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Positive selection of the peripheral B cell repertoire in gut-associated lymphoid tissues

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Gut-associated lymphoid tissues (GALTs) interact with intestinal microflora to drive GALT development and diversify the primary antibody repertoire; however, the molecular mechanisms that link these events remain elusive. Alicia rabbits provide an excellent model to investigate the relationship between GALT, intestinal microflora, and modulation of the antibody repertoire. Most B cells in neonatal Alicia rabbits express V_{H4} allotype immunoglobulin (Ig)M. Within weeks, the number of V_{H4} B cells decreases, whereas V_{H6} allotype B cells increase in number and become predominant. We hypothesized that the repertoire shift from V_{H4} to V_{H6} B cells results from interactions between GALT and intestinal microflora. To test this hypothesis, we surgically removed organized GALT from newborn Alicia pups and ligated the appendix to sequester it from intestinal microflora. Flow cytometry and nucleotide sequence analyses revealed that the V_{H4} to V_{H6} repertoire shift did not occur, demonstrating the requirement for interactions between GALT and intestinal microflora in the selective expansion of V_{H6} B cells. By comparing amino acid sequences of V_{H4} and V_{H6} Ig, we identified a putative V_{H4} ligand binding site for a bacterial or endogenous B cell superantigen. We propose that interaction of such a superantigen with V_{H6} B cells results in their selective expansion.

Vertebrates have developed two general strategies for generating a diverse primary B cell repertoire. In humans and mice, the primary B cell repertoire is generated by rearrangement of multiple V, D, and J gene segments in the bone marrow throughout the life of the animal. In other species, such as the rabbit (1–3), chicken (4, 5), and sheep (6, 7), this repertoire initially develops by rearrangement of a limited number of V genes in primary lymphoid tissue and further diversifies in gut-associated lymphoid tissues (GALTs). In rabbits, the D proximal V_{H}, V_{H6}, is preferentially rearranged during B cell development in the fetal liver and bone marrow (8). The VDJ genes undergo somatic diversification via somatic hypermutation and gene conversion in GALT in response to intestinal microflora (9). In the absence of appropriate intestinal microflora, GALT develops poorly, and both the number of B cells and the diversification of V_{H} genes are greatly inhibited (9).

Most (80–90%) rabbit serum Ig molecules express V_{H6} allotypic markers that are encoded by the predominantly rearranged gene, V_{H6} (1). The following three alleles of V_{H6} are found in laboratory rabbits: V_{H6}a, V_{H6}b, and V_{H6}c; they encode the V_{H6}a, V_{H6}b, and V_{H6}c allotypes, respectively. These V_{H6} allotypes differ in amino acid residues in framework region (FR)1 and FR3 (10). 10–20% of serum Ig does not react with anti-V_{H6}a, anti-V_{H6}b, or anti-V_{H6}c allotypic antibodies and is referred to as V_{H6} (V_{H6}-negative) Ig.

Kelus and Weiss (11) identified rabbits with a variant V_{H6} allotype-encoding allele, ali, which has a 10-kb deletion of DNA encompassing V_{H6} (1). In contrast with wild-type rabbits, nearly all Ig in young ali/ali rabbits (designated Alicia) is V_{H6}. V_{H6} Ig is encoded predominantly by V_{H6}a, V_{H6}c, and V_{H6} (12, 13), which reside >50 kb upstream of V_{H6} (1). In adult Alicia rabbits, high levels of serum Ig with the V_{H6} (a2) allotype are found (11). This increase in V_{H6} Ig is a result of increased numbers of V_{H6} B cells that use V_{H6}a, V_{H6}b, and V_{H6}c gene segments that encode several of the V_{H6} (a2) allotype-associated amino acids (14, 15). Pospisil et al. (16) found that, in the appendix, more V_{H6} B cells were proliferating and fewer were dying compared with V_{H6} B cells.
encoding gene segments deletion (brackets). Functional VH gene segments that encode either the gene segments. DH, D gene segments. rabbits.

Figure 2. Flow cytometric analysis of B cells from a 9-wk-old Alicia rabbit. Cells from the spleen, mesenteric lymph node (MLN), appendix, and PBLs were double stained with anti-IgM (FITC) and either anti-VHn (PE; top) or anti-VH,a (PE; bottom) antibodies. The numbers in parentheses indicate percentages of VHn or VH,a cells of total B cells.

Figure 1. Organization of the 3’-most VH gene segments in Alicia (ali/ali) rabbits. VH,a and VH,2 are absent in Alicia rabbits because of a 10-kb deletion (brackets). Functional VH gene segments that encode either the VH,a allotype (a) or the VH,n allotype (n) are indicated. The VH,n allotype-encoding gene segments VH,x, VH,y, and VH,z reside >50 kb upstream of VH,1. Their location respective to each other is unknown. VH,n, nonfunctional VH,n gene segments. Dn, D gene segments.

The molecular basis underlying the repertoire shift from VH,n to VH,a is unknown and is the subject of the current paper.

A shift in the B cell repertoire could arise from VH,n gene replacement or from secondary Ig gene rearrangements on the unexpressed Ig allele. Although it is generally believed that these events occur primarily in the bone marrow (17–19), there is evidence that VH,n gene replacement and secondary Ig gene rearrangement occur in peripheral tissues (20, 21). Another possible explanation for the B cell repertoire shift is that VH,a B cells are positively selected in the periphery. Positive selection of B cells in the periphery has been demonstrated in several transgenic mouse models (22–26).

In rabbit, intestinal microflora interacts with GALT to promote development of follicles containing proliferating B cells and to generate the primary B cell repertoire (9). We hypothesized that, in GALT of Alicia rabbits, interactions between GALT and the intestinal microflora also promote the development of follicles containing proliferating B cells and lead to the repertoire shift from VH,n to VH,a B cells. To test this possibility, we surgically disrupted the GALT–bacterial interaction in Alicia rabbits and tested whether the repertoire shift from VH,n to VH,a B cells was abrogated.

RESULTS
Kinetics of the B cell repertoire shift
The repertoire shift in Alicia rabbits, from the predominant expression of VH,a allotype early in life, was originally shown by Kelus and Weiss (11), who analyzed Ig allotypes in serum. Pospisil et al. (16) showed that a similar shift occurred in B cells in the appendix. By using antibodies to both VH,n and VH,a allotypes, we found that, in 9-wk-old rabbits, VH,a B cells represented 35–50% of the B cells in spleen, mesenteric lymph nodes, appendix, and PBLs (Fig. 2). We analyzed cells of various tissues from newborn to 2-yr-old Alicia rabbits to follow the appearance and disappearance of VH,a and VH,n B cells, respectively, throughout life. We found that, although 10–25% of B cells at birth are VH,a, at 3 wk of age essentially all B cells (~95%) in spleen, appendix, and PBLs were VH,n (Fig. 3 and not depicted). Subsequently, the percentage of VH,n cells steadily declined, so that by 2 yr of age, <20% of B cells were VH,n and >75% were VH,a. These data demonstrate that VH,a B cells accumulate throughout life, with a sharp increase between 4 and 10 wk of age. The VH,a B cells accumulate faster in the appendix than in spleen, suggesting that the B cell repertoire shift from VH,n to VH,a B cells may occur primarily in GALT.

B cell repertoire shift and GALT
Because GALT development and somatic diversification of Ig genes both require interaction between GALT and intestinal microflora (9, 27), we hypothesized that the repertoire shift in Alicia rabbits also requires this interaction. To investigate this possibility, we generated ligated appendix (LigApx) rabbits by surgically removing the Peyer’s patches and the saccula rotundus and ligating the lumen of the appendix to prevent bacterial colonization (9). If interactions between GALT and intestinal microflora are required for the repertoire shift from VH,n to VH,a B cells, we expected that the peripheral blood B cells in LigApx rabbits would be predominantly VH,n. In each of three 12-wk-old LigApx Alicia rabbits (94S, 353X2, 353X4), we found that the percentage of B cells was approximately eightfold less than in unmanipulated rabbits of that age and that almost all B cells (90–96%) were VH,a, at 3 wk of age and that almost all B cells (90–96%) were VH,a. These data demonstrate that VH,a B cells accumulate throughout life, with a sharp increase between 4 and 10 wk of age. The VH,a B cells accumulate faster in the appendix than in spleen, suggesting that the B cell repertoire shift from VH,n to VH,a B cells may occur primarily in GALT.
lated Alicia rabbits was 50%. We examined one of the LigApx Alicia rabbits (94S) at 8 mo of age and found that 90% of the B cells were still VHn, showing that the B cells remained predominantly VHn for many months. These results indicate that, without interactions between GALT and intestinal flora, the shift from VHn to VHa did not occur.

To confirm that the repertoire shift from VHn to VHa B cells was abrogated in LigApx rabbits, we examined the nucleotide sequences of VDJ genes cloned from peripheral blood of 12-wk-old LigApx Alicia rabbits. We expected that the VH genes used in the VDJ gene rearrangements would be primarily genes that encode VHn molecules rather than VHa molecules. From one LigApx Alicia rabbit (94S), shown in Fig. 4, and from three additional LigApx rabbits (32P2, 144T, 199T1) for which flow cytometry data are not available, we analyzed a total of 80 VDJ gene sequences. As predicted, most (76%) of the VDJ genes used VHn gene segments (Table I), whereas almost none (4%) of the VDJ genes from control (unmanipulated) Alicia rabbits of the same age used VHn gene segments. We think the PCR analysis underestimated the expression of VHn genes because, by FACS analysis, 50% of the peripheral B cells from 12-wk-old control Alicia rabbits were VHn, whereas only 4% of the PCR-amplified VDJ genes were VHn.

To determine whether the low percentage of VHn-encoding genes (Table I) resulted from preferential amplification of VHn cDNA, we conducted two independent experiments in which VDJ genes were PCR amplified from cDNA prepared from a pool of cells containing equivalent numbers of FACS-sorted VHn and VHa B cells from peripheral blood. In the two experiments, 67% (14 out of 21) and 81% (17 out of 21) of PCR-amplified VDJ genes used VHn gene segments. The reduced number of VHn PCR products was also observed with another 5′ VH primer, VHldr (5′-GGCTTCTCCTGTTGCGT-3′), which anneals to a different target site. The preferential amplification of VHn cDNA with independent primers suggests that, even though VHa and VHn B cells appear to express equivalent amounts of surface IgM (Fig. 4), VHa B cells might produce higher levels of IgM mRNA, possibly as a result of their stimulation in GALT (16).

Although we do not understand the molecular basis for the PCR skewing toward VHa-encoding VDJ genes, the data confirm the FACS analysis, which showed that most of the B cells of LigApx Alicia rabbits were VHn instead of VHa. We conclude that the repertoire shift from VHn to VHa B cells re-

Table I. VHn and VHa genes used in VDJ genes of LigApx and unmanipulated (control) Alicia rabbits

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>No. of VDJ sequences</th>
<th>VHn (%)</th>
<th>VHa</th>
</tr>
</thead>
<tbody>
<tr>
<td>LigApx, 12 wk</td>
<td>32P2</td>
<td>16 (70)</td>
<td>7</td>
</tr>
<tr>
<td>144T</td>
<td>18 (86)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>94S</td>
<td>17 (81)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>199T1</td>
<td>10 (67)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>LigApx, 8 mo</td>
<td>94S</td>
<td>13 (81)</td>
<td>3</td>
</tr>
<tr>
<td>Control, 12 wk</td>
<td>320W2</td>
<td>0 (0)</td>
<td>15</td>
</tr>
<tr>
<td>127W1</td>
<td>0 (0)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>199T3</td>
<td>0 (0)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>127W2</td>
<td>2 (20)</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*VDJ genes were PCR amplified from PBLs.

*aThe data were obtained by using VHRPS as the 5′ primer. Similar results were obtained in four additional experiments utilizing the VHldr primer as the 5′ primer: for rabbit 94S (12 wk), 5 out of 22 (23%) sequences were derived from VHn genes; for rabbit 127W1, 8 out of 9 (89%) were derived from VHa genes; for rabbit 199T3, 8 out of 8 (100%) sequences were derived from VHn genes; and for rabbit 127W2, 8 of 11 (73%) were derived from VHa genes.
quired interactions between GALT and the microflora and that expansion of V\(_{\text{Ha}}\) B cells requires such interactions.

**Rearrangement status of IgH alleles in V\(_{\text{Ha}}\) B cells**

The repertoire shift from V\(_{\text{Hn}}\) to V\(_{\text{Ha}}\) B cells in the periphery could occur by replacement of V\(_{\text{Hn}}\)-using VDJ genes with V\(_{\text{Ha}}\) gene segments (20), by rearrangement of a V\(_{\text{Ha}}\)-encoding V\(_{\text{Ha}}\) gene segment on the second IgH allele (17, 21), or by selective expansion of V\(_{\text{Ha}}\) B cells (16, 24). We think that V\(_{\text{H}}\) gene replacement is unlikely to explain the repertoire shift because the V\(_{\text{Hn}}\) genes (V\(_{\text{Hx}}\), V\(_{\text{Hy}}\), and V\(_{\text{Hz}}\)) used in VDJ gene rearrangements in V\(_{\text{Hn}}\) B cells reside upstream of the V\(_{\text{Ha}}\) genes (V\(_{\text{H4}}\), V\(_{\text{H7}}\), and V\(_{\text{H9}}\)) used in VDJ gene rearrangements in peripheral V\(_{\text{Ha}}\) B cells of Alicia rabbits (15). Accordingly, the rearrangement of V\(_{\text{Hn}}\) genes during VDJ gene rearrangements would likely result in deletion of the V\(_{\text{Ha}}\) genes (Fig. 1).

If the repertoire shift from V\(_{\text{Hn}}\) to V\(_{\text{Ha}}\) B cells is caused by gene rearrangements of V\(_{\text{Ha}}\) gene segments on the second IgH allele in V\(_{\text{Hn}}\) B cells, we expected to find VDJ gene rearrangements by single cell PCR. We used PCR primers that would detect rearranged VDJ genes and germline J\(_{\text{H1}}\) genes (Fig. 5 a). Of 26 single cells from which a rearranged VDJ PCR product was obtained, all but one had a product of the expected size for an unrearranged (second) IgH allele (Fig. 5 b). This result showed that essentially all V\(_{\text{Hn}}\) B cells rearranged only one IgH allele, indicating that the B cell repertoire shift from V\(_{\text{Hn}}\) to V\(_{\text{Ha}}\) B cells in Alicia rabbits is not due to secondary IgH gene rearrangements on the other allele. Instead, we propose that the B cell repertoire shift occurs through positive selection due to preferential expansion of V\(_{\text{Ha}}\) B cells.

**DISCUSSION**

The intestinal microflora are important in regulating many immune functions, including development of GALT (27), induction of oral tolerance (28), and induction of mucosal immunity (29). In rabbits, intestinal microflora are required not only for GALT to develop but also to generate a diverse primary B cell repertoire (9). Previously, we found that surgical disruption of GALT–bacterial interactions prevented GALT development, B cell expansion, and somatic diversification of the B cell repertoire (9). In the current paper, we

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**Figure 5.** Single cell PCR of V\(_{\text{Ha}}\) B cells from PBLs of unmanipulated adult Alicia rabbits. (a) Location of PCR primer binding sites. Sense primer 5’ of J\(_{\text{H2}}\) and antisense primer 3’ of J\(_{\text{H2}}\) region results in a band of ~380 bp for germline configuration; sense primer from conserved region 5’ of V\(_{\beta}\) promoters and antisense primer 3’ of the J\(_{\beta}\) region results in a band of ~800 bp for a rearranged VDJ gene. (b) PAGE of PCR products for rearranged and germline IgH alleles. 6 out of 26 samples are shown.

**Figure 6.** Intracellular calcium mobilization in V\(_{\text{Ha}}\) and V\(_{\text{Hn}}\) B cells after stimulation with anti-\(\kappa\) chain allotype antibodies, anti-\(\beta4\) and anti-\(\beta5\) (top). (solid line) V\(_{\text{Ha}}\) B cells; (dashed line) V\(_{\text{Hn}}\) B cells. PBLs from 12-wk-old control Alicia rabbits (b\(\kappa\)b\(\kappa\)) were loaded with Fluo-3 and Fura-red, and calcium mobilization in electronically gated V\(_{\text{Hn}}\) and V\(_{\text{Ha}}\) B cells (see Material and Methods) was determined. Relative concentrations of cytoplasmic calcium [Ca\(^{2+}\)] are shown as the mean of the ratio of Fluo-3 to Fura-red fluorescence. The release of [Ca\(^{2+}\)] after ionomycin is shown as control (bottom). Similar results were obtained in each of three experiments.
found that the repertoire shift from $V_{Hn}$ to $V_{Ha}$ B cells in Alicia rabbits also depends on GALT–bacterial interactions.

At birth, 10–25% of B cells in peripheral tissues of Alicia rabbits were $V_{i}$, and these B cells subsequently declined to nearly undetectable levels by week 3. Although these $V_{i}$ B cells could represent maternal B cells, we think the percentages are much higher than would be expected for maternal B cells. We also do not think these cells are $V_{i}$ B cells with maternal $V_{i}$ Ig bound through Fc receptors because, in this case, we would expect all B cells, rather than a subset, to be $V_{i}$. Instead, we think the decline in the percentage of $V_{i}$ B cells may be due to a dramatic increase in $V_{i}$ B cells from a second wave of B lymphopoiesis in the bone marrow. We recently identified a burst of both pre–B cells and B cells in bone marrow at 3 wk of age and we suggest that in Alicia rabbits, the newly generated B cells may be primarily $V_{i}$ (30).

The shift from $V_{i}$ to $V_{i}$ B cells after 3 wk of age likely occurs in GALT rather than in the bone marrow because the shift requires GALT–bacterial interactions. Therefore, we favor the idea that this shift is due to selective expansion of $V_{i}$ B cells as proposed by Pospisil et al. (16), who showed that more $V_{i}$ B cells proliferate and fewer die than $V_{i}$ B cells in the appendices of Alicia rabbits. We suggest that $V_{i}$ B cells are preferentially stimulated by interaction with a bacterial ligand or a bacterially induced GALT-derived ligand. Such preferential stimulation of $V_{i}$ B cells could be due to differences between $V_{i}$ and $V_{i}$ B cells in BCR density (31, 32), in localization of BCR in lipid rafts (33), or in BCR structure leading to differential stimulation and subsequent proliferation. We found no difference in surface IgM levels between $V_{i}$ and $V_{i}$ B cells, suggesting that differences in BCR density in $V_{i}$ and $V_{i}$ B cells do not contribute to the differential stimulation. Although we have not studied the localization of $V_{i}$ and $V_{i}$ BCR in lipid rafts, we suggest that $V_{i}$ and $V_{i}$ B cells are differentially stimulated by bacteria because of structural differences between the $V_{i}$ regions of $V_{i}$ and $V_{i}$ BCR. Differential stimulation of $V_{i}$ and $V_{i}$ B cells by bacteria will be investigated in future studies.

When we compared amino acid sequences encoded by $V_{i}$ and $V_{i}$ gene segments, we found many differences in FR1 and FR3. These differences include $V_{i}$ and $V_{i}$ allotype-associated amino acids, which Pospisil et al. (16, 34) proposed may interact with a ligand, leading to expansion of $V_{i}$ B cells. However, because the $V_{i}$ allotype-associated amino acids are not present in allelically encoded $V_{i}$ and $V_{i}$ allotypes (10), and because $V_{i}$ and $V_{i}$ B cells in $a^{1}/a^{1}$ and $a^{1}/a^{1}$ rabbits, respectively, also proliferate in GALT, we suggest that the $a^{1}$ allotype-associated amino acids are not critical for preferential expansion of $V_{i}$ B cells. Instead, we suggest that the nonallotype-associated amino acids present in $V_{i}$ molecules, but absent in $V_{i}$ molecules, are responsible for preferential expansion of $V_{i}$ B cells.

We examined the amino acid sequences encoded by $V_{i}$ and $V_{i}$ gene segments and found six positions in FR1 and FR3 (3, 19, 21, 23, 78, 82A) in which the same amino acids were encoded by all six $V_{i}$ gene segments known to encode $V_{i}$ molecules, but not by the three $V_{i}$ gene segments known to encode $V_{i}$ molecules (Fig. 7 a). In addition, we found that, at positions 79 and 82 (FR3), the same amino acids were encoded by five out of six $V_{i}$ gene segments, but not by the $V_{i}$ gene segments (Fig. 7 a). If selective expansion of $V_{i}$ B cells results from interaction of a ligand with $V_{i}$ molecules, the contacting amino acids are likely to be present on the exterior surface of the $V_{i}$ region. By three-dimensional modeling, we found that of these eight amino acids, five (19, 21, 23, 79, 82A) are clustered on the external face of the $V_{i}$ domain with their side chains exposed for potential interaction with a ligand (Fig. 7 b). Two out of the eight amino acids (78 and 82) are nonpolar and, thus, their side chains are not likely to be exposed to solvent. Another conserved amino acid (position 3) is located at a flexible region, making it difficult to predict whether this amino acid will participate in a ligand interaction. We propose that the five amino acids (19, 21, 23, 79, 82A) clustered on the exterior face of the $V_{i}$ molecules are part of a binding site for a bacterial ligand or a bacterially derived GALT-derived ligand. Closer examination of the putative binding site reveals two additional amino acids (at positions 77 and 81) that may contribute to ligand binding, even though they are present in both $V_{i}$ and $V_{i}$ molecules. We propose that a combination of seven $V_{i}$ amino acids at positions 19, 21, 23,
were as described previously (41). Similarly, we think that a putative bacterial cell superantigen (37) or a bacterially induced GALT-derived superantigen (38) preferentially binds to and stimulates V_{H}\alpha B cells. If a B cell superantigen promotes positive selection of V_{H}\alpha B cells in GALT, the interaction between such a B cell superantigen and the rabbit V_{H} region would be expected to stimulate the B cells in an antigen-nonspecific, polyclonal manner. Consistent with this idea, Sehgal et al. (39) found that the nature of somatic mutation in VDJ genes in the appendix of young rabbits differed from that which occurs in response to specific antigens in the spleen.

Furthermore, Casola et al. (40) demonstrated that anti-HEL transgenic mice had normal-sized Peyer’s patches, indicating that B cell expansion in GALT is specific-antigen independent. However, we cannot rule out the possibility that the microflora stimulate B cells in a non-BCR-dependent manner, rather than through interaction with the V_{H} region (40).

Using IgH-transgenic mice, it has been shown that peri toneal B-1 cells undergo antigen-specific B cell–positive selection (23). Evidence for positive selection of conventional B cells (B-2), whether dependent or independent of specific antigen, is more circumstantial (24). Here, we demonstrated in a nontransgenic model that B cells can be positively selected in the GALT during generation of the primary B cell repertoire, likely in an antigen-independent manner (37, 39).

Furthermore, this occurs as a result of interactions between GALT and the intestinal microflora. These data demonstrate the potential for commensal intestinal microflora to shape the B cell repertoire. The extent to which commensal microflora play a role in modifying the B cell repertoire in other species remains to be elucidated.

**MATERIALS AND METHODS**

Rabbits and antiallootype antibodies. *All/alb* rabbits (designated Alicia; reference 1), which are homozygous for the b5 κ-chain allotype (b/\textit{b})5, were maintained in the Comparative Medicine Facility at Loyola University Chicago, Maywood, IL. All experiments were performed following the guidelines of the Loyola University Chicago Institutional Animal Care and Use Committee. The anti-b4 and anti-b5 anti–κ chain allotype antisera were as described previously (41).

Anti-V_{H}\alpha\beta antibody directed against V_{H}\alpha and V_{H}y allotypes was produced by immunizing a homozygous b/\textit{b}\gamma–γ (IgH haplootype A/A) rabbit (L76–3) with IgG from a homozygous a2-suppressed a/\textit{a}\gamma–\gamma rabbit (42). Ig fractions of the anti-V_{H}\alpha and anti-V_{H}y antisera (41) obtained by precipita tion with 40% saturated ammonium sulfate were biotinylated for use in immunofluorescence analysis and in Ca^{2+} mobilization assays. By immunofluorescence, the anti-V_{H}y\alpha antibody reacted with <5% of peripheral B cells in adult homozygous a/\textit{a}\gamma–\gamma rabbits, as expected (unpublished data).

To confirm that the anti-V_{H}\alpha\β allotype antibodies reacted with V_{H}\α and V_{H}y Ig, we analyzed PCR-amplified VDJ genes from FACS-sorted splenic V_{H}y B cells from Alicia rabbits, using a 5′ conserved V_{H}y leader primer and a 3′ primer specific for J_{H}y. Nearly all of the VDJ genes (32 out of 34) encoded amino acids characteristic of the V_{H}y molecules encoded by the V_{H}y and V_{H}y gene segments (references 10, 13 and unpublished data). We also analyzed 12 VDJ genes PCR-amplified from splenic B cells that did not react with anti-V_{H}y\β antibodies and found that, as expected, all 12 genes encoded amino acids characteristic of those encoded by the V_{H}y,μ gene segments V_{H}y, J_{H}y,7, and V_{H}y,9 (10, 12).

**Immunofluorescence and flow cytometry.** 10^6 PBLs were prepared from buffy coat and stained with biotinylated rabbit anti-V_{H}y or biotinylated rabbit anti-V_{H}\alpha2 allotype antibodies followed by streptavidin–PE as a secondary reagent (Molecular Probes). CD4^+ T cells were stained with FITC-conjugated anti-CD4 mAb (clone KEN4; reference 43). B cells were detected using biotinylated affinity-purified goat anti-Igλ chain antibodies and streptavidin–PE or FITC-conjugated anti-IgM mAb (clone 367; reference 3). Cells within the side- and forward-scatter lymphocyte gate were analyzed using a FACS Calibur flow cytometer (BD Biosciences) in the FACS core facility at Loyola University Chicago.

**PCR analysis to determine rearrangement status of the IgH locus.** Single V_{H}\beta B cells were FACS sorted into 96-well V-bottom plates containing 1× lysis buffer as described previously (30). VDJ genes were PCR amplified using nested primers as follows: the 5′ primers were 5′-TG[GC]-GATAAT[GT]/GAAGGG[TC][G]/CACACA-3′ (sense-inside primer) and 5′-CATAAAATT[CA][T]/CATGAT[TC]-3′ (sense-inside primer); taken from conserved sequences 5′ of V_{H} promoter regions; the 3′ primers were 5′-AGTTGAGTAGGAGAGAGAGA-3′ (antisense-outside primer) and 5′-GAGTTGCGAAGGGCTCACC-3′ (antisense-inside primer); taken from conserved sequences 3′ of J_{H}4 (J_{H}4 is used in 80–90% of VDJ gene rearrangements) and J_{H}2. To determine whether rearrangements in the J_{H} region had occurred, nested PCR amplification was performed by using the 5′ primers 5′-TGAGTGCTGGTTGGAGCTTGGC-3′ (sense-inside primer) and 5′-CAGACGCTGGAGGCTGTGGCAT-3′ (antisense-inside primer); taken from a region 5′ of the J_{H} locus; the antisense primers were the same as those used for VDJ gene rearrangements.

Development of rabbits with a LigApx. The LigApx rabbits were developed as described previously (9). In brief, we removed the sacculus rotundus from newborn rabbits and ligated the lumen of the appendix to prevent bacterial colonization. The vasculature to the appendix was left intact. Peyer’s patches were removed at 4 wk of age, when they became macroscopically visible.

**Cloning and nucleotide sequence analysis of VDJ cDNA.** VDJ genes were PCR amplified from splenic and PBL-derived cDNA (44). For the PCR, we used a 5′ conserved V_{H} leader primer (V_{H}lRPS; reference 45) and a 3′ primer specific for exon 1 of C_{\mu} (primer C_{\mu}1-μ; reference 46). The PCR products were cloned into pGEM-T Easy (Promega), and the nucleotide sequences were determined using an automated ABI Prism 310 sequencer with Big Dye–labeled terminators (PerkinElmer and Applied Biosystems). The V_{H}1 gene segments used in the VDJ genes were identified by comparing the nucleotide sequences to those of known germine V_{H} gene segments. The germine V_{H}1 gene segment sequences most similar to those of the VDJ genes used in this study were designated V_{H}1-μ, V_{H}1-μ. All V_{H}1 gene segments were submitted to GenBank/EMBL/DDJB and are available under the following accession nos.: rabbit, no. 320P (AY667679–AY667681); no. 144T (AY676782–AY676802); no. 94S (12 wk) (AY676783–AY676785); no. 199T1 (AY676824–AY676838); no. 94S (8 mo) (AY676895–AY676710); no. 320W2 (AY676711–AY676723); no. 127W2 (AY676726–AY676730); no. 199T3 (AY676737–AY676748); and no. 127W2 (AY676749–AY676758).

**Ca^{2+} mobilization.** PBLs isolated with LSM R (ICN Biomedicals) were stained with anti–rabbit T cell mAb (clone KEN5; reference 43) and biotinylated anti-V_{H}y or anti-V_{H}\alpha2 allotype antibodies. Secondary reagents
were biotinylated Fab goat anti-mouse IgG (Jackson Immunoresearch Laboratories) and streptavidin-APC (BD Biosciences). The stained cells were suspended in phenol red-free HBSS containing CaCl2 and MgCl2 (GIBCO BRL) and were incubated with rotation for 45 min at room temperature in 10 μM Fura-2, 5 μM Fluo-3 (prepared as 1 mM stocks in 100% DMSO; Molecular Probes), and 2.8 μl 20% pluronic F-127 (Molecular Probes). Vβ4 or Vβ8 B cells were electronically gated as follows: Vβ4 B cells were those cells in the lymphocyte gate that did not react with anti-Vβ4 or anti–T cell antibodies, and the Vβ8 B cells were cells that did not react with anti-Vβ8 or anti–T cell antibodies. The electronically gated Vβ4 and Vβ8 B cells were FACS sorted and, upon reanalysis by FACS Calibur, were shown to be at least 90% pure. The calcium flux of the Vβ4 and Vβ8 B cells in response to anti-b4 and anti-b5 κ-chain allotype antisera was measured essentially as described previously (47). The fluorescence of Fluo-3 and Fura-red was measured over time, in a linear format. The baseline was determined from data collected 30 s before the addition of antiallotype antibody. The ratio of Fluo-3 to Fura-red and the corresponding mean intracellular calcium ([Ca2+]i) levels were calculated and analyzed using Flowjo software (Tree Star, Inc.).

Three-dimensional modeling of rabbit Vβ domain. The crystal structure of a Fab fragment of a human IgM antibody-encoding IgM rearrangement factor (Vβ3.3-30.1FII; reference 35) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) and used as a modeling template for the rabbit Vβ domain. Modeling was performed using DeepView/Swiss-ModelView (http://www.expasy.org/spdbv), and images were rendered using Pov-Ray for Windows v3.5 (http://www.povray.org).

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