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Only VpreB1, but not VpreB2, is expressed at levels which allow normal development of B cells

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Abstract

The surrogate light chain (SLC) consists of the polypeptides λ5 and, in the mouse, either VpreB1 or VpreB2. SLC associates with BILL-Cadherin and other glycoproteins to form the pro-B cell receptor (pro-BCR) at the pre-BI cell stage, and with the immunoglobulin μ heavy chain to form the pre-BCR at the pre-BII cell stage. The function of the pro-BCR, if any, is unknown, whereas the pre-BCR is crucial for proliferative expansion of pre-BII cells. To shed light on the functional properties of VpreB1 and VpreB2 *in vivo*, mice with either one or two VpreB1, or one or two VpreB2, alleles have been investigated. We show that B cell development in mice with two VpreB1 alleles is indistinguishable from that of normal mice. In contrast, mice with two VpreB2 alleles show an ~1.6-fold increase in pre-BI and a 35% decrease in pre-BII cell numbers, while mice with only one VpreB2 allele show a reduction in B cell development manifested in a 2-fold enrichment in pre-BI cells and a 75% reduction in pre-BII cells. However, such a gene dosage effect is not observed for VpreB1. Our results suggest that the difference between *VpreB1*- and *VpreB2*-deficient mice is due to lower VpreB2 protein expression, thus limiting the formation of pre-BCRs and thereby the number of large, cycling pre-BII cells.

Introduction

The surrogate light chain (SLC), encoded by the VpreB and λ5 genes, is specifically expressed in precursor B cells (1, 2). During early B cell development, SLC is initially expressed in pre-BI (DJ_H-recombined) cells, where it associates with BILL-Cadherin and other glycoproteins in a complex termed the pro-B cell receptor (pro-BCR) (3). At the following, large pre-BII (VDJ_H-recombined) cell stage, SLC together with Ig μ heavy (μH) chain forms the pre-BCR (4, 5). Pre-BCR expression has been proposed to act as a checkpoint to monitor expression of a functional μH chain, developmental progression and clonal expansion resulting later in a broad Ig repertoire (6).

In contrast to humans who only have one *VpreB* gene (7), mice have two, *VpreB1* and *VpreB2* (1, 8). The mouse *VpreB* genes, located on chromosome 16, are 97% identical at the nucleotide level, resulting in a difference of four amino acids in the corresponding protein. *VpreB1* is located ~4.5 kb upstream of λ5 while *VpreB2* is situated ~1 Mb downstream and in the opposite transcriptional orientation. Both VpreB1 and VpreB2 can associate with λ5 to form the SLC (9, 10).

Mice lacking either the entire SLC ($SL^{-/-}$) or its components, i.e. λ5T and $VpreB1^{-/-}VpreB2^{-/-}$ mice, have a mild enrichment in the number of pre-BI cells and greatly reduced numbers of pre-BII cells (11–13). The enrichment of pre-BI cells is currently not understood but may be explained for example by an unknown function of the pro-BCR, because the lack of BILL-Cadherin also results in an enrichment of pro-/pre-BI cells (14). The reduction in pre-BII cells is explained by the essential role of the pre-BCR for proliferative expansion of these cells (15–17). The amount of SLC that is required to generate normal-sized pre-BI and pre-BII cell populations and consequently normal B cell numbers is currently unknown. B cell development is unaffected in haplo-deficient SLC mice, i.e. $λ5^{+/-}$, $VpreB1^{+/-}VpreB2^{+/-}$ and $SL^{+/-}$ (11–13), which suggests that half the normal amount of protein is sufficient, provided that the expression from one allele results in a 50% reduction in protein levels.

We have previously shown that *VpreB1* deficiency results in a partial impairment of B cell development (18). Here we demonstrate, somewhat unexpectedly, that B cell development

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is normal in *VpreB2*-deficient mice. In pursuing the reason for this discrepancy we have also analyzed mice carrying either only one functional *VpreB1* or one functional *VpreB2* allele.

Methods

Generation of VpreB2-deficient mice

The generation and analysis of *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice have been described previously (12). Briefly, the *VpreB2* gene was replaced with the hygromycin resistance gene by homologous integration in ES cells, already heterozygous for *VpreB1* deficiency. As the two genes are located on the same chromosome, this resulted in 40% of the clones having undergone homologous recombination on the same chromosome as the *VpreB1*-targeting event and the other 60% on the non-targeted chromosome. Thus, this resulted in both single- and double-deficient mice. The *VpreB2* heterozygous mutant mice were intercrossed to establish *VpreB2*$^{-/-}$ mice. Two (out of three) *VpreB2*$^{-/-}$ mouse lines were analyzed in detail and as both phenotypes were identical the results of only one line are shown. These initial analyses were carried out on mice of mixed genetic background. The *VpreB2*, *VpreB1* and *VpreB1/VpreB2* double-deficient mice were then backcrossed for >10 generations onto the C57BL/6 background. *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ were thereafter crossed with either *VpreB1*$^{-/-}$ or *VpreB2*$^{-/-}$ mice to generate offspring with either one VpreB1 or one VpreB2 allele. Mice were bred in the Babraham Institute's animal facility under home office project licenses 80/1501, 1143 and 1763. Gene targeted mice were genotyped by PCR as previously described (12, 18) and analyzed at 3–8 weeks of age.

FACS analysis of lymphoid organs

Bone marrow (BM) and spleen cell suspensions were prepared and stained with antibodies as described previously (12) except anti-Ki-67 (clone B56) and PE-labeled anti-CD25 (clone 7D4) (BD PharMingen, Heidelberg, Germany). To investigate proliferation, BM B220$^+$CD25$^+$ cells were sorted on a FACSAria (Becton Dickinson), fixed in ethanol, stained with propidium iodide (Sigma) in the presence of RNaseA (Sigma) and analyzed using CellQuest (Becton Dickinson), as previously described (19).

For enzymatic amplification staining of cell surface molecules, *ex vivo* BM cells were incubated at 37°C for 1 h followed by a B cell-enrichment step using anti-CD19 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were thereafter stained with biotinylated antibodies recognizing λ5 (LM34), VpreB1/VpreB2 (VP245) (3) or the pre-BCR (SL156) (20). The enzymatic amplification was performed using a kit for biotinylated primary antibodies (EAS-B, Flow-Amp, Cleveland, USA), according to the manufacturer's instructions. In some instances markers for additional cell-surface molecules were included in the last step of the amplification procedure.

For intracellular antibody staining, BM cells from 2 to 5 mice were pooled, enriched for B-lineage cells using anti-CD19 magnetic beads and stained for the expression of cell surface markers as described above. After fixation with formaldehyde the cells were permeabilized and stained intracellularly, according to the manufacturer's protocol (Fix & Perm, Cell Permeabilization Kit, Caltag Laboratories, Burlingame, CA, USA). The biotinylated antibodies used were LM34 and VP245 or an isotype control, biotinylated rat IgG2a (clone R35-95, BD PharMingen), as well as polyclonal FITC-labeled goat anti-mouse IgM or goat IgG used as an isotype control (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

Immunofluorescence microscopy

For immunofluorescence microscopy, BM cells from *VpreB1*$^{-/-}$, *VpreB2*$^{-/-}$ and *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice were cultured on irradiated stromal cells in the presence of IL-7 for 7 days. Cells were fixed onto coverslips pre-coated with 0.01% poly-L-lysine solution and stained with LM34 or VP245, followed by FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch, Baltimore, MD, USA). Coverslips were analyzed (×63 magnification) under a Zeiss Axiophot fluorescent microscope (Carl Zeiss Ltd, Jena, UK). Using this protocol, both intracellular and surface antigens are stained.

Reverse transcription–PCR analysis

BM cells were enriched for CD19-positive cells using magnetic beads and sorted for CD19$^+$c-kit$^+$ cells. Total RNA was prepared from 2×10^5 sorted cells using RNA-Bee (AMS Biotechnology, Oxon, UK) followed by cDNA synthesis using random primers (Life Technologies). The cDNAs were analyzed by PCR using primers specific for *VpreB1*, *VpreB2,* λ*5* and *HGPRT* or with primers that detect both VpreB1 and VpreB2 (11).

Results

Normal B cell development in VpreB2-deficient mice

To investigate whether the absence of VpreB2 resulted in any effect on B cell development, *VpreB2*-deficient (*VpreB2*$^{-/-}$) mice were generated (12). Successful targeting of the *VpreB2* gene was confirmed by Southern blotting (12) and by the fact that mRNA encoding *VpreB2* was not detected in homozygous *VpreB2*-deficient mice, whereas mRNA from both *VpreB1* and λ*5* were detected (Supplementary Figure 1, available at *International Immunology* Online). After confirming that targeting of the *VpreB2* gene resulted in a loss of *VpreB2* but not *VpreB1* and λ*5*, BM B cell development was investigated for possible lesions. There was no difference between *VpreB2*$^{+/+}$, *VpreB2*$^{+/-}$ and *VpreB2*$^{-/-}$ mice in the number of pre-BI (B220$^+$CD19$^+$c-kit$^+$), pre-BII (B220$^+$CD25$^+$) and immature/mature (B220$^+$IgM$^+$IgD$^{+/-}$) B cells (Table 1 and data not shown). Although there was no obvious effect on BM B cell development in *VpreB2*$^{-/-}$ mice, it could be that there was some effect on the peripheral B cell pool. However, similar numbers of IgM$^+$ and IgD$^+$ B cells were observed in the spleen of *VpreB2*$^{+/-}$ and *VpreB2*$^{-/-}$ mice (Table 1). Analysis of peritoneal B cells revealed that the numbers of B-1 and B-2 B cells were also similar in these mice (data not shown). Furthermore, serum IgM levels were normal as were the responses to T-cell-dependent (NP-OVA) and T-cell-independent (NP-Ficoll) antigens (data not shown). These results demonstrate that BM B cell development in *VpreB2*-deficient mice is normal, resulting in the generation of normal numbers of

peripheral B-1 and B-2 B cells. Furthermore, these B cells are functional, as reflected in normal serum IgM levels as well as normal T cell-dependent and -independent immune responses.

Comparison of VpreB2$^{-/-}$ and VpreB1$^{-/-}$ mice

The above results demonstrate that the absence of *VpreB2* has no effect on BM B cell development, which is in contrast to mice lacking *VpreB1* (*VpreB1*$^{-/-}$) (18), in which there is an impairment at the transition from the pre-BI to the pre-BII cell stage. This difference was confirmed after backcrossing the two lines onto the C57BL/6 background (Fig. 1A). In the BM, a 1.6-fold enrichment of pro-/pre-BI (B220$^+$c-kit$^+$) cells was observed in *VpreB1*$^{-/-}$ compared with *VpreB2*$^{-/-}$ mice. At the following pre-BII stage (B220$^+$CD25$^+$), cell numbers were reduced by ~35% resulting in fewer (reduced by ~40%) IgM$^+$ cells in *VpreB1*$^{-/-}$ compared with *VpreB2*$^{-/-}$ mice. Thus, in the presence of only VpreB1 B cell development is normal, whereas in mice expressing only VpreB2 it is not.

The proliferation of pre-BII cells is unaffected in mice expressing only VpreB2

The lack of either *VpreB1* and *VpreB2*, *λ5* or the entire SLC results in a 2- to 3-fold enrichment of pre-BI and a 20- to 40-fold reduction of pre-BII cells (11–13), the latter ascribed to a lack of pre-BII cell proliferation (15–17). Because the deficiency of *VpreB1* also resulted in an enriched pre-BI and a mildly decreased pre-BII cell population, we reasoned that this may be the result of an effect on pre-BII cell proliferative expansion. Based on forward scatter analysis, the pre-BII population can be divided into large cycling and small resting cells in a ratio of ~20–25% large to 75–80% small cells (21). This ratio changes to ~40:60% in *VpreB1/VpreB2* double-deficient mice but was previously found to be unaffected in *VpreB1*$^{-/-}$ mice (12, 18). A similar analysis performed on the backcrossed *VpreB1*$^{-/-}$ as well as *VpreB2*$^{-/-}$ mice showed that the ratio of large to small pre-BII cells in the two genotypes was normal (data not shown). To further investigate proliferation, levels of the proliferation-associated antigen, Ki-67, and the proportion of cells in S/G_2/M of the cell cycle were analyzed. Independent of genotype, the vast majority of pre-BII cells expressed Ki-67 (Fig. 1B). Given that large cycling pre-BII cells are by definition Ki-67$^+$, this indicates that the majority of small resting pre-BII cells must also be positive. Thus, recently cycling cells retain expression of Ki-67, in contrast to the majority of re-circulating, mature B cells which are Ki-67 negative (data not shown). Furthermore, determination of the proportion of pre-BII cells in S/G_2/M phase of the cell cycle revealed a similar percentage (12–13%) in both *VpreB1*$^{-/-}$ and *VpreB2*$^{-/-}$ mice (Fig. 1B). Thus, these data would suggest that the proportion of cycling pre-BII cells is similar, regardless of whether they express either VpreB1 or VpreB2. Because the total number of pre-BII cells is reduced in VpreB1-deficient mice (Fig. 1A), there is a consequent reduction in the number of cycling pre-BII cells.

Table 1. Cell populations in *VpreB2*$^{-/-}$ mice

	+/–	–/–
BM[a]		
Nucleated cells	32.3 ± 7.3	33.4 ± 4.0
B220$^+$	7.1 ± 2.1	7.9 ± 1.7
CD19$^+$	6.9 ± 1.9	7.6 ± 1.6
B220$^+$c-kit$^+$	0.5 ± 0.1	0.6 ± 0.2
B220$^+$CD25$^+$	4.6 ± 1.9	4.9 ± 1.2
B220$^+$IgM$^+$	1.8 ± 0.5	1.8 ± 0.4
Spleen[b]		
Nucleated cells	161.0 ± 58.0	173.0 ± 54.0
B220$^+$	49.0 ± 7.4	48.0 ± 13.8
B220$^+$IgM$^+$	43.0 ± 5.4	43.0 ± 12.1
B220$^+$IgD$^+$	34.0 ± 4.8	36.0 ± 13.5

The mean and SD of absolute cell numbers ($\times 10^6$) are shown (two femurs for BM).
[a]$N = 8$. [b]$N = 3$. Mice were 18–21 days old.

VpreB2 mRNA levels are lower than those of VpreB1

The above results show that *VpreB1* and *VpreB2* differ in their ability to support early BM B cell development. There are several explanations that could account for this difference. For instance, different μH chains differ in their ability to pair with SLC (22) and some of these μH chains are able to associate with VpreB in the absence of λ5. However, we were unable to find any difference between the VpreB1 and VpreB2 polypeptides in their ability to pair with different μH chains (22).

Another possibility could be an effect on the level of SLC, due to a change in λ5 levels. In this scenario, the neomycin cassette, which replaces the deleted *VpreB1* gene, might affect the expression of the neighboring *λ5* gene. However, the *λ5* mRNA steady-state level in both total BM and sorted pre-BI (CD19$^+$c-kit$^+$) cells from *VpreB1*$^{-/-}$ and *VpreB2*$^{-/-}$ mice was similar as determined by semi-quantitative reverse transcription (RT)-PCR (Fig. 2 and data not shown). Thus, the difference

Table 2. *VpreB* gene dosage effect on BM cell populations

VpreB1 *VpreB2*	+/– +/–[a]	+/– –/–	–/– +/–	–/– –/–
Lymphocytes	10.57 ± 0.80	10.10 ± 3.12	7.96 ± 2.46	7.16 ± 1.53
B220$^+$CD19$^-$c-kit$^+$	n.d.	0.016 ± 0.008	0.014 ± 0.005	0.01 ± 0
B220$^+$c-kit$^+$	0.36 ± 0.06	0.33 ± 0.14	0.71 ± 0.22	0.67 ± 0.22
B220$^+$CD25$^+$	3.30 ± 0.61	3.60 ± 1.36	0.94 ± 0.29	0.07 ± 0.02
B220$^+$IgM$^+$	1.70 ± 0.24	1.33 ± 0.42	0.31 ± 0.12	0.04 ± 0.01

The mean and SD of absolute cell numbers ($\times 10^6$) from one femur are shown (6–10 mice per genotype). Mice were 3–4 weeks old. n.d., not done.
[a]Mice were 8 weeks old.

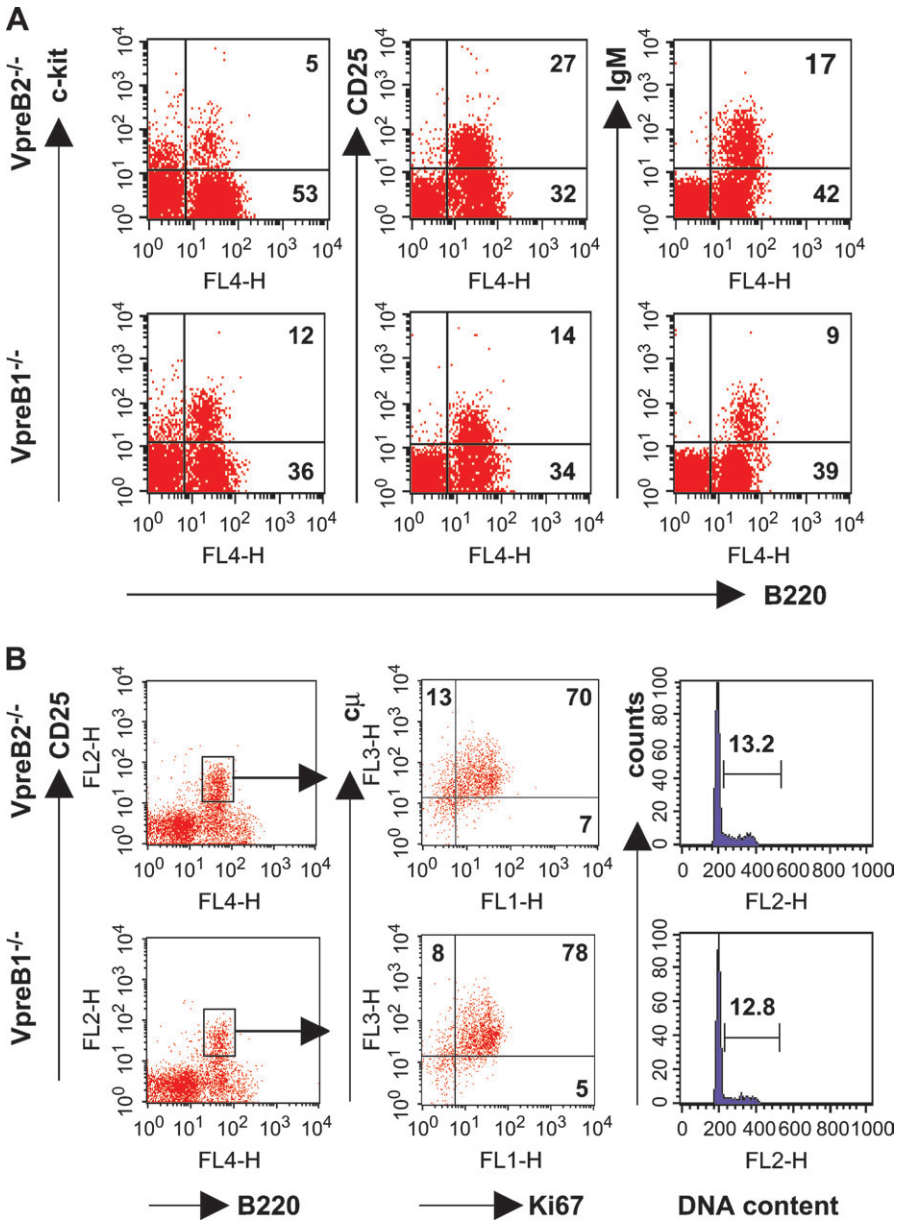

Fig. 1. Comparative FACS analysis of BM cells from *VpreB2*$^{-/-}$ and *VpreB1*$^{-/-}$ mice. (A) Analysis of BM cell populations after staining for the indicated markers. Viable cells within the lymphocyte gate are shown. Numbers indicate the percentage of cells within the quadrant. The mean absolute numbers ($\times 10^6$) for 5–8 mice (3 weeks) of each genotype were for VpreB2$^{-/-}$ mice: lymphocytes, 8.6 ± 1.3; B220$^+$c-kit$^+$, 0.36 ± 0.13; B220$^+$CD25$^+$, 2.0 ± 0.70; B220$^+$IgM$^+$, 0.7 ± 0.16; and for VpreB1$^{-/-}$ mice: lymphocytes, 8.0 ± 2.2; B220$^+$c-kit$^+$, 0.56 ± 0.22; B220$^+$CD25$^+$, 1.32 ± 0.60; B220$^+$IgM$^+$, 0.4 ± 0.21. (B) Analysis of pre-BII cell proliferation. Cells were stained for indicated markers. Middle panels, expression levels of cytoplasmic μ (cμ) and nuclear proliferation antigen Ki-67 after gating on pre-BII (B220$^+$CD25$^+$) cells. The percentages of cells in the relevant quadrants are shown. Right panels, DNA content in nuclei after sorting B220$^+$CD25$^+$ cells. The marker indicates the percentage of cells in S/G_2/M phases of the cell cycle determined using propidium idodide.

in phenotype between mice expressing either VpreB1 or VpreB2 is not due to an effect on λ*5* mRNA levels.

Since *VpreB2* is located 1 Mb downstream of *VpreB1*, it is unlikely that targeting of *VpreB1* would have an effect on the expression of *VpreB2*. However, several lines of evidence suggest that *VpreB1* and *VpreB2* are both expressed albeit at different levels. Firstly, in a panel of 10 pre-B cell lines all express both *VpreB* genes (10), although the level of VpreB2 may be lower than that of VpreB1 (23). Secondly, single-cell RT-PCR analysis of B220$^+$c-kit$^+$ cells showed that all express VpreB1 and λ5, whereas VpreB2 mRNA was detectable in ~30% of these cells. However, we suggested this as a minimum figure on account of assay sensitivity limits (10). Thirdly, the activity of the VpreB2 promoter is ~3-fold lower compared with that of the VpreB1 (10). Taken together, this suggested that VpreB2 is expressed in all pre-BI cells, but at a lower level

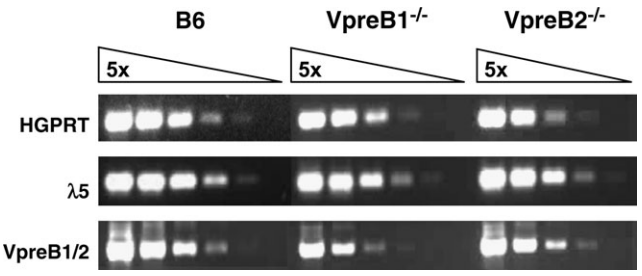

Fig. 2. Semi-quantitative RT-PCR. cDNA from BM pre-BI ($CD19^+$ c-kit^+) cells from mice of the indicated genotypes (C57BL/6; B6) was amplified for HGPRT, λ5 and VpreB (using a primer pair amplifying VpreB1 and VpreB2). Five-fold serial cDNA dilutions were used.

than VpreB1, although it was possible that only a proportion of cells express VpreB2. We therefore set out to investigate VpreB1 and VpreB2 mRNA levels using a primer pair which is equally efficient in detecting both. For this pre-BI cell ($CD19^+$c-kit^+) RNA from *VpreB1*$^{-/-}$ and *VpreB2*$^{-/-}$ mice was analyzed. As shown in Fig. 2, the amount of VpreB2 mRNA is about one-third that of VpreB1 (normalized against HGPRT). Thus, in pre-BI cells from normal mice, VpreB2 mRNA levels correspond to ~25% of total VpreB levels.

VpreB2 protein levels are lower than those of VpreB1 on the surface of pre-BI cells

To investigate whether the low *VpreB2* mRNA level was reflected in protein level, and if so, whether this was due to an overall lower *VpreB2* level or only a fraction of cells expressing *VpreB2*, *ex vivo* BM pre-BI ($B220^+CD19^+$c-kit^+) cells from *VpreB1*$^{-/-}$ and *VpreB2*$^{-/-}$ mice were analyzed for surface VpreB and λ5 protein expression. As shown in Fig. 3(A) (top panel), in *VpreB2*$^{-/-}$ mice, VpreB1 levels varied greatly between cells, with fluorescence levels spanning almost two orders of magnitude. Furthermore, two populations were observed, one with high and the other with low levels. Similarly, two VpreB2-expressing cell populations were observed in *VpreB1*$^{-/-}$ mice, one expressing low and the other higher levels (middle panel). However, the VpreB2 'high' levels were lower than those of the VpreB1 'high'-expressing population, whereas those of the low-expressing populations were similar (bottom panel). Gating on the positive cells showed that 81% expressed VpreB1 and 59% VpreB2. Two sub-populations were also observed in terms of λ5, with a similar proportion of VpreB1- and λ5-expressing (~80%) cells, whereas there were more cells expressing λ5 (77%) than VpreB2 (59%). In addition, independent of genotype, the mean fluorescence intensity (MFI) was higher for λ5 than VpreB (126 versus 83 and 58 versus 36 in *VpreB2*$^{-/-}$ and *VpreB1*$^{-/-}$ mice, respectively). This may indicate that λ5 is expressed in a protein complex lacking VpreB which would be supported by a previous observation that λ5 is detected on the surface of pre-B cells from *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice (24). Nevertheless, this shows that pre-BI cells can be divided into two sub-populations in terms of surface SLC expression, whether they express VpreB1 or VpreB2, although when it comes to VpreB2, the level on the 'high'-expressing subpopulation is lower compared with VpreB1.

VpreB2 and VpreB1 protein levels are similar on the surface of pre-BII cells

The pre-BCR is expressed at very low levels on large pre-BII ($CD19^+CD25^+$) cells, while small pre-BII cells do not express SLC, i.e. are pre-BCR negative (25, 26). In agreement with this, analysis of pre-BII cells from *VpreB2*$^{-/-}$ mice confirmed that the surface levels of pre-BCR, as well as VpreB1 and λ5, were very low (Fig. 3B). In *VpreB1*$^{-/-}$ mice, the levels of pre-BCR, VpreB2 and λ5 were also very low, although it was not obvious from this analysis whether there was a difference between cells expressing either VpreB1 or VpreB2. Nevertheless, at the pre-BII cell stage, there is no distinct difference between VpreB1 and VpreB2, although this may be due to poor resolution since the SLC levels are much lower on pre-BII compared with pre-BI cells. Alternatively, VpreB1 and VpreB2 expression levels are similar at the large pre-BII stage.

Lower surface/intracellular levels of VpreB2 in in vitro cultured pre-BI cells

Because the surface staining suggested similar levels of VpreB1 and VpreB2 in pre-BII but not in pre-BI cells, it was important to determine whether the low VpreB2 levels in pre-BI cells were due to a sub-population not expressing VpreB2 or alternatively to overall lower VpreB2 levels. We therefore cultured BM cells from the mutant mice short term (1 week) *in vitro* (27). The cells were then analyzed by immunofluorescence microscopy after staining for both surface and intracellular VpreB and λ5. As shown in Fig. 4 (in color as Supplementary Figure 2, available at *International Immunology* Online), the vast majority of cells stained positive for VpreB2 although expressing lower levels compared with VpreB1. The lack of VpreB signal in pre-BI cells from *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice, cultured in parallel, demonstrates that the low level of VpreB2 in pre-BI cells from *VpreB1*$^{-/-}$ mice is the result of specific staining. The cells from the double-deficient mice stained, as expected, positive for λ5. This shows therefore that, in pre-BI cells, *VpreB1* is expressed at higher levels than VpreB2.

VpreB protein levels in mice with either one VpreB1 or one VpreB2 allele

To investigate the levels of VpreB1 and VpreB2 further, BM pre-BI and pre-BII cells from *VpreB1*$^{-/-}$ and *VpreB2*$^{-/-}$ mice were analyzed for intracellular levels of VpreB and λ5 (Fig. 5). In contrast to surface expression and because the bulk of VpreB2-expressing cells shifted compared with that of the negative control, not only VpreB1 but also VpreB2 was found to be expressed in the majority of pre-BI cells. A division into two sub-populations was not obvious. However, the intracellular levels of VpreB2 were lower than those of VpreB1. Therefore, the intracellular VpreB1 and λ5 levels seemed to better reflect that of the mRNA levels. Thus, the majority of pre-BI cells express VpreB2 but at a lower level than that of VpreB1.

The results from pre-BI cells suggested a correlation between *VpreB1* and *VpreB2* mRNA and protein levels, implying a dosage effect between the two genes. To investigate whether there is also a gene dosage effect, *VpreB1/VpreB2* double-deficient (*VpreB1*$^{-/-}$*VpreB2*$^{-/-}$) mice were crossed

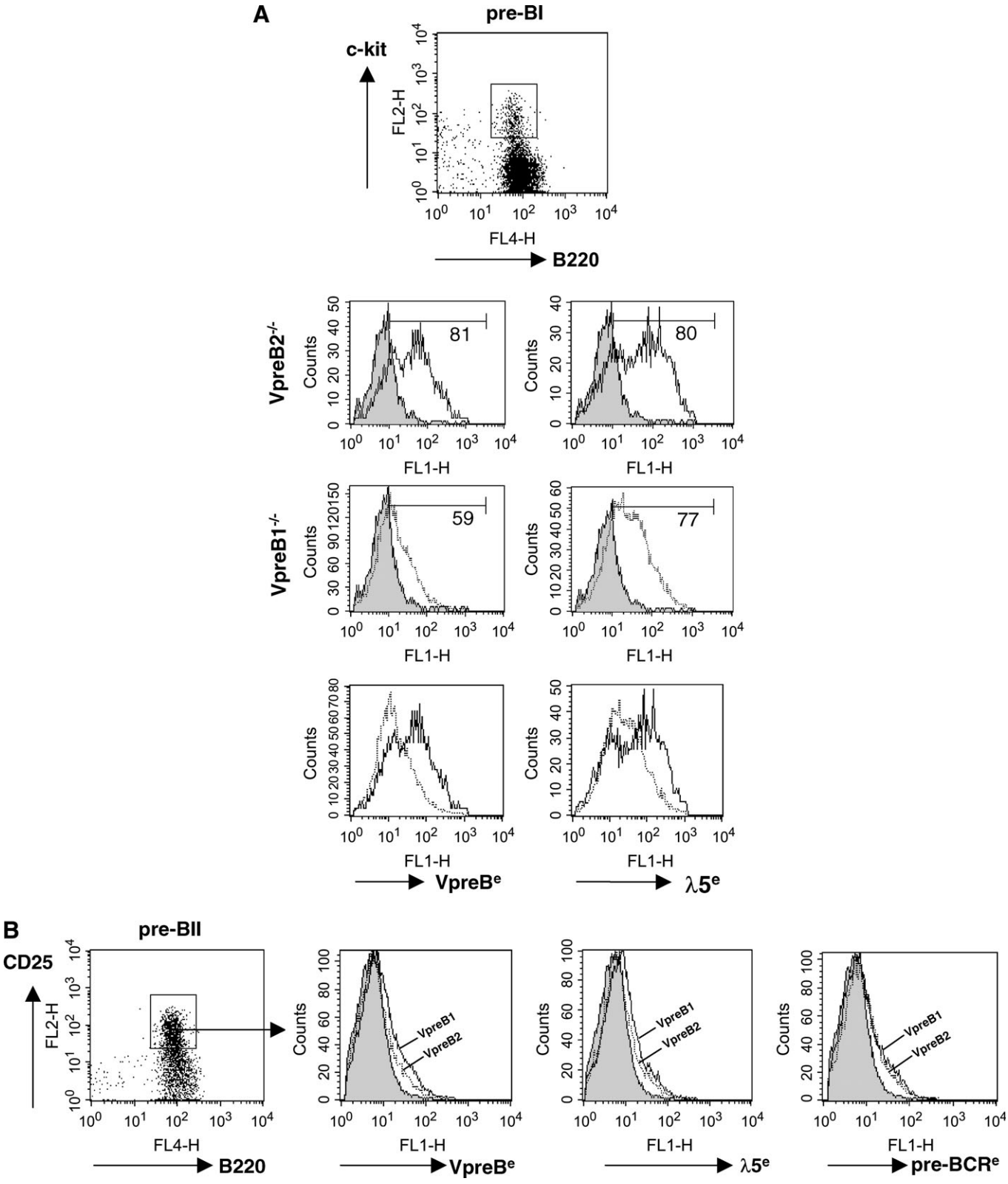

Fig. 3. Comparative FACS analysis of surface expression levels of VpreB, λ5 and pre-BCR in *VpreB2*$^{-/-}$ and *VpreB1*$^{-/-}$ mice. BM cells were enriched for CD19-positive cells and stained for the indicated markers. Cells were gated (shown for *VpreB2*$^{-/-}$) as (A) pre-BI (B220^{+}c-kit^{+}) or (B) pre-BII (B220^{+}CD25^{+}) cells. Bold lines, VpreB1 protein (*VpreB2*$^{-/-}$ mice); dotted lines, VpreB2 protein (*VpreB1*$^{-/-}$ mice); filled histograms, isotype controls. Representative plots from at least three experiments performed on individual mice are shown. Superscript e indicates enzymatically enhanced stains.

with *VpreB2*$^{-/-}$ and *VpreB1*$^{-/-}$ mice, resulting in mice with either one VpreB1 (*VpreB1*$^{+/-}$*VpreB2*$^{-/-}$) or one VpreB2 (*VpreB1*$^{-/-}$*VpreB2*$^{+/-}$) allele. Initially, BM pre-BI cells from mice with two (*VpreB1*$^{+/+}$*VpreB2*$^{-/-}$) or one (*VpreB1*$^{+/-}$ *VpreB2*$^{-/-}$) *VpreB1* allele were compared for intracellular levels of VpreB and λ5 in combination with μH chain. As shown in Fig. 5, no obvious gene dosage effect was observed in terms of VpreB1 expression levels (MFI 26 and 27, respectively). In contrast, a gene dosage effect was observed for VpreB2: mice with two *VpreB2* alleles (*VpreB1*$^{-/-}$*VpreB2*$^{+/+}$)

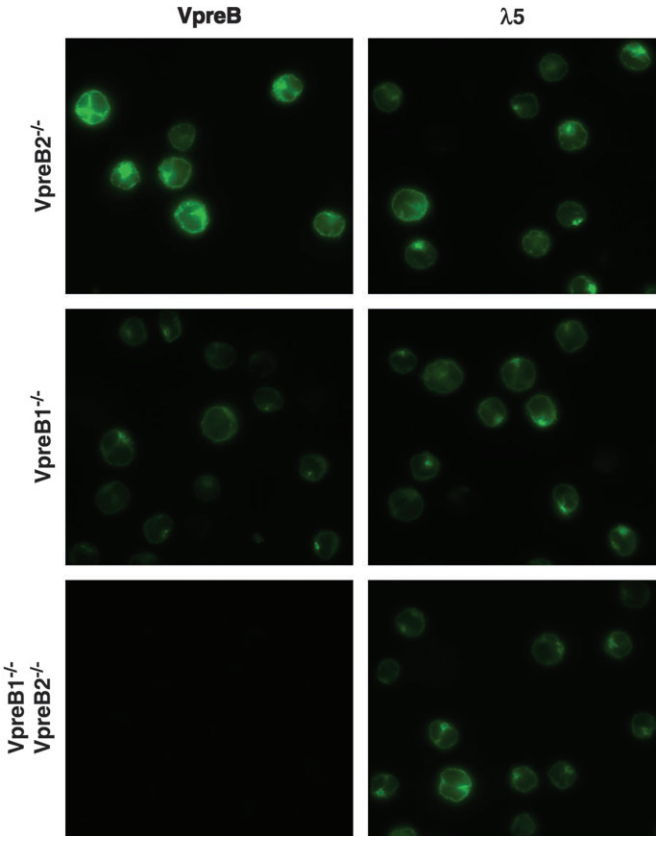

Fig. 4. Comparative immunofluorescence microscopy of VpreB1 and VpreB2 protein levels in *in vitro* cultured pre-BI cells. BM cells from *VpreB1*$^{-/-}$, *VpreB2*$^{-/-}$ and *VpreB1*$^{-/-}$*VpreB2*$^{-/-}$ mice were cultured on irradiated stromal cells in the presence of IL-7. After 7 days cells were analyzed for a combination of intracellular and surface expression of λ5 or VpreB using immunofluorescence microscopy. Representative figures from at least three experiments performed on individual mice are shown.

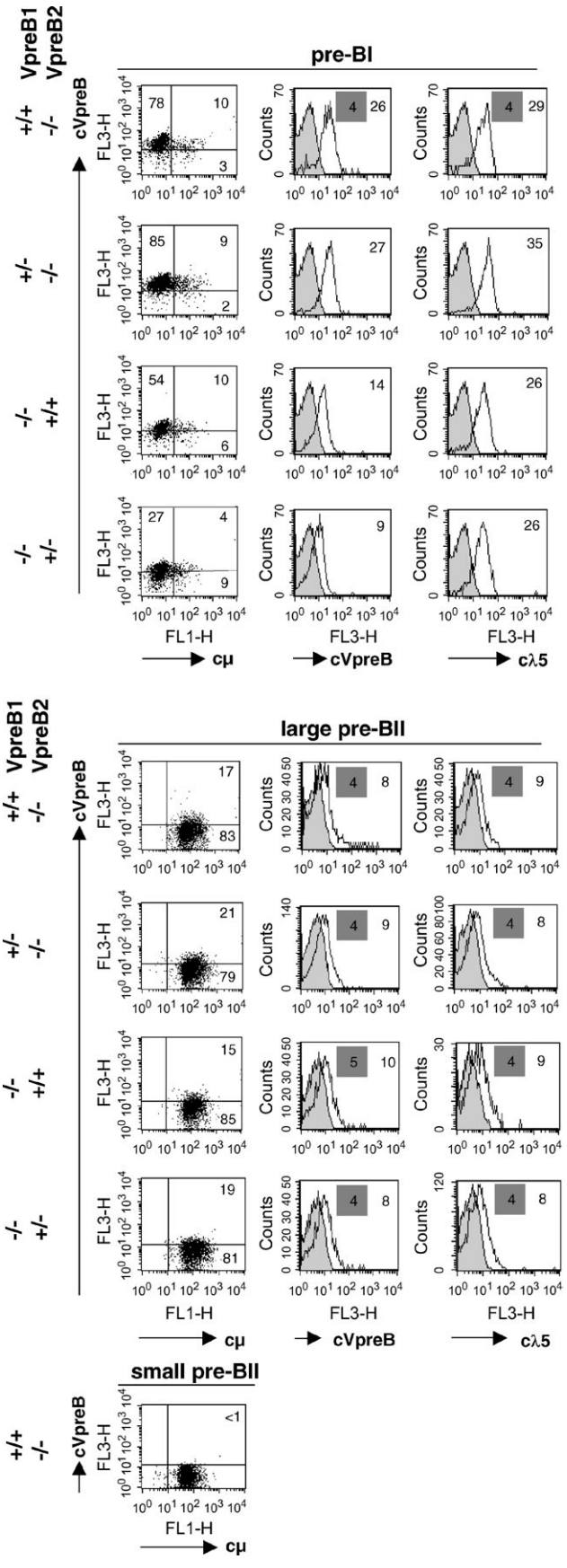

Fig. 5. Comparative FACS analysis of intracellular expression levels of VpreB, λ5 and μH chain. BM preparations of the indicated genotypes were enriched for CD19-positive cells and stained for surface, followed by intracellular, expression of the indicated markers. Cells were gated as pre-BI (B220^{+}c-kit^{+}) and large pre-BII (large B220^{+}CD25^{+}) cells. Because small pre-BII (small B220^{+}CD25^{+}) cells do not express VpreB and λ5, they were used as negative controls (gray histograms, dot plot in last row). Numbers in histograms represent the mean fluorescence levels; gray boxes represent negative control. In dot plots the percentage of cells in the relevant quadrants are shown. Representative plots from two experiments, each using pooled cells from 2 to 6 mice, are shown.

showing higher expression levels (MFI 14 versus 9) than those with one (*VpreB1*$^{-/-}$ *VpreB2*$^{+/-}$). Although the levels were very low in the latter, because most of the population shifted as compared with the negative control, this suggested that the majority of cells did express VpreB2. Furthermore, within each genotype, the levels of VpreB, regardless of it being VpreB1 or VpreB2, were similar when comparing μH-positive or -negative cells (Fig. 4).

Thereafter the intracellular levels of VpreB and λ5, in combination with μH chain, were analyzed in large pre-BII cells (Fig. 5). Since small pre-BII cells do not express SLC, these cells from the same individual stain were used as negative controls. As expected, the levels of both VpreB and λ5 were low at the large pre-BII stage. Somewhat unexpectedly, though in agreement with the surface expression (Fig. 3), there was no obvious difference in VpreB levels when comparing mice expressing VpreB1 or VpreB2 or when comparing mice with either one or two alleles (Fig. 5). In accordance with this, the levels of λ5 were also very similar independent of genotype. Taken together, these data show that the number of VpreB2 but not VpreB1 alleles affects VpreB protein levels at the pre-BI cell stage. However, at the large pre-BII stage, cells express similar levels of VpreB, irrespective of whether either VpreB1 or VpreB2, or either one or two alleles are present.

B cell development in mice with only one VpreB1 or one VpreB2 allele

As we show above, BM B cell numbers are normal in mice with two *VpreB1* but not in mice with two *VpreB2* alleles (Fig. 1 and Table 1). This discrepancy may be due to lower VpreB2 levels resulting in lower surface SLC expression at the pre-BI cell stage. If a lower level of VpreB protein ultimately results in the production of fewer BM B cells, we would also predict, based on the above FACS analyses (Fig. 5), an effect in mice with only one *VpreB2* but not in mice with only one *VpreB1* allele. To investigate this, the size of the different BM B cell populations was determined in the various mouse lines. As B cell development is normal in *VpreB1/VpreB2* haplo-deficient (*VpreB1*$^{+/-}$ *VpreB2*$^{+/-}$) mice, they were used as controls (12). The sizes of the pre-BI and pre-BII cell populations were similar in *VpreB1/VpreB2* heterozygous-deficient mice and mice with one VpreB1 allele (*VpreB1*$^{+/-}$ *VpreB2*$^{-/-}$). The number of B220^{+}IgM^{+} cells in the former was slightly higher due to more re-circulating B cells, in agreement with these mice being older (8 weeks compared with 3–4 weeks). Nevertheless, the presence of only one VpreB1 allele results in normal BM B cell development. In contrast, in mice with only one *VpreB2* allele (*VpreB1*$^{-/-}$ *VpreB2*$^{+/-}$), an ~2-fold enrichment in pre-BI, an ~75% decrease in pre-BII and a similar reduction in the number of B220^{+}IgM^{+} cells was observed compared with mice with only one *VpreB1* allele. The effect on the pre-BI and pre-BII cell populations is therefore more pronounced in mice with one as compared with two VpreB2 alleles. Even so, in *VpreB1*$^{-/-}$ *VpreB2*$^{+/-}$ mice, the ratio of large to small pre-BII cells was normal and ~13% of cells at the pre-BII stage were found to be in S/G_2/M phase of the cell cycle (data not shown), i.e. a proportion similar to those observed for mice with either two *VpreB1* or *VpreB2* alleles (Fig. 1B). Taken together, these data show that only one *VpreB1* allele is still capable of allowing normal BM B cell development whereas neither one nor two *VpreB2* alleles can do so.

Discussion

Here we demonstrate that BM B cell development is normal in mice carrying at least one functional *VpreB1* allele. In contrast, the production of BM B cells is reduced in mice with only two and, even more so, in mice with only one *VpreB2* allele. Our results suggest that the difference between VpreB1 and VpreB2 is due to unequal expression levels rather than to the proteins themselves. Thus, *VpreB2* mRNA levels are approximately one-third of those of *VpreB1* resulting in lower protein levels and, at the pre-BI cell stage, lower surface SLC levels. Thus, these data suggest that the level of SLC is critical for normal B cell development.

The results described herein suggest that VpreB2 is expressed in the majority of pre-BI cells but at a lower level than VpreB1. This is supported by our previous observation that all pre-B cell lines analyzed so far express both VpreB genes (10), possibly at different levels (23). However, these data seem to contradict those of our previously reported single-cell RT-PCR analyses in which *VpreB2* was only detectable in about a third of those pre-BI cells transcribing *VpreB1* (10). The present results suggest that this discrepancy can be explained by overall lower *VpreB2* compared with *VpreB1* mRNA levels. This is further supported by our observation that in the single-cell RT-PCR analyses, dilution of the cDNA often abolished the VpreB2 but not VpreB1 signal (10). Furthermore, if VpreB2 is only expressed in a third of pre-BI cells, we would have expected the FACS analyses to reveal two distinct populations, one with VpreB2 high and one with undetectable levels. In terms of surface expression, we observed two populations, although we suggest that both populations are positive, one expressing high and the other low levels. We base this on the observation that two populations were also observed for VpreB1, which is transcribed in all pre-BI cells (10), and because the low-expressing VpreB1 and VpreB2 sub-populations overlapped. Furthermore, if we consider the intracellular staining patterns, these suggest that the VpreB2 levels are overall lower than those of VpreB1. It may therefore be that VpreB2 is not only expressed at lower levels than VpreB1 but also that the single-cell RT-PCR assay was less sensitive for VpreB2 than for VpreB1. We propose therefore that, under normal circumstances, the majority of pre-BI cells express both VpreB1 and VpreB2.

Lower levels of *VpreB2* mRNA may be the result of a lower transcription rate due to the regulatory regions of *VpreB1* and *VpreB2* not being equally efficient. While the ~350 bp immediately 5′ of the coding sequences are 97% identical at the nucleotide level, this region of the *VpreB2* gene is ~3-fold less active in transient transfection assays (10). Another explanation may be the physical location of *VpreB2*, which is located 1 Mb downstream of the *VpreB1* and *λ5* genes. Although all three genes have their own promoter and enhancer elements (28), they may still be co-regulated by a locus control region (LCR) located just downstream of λ5 (29). If this LCR also acts on *VpreB2*, it may be less efficient due to its being further away, resulting in a lower rate of transcription. Alternatively, it may also be a combination of the

two, since the LCR seems to act in concert with the individual promoter/enhancer regions (29).

The VpreB1 and VpreB2 proteins differ in four amino acids with one located in the leader sequence, one in the unique non-Ig domain and the other two in the IgV-domain-like region (1, 30). However, this structural difference does not appear to bias the selection of different repertoires of V_H-, D_H- or J_H-containing μH chains (22). Therefore, this supports our conclusion that the functional difference between VpreB1 and VpreB2 is due to unequal expression levels rather than to the proteins themselves. In mice expressing only VpreB2, VpreB mRNA levels are ~25% those of normal, resulting in lower amounts of VpreB protein and consequently surface SLC expression at the pre-BI cell stage. However, somewhat surprisingly, at the pre-BII cell stage SLC expression levels are similar independent of which and how many VpreB alleles are expressed. Based on these results, we hypothesize that a pre-BI cell is only 'allowed' to become a large pre-BII cell if it expresses a certain threshold level of SLC and, consequently, pre-BCR. In mice, which express only *VpreB2*, and even more so in mice with only one functional *VpreB2* allele, just a proportion of pre-BI cells reach this threshold level. Because of this, fewer cells enter the proliferating large pre-BII cell compartment resulting in fewer pre-BII and, consequently, fewer immature B cells. Thus, the SLC level at the pre-BI cell stage determines the progression from the pre-BI to the pre-BII stage, ensuring that the cells that enter the pre-BII cell compartment express sufficient levels of pre-BCR to proliferate normally.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Abbreviations

BCR	B cell receptor
BM	bone marrow
LCR	locus control region
MFI	mean fluorescence intensity
RT	reverse transcription
SLC	surrogate light chain
μH	μ heavy

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