $VpreB1$ and $VpreB2$, two other candidates could possibly be part of a modified pre-BCR. One is the chaperone BiP, which is known to associate with μH chains before SL chain association (43). The other is the $8HS20$-encoded VpreB3 protein (42). We will have to combine defects in these genes with the $VpreB1$, $VpreB2$, and $\lambda5$ defects to investigate these possible pre-BCR–like associations and their roles in allelic exclusion. This is experimentally complicated, as $VpreB1$, $VpreB2$, $\lambda5$, and $VpreB3$ all appear located on the same chromosome (44).

Yet another scenario for allelic exclusion at the μH chain loci is suggested from the finding that in $\lambda5^{-/-}$ mice IgL chains take the place of SL chain to change the repertoire of V_H-representation during B cell development. In the absence of proliferative expansion of pre-BII cells in these $\lambda5^{-/-}$ mice, differentiation from pre-BI to small pre-BII cells might occur much more rapidly than in wild-type mice, so that V_L to J_L rearrangements might occur immediately following V_H to DJ_H-rearrangements, so that a BCR could signal the cell to turn off the recombination machinery and close the IgH chain loci. In line with such functional roles of IgL chains in precursor B cells, it has been observed that the abnormally early expression of transgenic IgL can repair the $\lambda5$ defect in B cell development, whereas in the absence of both $\lambda5$ and IgL there is a complete block in B-lymphocyte production (45, 46). Early expression of L chain would also predict that signaling by the pre-BCR of proliferative expansion of large pre-BII cells would, in fact, also signal inhibition of IgL chain gene expression in these wild-type cells. If such a scenario were correct, we would predict that a $VpreB1$-, $VpreB2$-, $VpreB3$-, and $\lambda5$-deficient mouse would still show allelic exclusion at the IgH chain loci.

References

1. Hardy, R., C. Carmack, S. Shinton, J. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213–1225.
2. Rolink, A., U. Grawunder, T.H. Winkler, H. Karasuyama, and F. Melchers. 1994. IL-2 receptor alpha chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. *Int. Immunol.* 6:1257–1264.
3. Osmond, D.G., A. Rolink, and F. Melchers. 1998. Murine B lymphopoiesis: towards a unified model. *Immunol. Today.* 19:65–68.
4. Ehlich, A., V. Martin, W. Muller, and K. Rajewsky. 1994. Analysis of the B cell progenitor compartment at the level of single cells. *Curr. Biol.* 4:573–583.
5. ten Boekel, E., F. Melchers, and A. Rolink. 1995. The status of Ig loci rearrangements in single cells from different stages of B cell development. *Int. Immunol.* 7:1013–1019.
6. Meffre, E., R. Casellas, and M.C. Nussenzweig. 2000. Antibody regulation of B cell development. *Nat. Immunol.* 1:379–385.
7. Sakaguchi, N., and F. Melchers. 1986. Lambda 5, a new light-chain–related locus selectively expressed in pre-B lymphocytes. *Nature.* 324:579–582.
8. Kudo, A., and F. Melchers. 1987. A second gene, VpreB in the lambda 5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2267–2272.
9. Karasuyama, H., A. Kudo, and F. Melchers. 1990. The proteins encoded by the VpreB and lambda 5 pre-B cell–specific genes can associate with each other and with mu heavy chain. *J. Exp. Med.* 172:969–972.
10. Pillai, S., and D. Baltimore. 1987. Formation of disulfide-linked $\mu 2 \omega 2$ tetramers in pre-B cells by the 18k ω-immunoglobulin light chain. *Nature.* 329:172–174.
11. Tsubata, T., and M. Reth. 1990. The products of pre-B cell-specific genes (lambda 5 and VpreB) and the immunoglobulin μ chain form a complex that is transported onto the cell surface. *J. Exp. Med.* 172:973–976.
12. Dul, J.L., Y. Argon, T. Winkler, E. ten Boekel, F. Melchers, and I.L. Mårtensson. 1996. The murine VpreB1 and VpreB2 genes both encode a protein of the surrogate light chain and are co-expressed during B cell development. *Eur. J. Immunol.* 26:906–913.
13. Karasuyama, H., A. Rolink, and F. Melchers. 1993. A complex of glycoproteins is associated with VpreB/lambda 5 surrogate light chain on the surface of mu heavy chain-negative early precursor B cell lines. *J. Exp. Med.* 178:469–478.
14. Mårtensson, I.-L., F. Melchers, and T.H. Winkler. 1997. A transgenic marker for mouse B lymphoid precursors. *J. Exp. Med.* 185:653–661.
15. Ogawa, M., E. ten Boekel, and F. Melchers. 2000. Identification of CD19-B220$^+$c-kit$^+$Flt3/Flk-2$^+$ cells as early B lymphoid precursors before pre-B-I cells in juvenile mouse bone marrow. *Int. Immunol.* 12:313–324.
16. Ohnishi, K., T. Shimizu, H. Karasuyama, and F. Melchers. 2000. The identification of a non-classical cadherin expressed during B cell development and its interaction with surrogate light chain. *J. Biol. Chem.* 275:31134–31144.
17. Kitamura, D., A. Kudo, S. Schaal, W. Muller, F. Melchers, and K. Rajewsky. 1992. A critical role of λ5 protein in B cell development. *Cell.* 69:823–831.
18. ten Boekel, E., F. Melchers, and A.G. Rolink. 1997. Changes in the V(H) gene repertoire of developing precursor B lymphocytes in mouse bone marrow mediated by the pre-B cell receptor. *Immunity.* 7:357–368.
19. Melchers, F. 1999. Fit for life in the immune system? Surrogate L chain tests H chains that tests L chains. *Proc. Natl. Acad. Sci. USA.* 96:2571–2573.
20. Grawunder, U., T.M. Leu, D.G. Schatz, A. Werner, A.G. Rolink, F. Melchers, and T.H. Winkler. 1995. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity.* 3:601–608.
21. Melchers, F., E. ten Boekel, T. Seidl, X.C. Kong, T. Yamagami, K. Onishi, T. Shimizu, A.G. Rolink, and J. Andersson. 2000. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. *Immunol. Rev.* 175:33–46.
22. Nussenzweig, M.C., A.C. Shaw, E. Sinn, D.B. Danner, K.L. Holmes, H.C.L. Morse, and P. Leder. 1987. Allelic exclusion in transgenic mice that express the membrane form of immunoglobulin μ. *Science.* 236:816–819.
23. Manz, J., K. Denis, O. Witte, R. Brinster, and U. Storb. 1988. Feedback inhibition of immunoglobulin gene rearrangement by membrane mu, but not by secreted mu heavy chains. *J. Exp. Med.* 168:1363–1381.
24. Nussenzweig, M.C., E.V. Schmidt, A.C. Shaw, E. Sinn, J. Campos-Torres, B. Mathey-Prevot, P.K. Pattengale, and P. Leder. 1988. A human immunoglobulin gene reduces the incidence of lymphomas in c-Myc-bearing transgenic mice. *Nature.* 336:446–450.
25. ten Boekel, E., F. Melchers, and A.G. Rolink. 1998. Precursor B cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B cell receptor surface expression. *Immunity.* 8:199–207.
26. Rolink, A.G., T. Winkler, F. Melchers, and J. Andersson. 2000. Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J. Exp. Med.* 191:23–32.
27. Potter, M., and F. Melchers. 2000. Opinions on the nature of B-1 cells and their relationship to B cell neoplasia. *Curr. Top. Microbiol. Immunol.* 252:307–324.
28. Rolink, A.G., T. Brocker, H. Bluethmann, M.H. Kosco-Vilbois, J. Andersson, and F. Melchers. 1999. Mutations affecting either generation or survival of cells influence the pool size of mature B cells. *Immunity.* 10:619–628.
29. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature.* 350:423–426.
30. Kitamura, D., and K. Rajewsky. 1992. Targeted disruption of m chain membrane exon causes loss of heavy-chain allelic

exclusion. *Nature*. 356:154–156.

31. Rolink, A., H. Karasuyama, U. Grawunder, D. Haasner, A. Kudo, and F. Melchers. 1993. B cell development in mice with a defective lambda 5 gene. *Eur. J. Immunol.* 23:1284–1288.

32. Mårtensson, A., Y. Argon, F. Melchers, J. Dul, and I.-L. Mårtensson. 1999. Partial block in B lymphocyte development at the transition into the pre-B cell receptor stage in VpreB1 deficient mice. *Int. Immunol.* 11:453–460.

33. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

34. Rolink, A., A. Kudo, H. Karasuyama, Y. Kikuchi, and F. Melchers. 1991. Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:327–336.

35. Shinjo, F., R.R. Hardy, and J. Jongstra. 1994. Monoclonal anti-lambda 5 antibody FS1 identifies a 130 kDa protein associated with lambda 5 and Vpre-B on the surface of early pre-B cell lines. *Int. Immunol.* 6:393–399.

36. Rolink, A., E. ten Boekel, F. Melchers, D.T. Fearon, I. Krop, and J. Andersson. 1996. A subpopulation of B220^{+} cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J. Exp. Med.* 183:187–194.

37. Hayakawa, K., R.R. Hardy, L.A. Herzenberg, and L.A. Herzenberg. 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med.* 161:1554–1568.

38. Bosma, G.C., R.P. Custer, and M.J. Bosma. 1983. A severe combined immunodeficiency mutation in the mouse. *Nature*. 301:527–530.

39. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1 deficient mice have no mature B and T lymphocytes. *Cell*. 68:869–877.

40. Shinkai, Y., G. Rathbun, K.-P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 68:855–867.

41. Ceredig, R., E. ten Boekel, A. Rolink, F. Melchers, and J. Andersson. 1998. Fetal liver organ cultures allow the proliferative expansion of pre-B receptor-expressing pre-B-II cells and the differentiation of immature and mature B cells in vitro. *Int. Immunol.* 10:49–59.

42. Ohnishi, K., and T. Takemori. 1994. Molecular components and assembly of mu.surrogate light chain complexes in pre-B cell lines. *J. Biol. Chem.* 269:28347–28353.

43. Thorens, B., M.F. Schulz, and P. Vassalli. 1985. Bone marrow pre-B lymphocytes synthesize immunoglobulin mu chains of membrane type with different properties and intracellular pathways. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:361–368.

44. Dunham, I., N. Shimizu, B.A. Roe, S. Chissoe, A.R. Hunt, J.E. Collins, R. Bruskiewich, D.M. Beare, M. Clamp, L.J. Smink, et al. 1999. The DNA sequence of human chromosome 22. *Nature*. 402:489–495.

45. Papavasiliou, F., M. Jankovic, and M.C. Nussenzweig. 1996. Surrogate or conventional light chains are required for membrane immunoglobulin mu to activate the precursor B cell transition. *J. Exp. Med.* 184:2025–2029.

46. Pelanda, R., S. Schaal, R.M. Torres, and K. Rajewsky. 1996. A prematurely expressed Ig(kappa) transgene, but not V(kappa)J(kappa) gene segment targeted into the Ig(kappa) locus, can rescue B cell development in lambda5-deficient mice. *Immunity*. 5:229–239.