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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\textsubscript{H}n allotype immunoglobulin (Ig)M. Within weeks, the number of V\textsubscript{H}n B cells decreases, whereas V\textsubscript{H}a allotype B cells increase in number and become predominant. We hypothesized that the repertoire shift from V\textsubscript{H}n to V\textsubscript{H}a 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\textsubscript{H}n to V\textsubscript{H}a repertoire shift did not occur, demonstrating the requirement for interactions between GALT and intestinal microflora in the selective expansion of V\textsubscript{H}a B cells. By comparing amino acid sequences of V\textsubscript{H}n and V\textsubscript{H}a Ig, we identified a putative V\textsubscript{H}n ligand binding site for a bacterial or endogenous B cell superantigen. We propose that interaction of such a superantigen with V\textsubscript{H}a 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\textsubscript{H} gene, V\textsubscript{H}4, 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\textsubscript{H} genes are greatly inhibited (9).

Most (80–90%) rabbit serum Ig molecules express V\textsubscript{H}a allotypic markers that are encoded by the predominantly rearranged gene, V\textsubscript{H}1 (1). The following three alleles of V\textsubscript{H}1 are found in laboratory rabbits: V\textsubscript{H}1-a\textsuperscript{1}, V\textsubscript{H}1-a\textsuperscript{2}, and V\textsubscript{H}1-a\textsuperscript{3}; they encode the V\textsubscript{H}a1, V\textsubscript{H}a2, and V\textsubscript{H}a3 allotypes, respectively. These V\textsubscript{H}a 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\textsubscript{H}a1, anti-V\textsubscript{H}a2, or anti-V\textsubscript{H}a3 allotypic antibodies and is referred to as V\textsubscript{H}a (V\textsubscript{H}a-negative) Ig.

Kelus and Weiss (11) identified rabbits with a variant V\textsubscript{H}a2 allotype-encoding allele, ali, which has a 10-kb deletion of DNA encompassing V\textsubscript{H}1 (Fig. 1 and reference 1). In contrast with wild-type rabbits, nearly all Ig in young ali/ali rabbits (designated Alicia) is V\textsubscript{H}a\textsuperscript{n}. V\textsubscript{H}a\textsuperscript{n} Ig is encoded predominantly by V\textsubscript{H}a, V\textsubscript{H}x, and V\textsubscript{H}z (12, 13), which reside >50 kb upstream of V\textsubscript{H}1 (1). In adult Alicia rabbits, high levels of serum Ig with the V\textsubscript{H}a (a2) allotype are found (11). This increase in V\textsubscript{H}a Ig is a result of increased numbers of V\textsubscript{H}a B cells that use V\textsubscript{H}a4, V\textsubscript{H}a7, and V\textsubscript{H}a9 gene segments that encode several of the V\textsubscript{H}a (a2) allotype-associated amino acids (14, 15). Pospísil et al. (16) found that, in the appendix, more V\textsubscript{H}a B cells were proliferating and fewer were dying compared with V\textsubscript{H}n B cells.
The repertoire shift in Alicia rabbits, from the predominant expression of V_{H\alpha} allotype later 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 V_{H\alpha} and V_{H\beta} allotypes, we found that, in 9-wk-old rabbits, V_{H\alpha} 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 V_{H\alpha} and V_{H\beta} B cells, respectively, throughout life. We found that, although 10–25% of B cells at birth are V_{H\alpha}, at 3 wk of age essentially all B cells (~95%) in spleen, appendix, and PBLs were V_{H\beta} (Fig. 3 and not depicted). Subsequently, the percentage of V_{H\alpha} cells steadily declined, so that by 2 yr of age, <20% of B cells were V_{H\alpha} and >75% were V_{H\beta}. These data demonstrate that V_{H\alpha} B cells accumulate throughout life, with a sharp increase between 4 and 10 wk of age. The V_{H\beta} B cells accumulate faster in the appendix than in spleen, suggesting that the B cell repertoire shift from V_{H\alpha} to V_{H\beta} 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 saccular 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 V_{H\alpha} to V_{H\beta} B cells, we expected that the peripheral blood B cells in LigApx rabbits would be predominantly V_{H\beta}. 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 V_{H\beta} (Fig. 4). As expected, the percentage of V_{H\alpha} B cells in unmanipulated rabbits was approximately 10–25%. The repertoire shift in Alicia rabbits was 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 development of follicles containing proliferating B cells and lead to the repertoire shift from V_{H\alpha} to V_{H\beta} B cells. To test this possibility, we surgically disrupted the GALT–bacterial interaction in Alicia rabbits and tested whether the repertoire shift from V_{H\alpha} to V_{H\beta} B cells was abrogated.
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 V Hn 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 V Hn molecules rather than V Ha 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 V Hn gene segments (Table I), whereas almost none (4%) of the VDJ genes from control (unmanipulated) Alicia rabbits of the same age used V Hn gene segments. We think the PCR analysis underestimated the expression of V Hn 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 V Hn.

To determine whether the low percentage of V Hn-encoding genes (Table I) resulted from preferential amplification of V Hn 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 V Hn and V Hn 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 V Hn gene segments. The reduced number of V Hn PCR products was also observed with another 5' V H primer, V Hldr (5'GGCTTCTCCTGTCGCTG-3'), which anneals to a different target site. The preferential amplification of V Hn cDNA with independent primers suggests that, even though V Hn B cells appear to express equivalent amounts of surface IgM (Fig. 4), V Hn 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 V Hn-encoding VDJ genes, the data confirm the FACS analysis, which showed that most of the B cells of LigApx Alicia rabbits were V Hn instead of V Hn. We conclude that the repertoire shift from V Hn to V Hn 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>VHn (%)</th>
<th>VHa</th>
</tr>
</thead>
<tbody>
<tr>
<td>LigApx, 12 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32P2</td>
<td>16 (70)</td>
<td>7</td>
</tr>
<tr>
<td>144T</td>
<td>18 (86)</td>
<td>3</td>
</tr>
<tr>
<td>94S</td>
<td>17 (81)</td>
<td>4</td>
</tr>
<tr>
<td>199T1</td>
<td>10 (67)</td>
<td>5</td>
</tr>
<tr>
<td>LigApx, 8 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>94S</td>
<td>13 (81)</td>
<td>3</td>
</tr>
<tr>
<td>Control, 12 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>320W2</td>
<td>0 (0)</td>
<td>15</td>
</tr>
<tr>
<td>127W1</td>
<td>0 (0)</td>
<td>11</td>
</tr>
<tr>
<td>199T3</td>
<td>0 (0)</td>
<td>12</td>
</tr>
<tr>
<td>127W2</td>
<td>2 (20)</td>
<td>8</td>
</tr>
</tbody>
</table>

*VDJ genes were PCR amplified from PBLs.
*The data were obtained using V HnR as the 5' primer. Similar results were obtained in four additional experiments utilizing the V H nR primer as the 5' primer: for rabbit 94S (12 wk), 5 out of 22 (23%) sequences were derived from V H nR genes; for rabbit 127W1, 8 out of 9 (89%) were derived from V H a genes; for rabbit 127W2, 8 out of 11 (73%) were derived from V H n genes.
quired interactions between GALT and the microflora and that expansion of V_{Ha} B cells requires such interactions.

**Rearrangement status of IgH alleles in V_{Ha} B cells**

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

If the repertoire shift from V_{Hn} to V_{Ha} B cells is caused by gene rearrangements of V_{Ha} gene segments on the second IgH allele in V_{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_{H} 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_{Ha} B cells rearranged only one IgH allele, indicating that the B cell repertoire shift from V_{Hn} to V_{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_{Ha} B cells.

**B cell receptor (BCR) signaling in V_{Ha} and V_{Hn} B cells**

One possible explanation for the preferential expansion of V_{Ha} B cells and the concomitant decrease in V_{Hn} B cells in Alicia rabbits is that V_{Ha} B cells are more responsive to BCR stimulation than V_{Hn} B cells. To test this possibility, we assessed the release of intracellular calcium after BCR cross-linking on V_{Ha} and V_{Hn} B cells from 12-wk-old Alicia rabbits. The Alicia rabbits had the b5 \( \kappa \) chain allotype; therefore, we incubated PBLs with anti-b5 antibody and measured the release of intracellular calcium, as described in Materials and Methods. We found that the V_{Hn} B cells responded to anti-b5 antibody as well as the V_{Ha} B cells when the differences in baseline stimulation were taken into account (Fig. 6). Anti-b4 \( \kappa \) chain allotype antibody served as a negative control. Although we cannot explain the different baseline stimulation of V_{Ha} and V_{Hn} B cells, we conclude that the inherent signaling capacity of V_{Ha} and V_{Hn} B cells is similar and, therefore, does not explain the selective expansion of V_{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...
found that the repertoire shift from V_\text{H}n to V_\text{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_\text{Ha}, and these B cells subsequently declined to nearly undetectable levels by week 3. Although these V_\text{Ha} 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_\text{H}n B cells with maternal V_\text{Ha} Ig bound through Fc receptors because, in this case, we would expect all B cells, rather than a subset, to be V_\text{Ha}. Instead, we think the decline in the percentage of V_\text{Ha} B cells may be due to a dramatic increase in V_\text{H}n 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_\text{H}n (30).

The shift from V_\text{Ha} to V_\text{H}n 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_\text{Ha} B cells as proposed by Pospisil et al. (16), who showed that more V_\text{Ha} B cells proliferate and fewer die than V_\text{H}n B cells in the appendices of Alicia rabbits. We suggest that V_\text{Ha} B cells are preferentially stimulated by interaction with a bacterial ligand or a bacterially induced GALT-derived ligand. Such preferential stimulation of V_\text{Ha} B cells could be due to differences between V_\text{Ha} and V_\text{H}n 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_\text{Ha} and V_\text{H}n B cells, suggesting that differences in BCR density in V_\text{Ha} and V_\text{H}n B cells do not contribute to the differential stimulation. Although we have not studied the localization of V_\text{Ha} and V_\text{H}n BCR in lipid rafts, we suggest that V_\text{Ha} and V_\text{H}n B cells are differentially stimulated by bacteria because of structural differences between the V_\text{H} regions of V_\text{Ha} and V_\text{H}n BCR. Differential stimulation of V_\text{Ha} B cells by bacteria will be investigated in future studies.

When we compared amino acid sequences encoded by V_\text{Ha} and V_\text{H}n gene segments, we found many differences in FR.1 and FR.3. These differences include V_\text{Ha}2 allotype-associated amino acids, which Pospisil et al. (16, 34) proposed may interact with a ligand, leading to expansion of V_\text{Ha}2 B cells. However, because the V_\text{Ha}2 allotype-associated amino acids are not present in allotypically encoded V_\text{Ha}1 and V_\text{Ha}3 allotypes (10), and because V_\text{Ha}1 and V_\text{Ha}3 B cells in a1/a1 and a1/a3 rabbits, respectively, also proliferate in GALT, we suggest that the a2 allotype-associated amino acids are not critical for preferential expansion of V_\text{Ha} B cells. Instead, we suggest that the nonallotype-associated amino acids present in V_\text{H}n molecules, but absent in V_\text{Ha} molecules, are responsible for preferential expansion of V_\text{Ha} B cells.

We examined the amino acid sequences encoded by V_\text{Ha} and V_\text{H}n gene segments and found six positions in FR.1 and FR.3 (3, 19, 21, 23, 78, 82A) in which the same amino acids were encoded by all six V_\text{H} gene segments known to encode V_\text{Ha} molecules, but not by the three V_\text{H} gene segments known to encode V_\text{H}n molecules (Fig. 7 a). In addition, we found that, at positions 79 and 82 (FR.3), the same amino acids were encoded by five out of six V_\text{Ha} gene segments, but not by the V_\text{H}n gene segments (Fig. 7 a). If selective expansion of V_\text{Ha} B cells results from interaction of a ligand with V_\text{H}n molecules, the contacting amino acids are likely to be present on the exterior surface of the V_\text{H}n 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_\text{H}n 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_\text{H}n molecules are part of a binding site for a bacterial ligand or a bacterially induced 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_\text{Ha} and V_\text{H}n molecules. We propose that a combination of seven V_\text{H} amino acids at positions 19, 21, 23,
77, 79, 81, and 82A constitutes a ligand binding site and, furthermore, that the ligand interacts more strongly with \( V_{H3} \) than with \( V_{H2} \) molecules, leading to the differential stimulation and subsequent expansion of \( V_{H3} \) B cells.

The putative \( V_{H} \) ligand binding site is on the exterior surface of the \( V_{H} \) region, similar to the \( V_{H} \) binding site of *Staphylococcus aureus* protein A in human \( V_{H} \) Ig molecules (35). Protein A binds to and preferentially stimulates B cells that use \( V_{H} \) gene segments of the \( V_{H3} \) family (36). Similarly, we think that a putative bacterial \( C_{H} \) cell superantigen (37) or a bacterially induced GALT-derived superantigen (38) preferentially binds to and stimulates \( V_{H3} \) B cells. If a \( C_{H} \) cell superantigen promotes positive selection of \( V_{H3} \) B cells in GALT, the interaction between such a \( C_{H} \) 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-tonal 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 antiallotype antibodies.

*Ali*/*ali* rabbits (designated Alicia; reference 1), which are homozygous for the b5 κ-chain allotype (b5/b5), 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. Anti-\( V_{H4} \) and anti-\( V_{H2} \) anti–\( \kappa \) chain allotype antisera were as described previously (41).

Anti-\( V_{H4} \) antibody directed against \( V_{H3} \) and \( V_{H4} \) allotypes was produced by immunizing a homozygous b5/b5 (IgG haplotype A/A) rabbit (L76–3) with IgG from a homozygous a2-suppressed a232y33 rabbit (42). Ig fractions of the anti-\( V_{H4} \) and anti-\( V_{H2} \) antisera (41) obtained by precipitation with 40% saturated ammonium sulfate were biotinylated for use in immunofluorescence analysis and in Ca\(^{2+}\) mobilization assays. By immunofluorescence, the anti-\( V_{H4} \) antibody reacted with ≤5% of peripheral B cells in adult homozygous a232y33 rabbits, as expected (unpublished data).

To confirm the anti-\( V_{H4} \) allotype antibodies reacted with \( V_{H4} \) and \( V_{H4} \) Ig, we analyzed PCR-amplified VDJ genes from FACS-sorted splenic \( V_{H4} \) B cells from Alicia rabbits, using a 5′ conserved \( V_{H4} \) leader primer and a 3′ primer specific for \( J_{H4} \). Nearly all of the VDJ genes (32 out of 34) encoded amino acids characteristic of the \( V_{H4} \) molecules encoded by the \( V_{H4} \) and \( V_{H4} \) 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-\( V_{H4} \) antibodies and found that, as expected, all 12 genes encoded amino acids characteristic of those encoded by the \( V_{H4} \) gene segments \( V_{H4} \), \( V_{H4} \), and \( V_{H4} \) (10, 12).

#### Immunofluorescence and flow cytometry.

10⁶ PBLs were prepared from buffy coat and stained with biotinylated rabbit anti-\( V_{H4} \) or biotinylated rabbit anti-\( V_{H4} \) 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-IgG 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 FACSCalibur flow cytometer (BD Biosciences) in the FACS core facility at Loyola University Chicago.

#### PCR analysis to determine rearrangement status of the IgH locus.

Single \( V_{H4} \) B cells were FACS sorted into 96-well V-bottom plates containing 1X lysis buffer as described previously (30). VDJ genes were PCR amplified using nested primers as follows: the 5′ primers were 5′-T(G/C)GTAGATT(G/C)AGGGTT/CJAGACA-3′ (sense-inside primer) and 5′-CATAAAAATC(T/C)ATGATGC-3′ (sense-inside primer), taken from conserved sequences 5′ of \( V_{H4} \) promoter regions; the 3′ primers were 5′-AGTTGAGTAGAGAGAGAGA-3′ (antisense-outside primer) and 5′-GAGTTCGCAAGGGACTCAC-3′ (antisense-inside primer), taken from conserved sequences 3′ of \( J_{H4} \) ( \( J_{H4} \) is used in 80–90% of VDJ gene rearrangements) and \( J_{H1} \). To determine whether rearrangements in the \( J_{H1} \) region had occurred, nested PCR amplification was performed by using the 5′ primers 5′-TGAGTGGCTGTTGGAGACT-3′ (sense-inside primer) and 5′-CAGACGCAGGCTGGTGCATAT-3′ (antisense-inside primer), taken from a region 5′ of the \( J_{H1} \) locus; the antisense primers were the same as those used for \( V_{H4} \) VDJ gene rearrangement.

#### 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 \( V_{H4} \) leader primer (\( V_{H} \)RPS; reference 45) and a 3′ primer specific for exon 1 of \( C_{\mu} \) (primer \( C_{\mu}-1\mu \); reference 46). The PCR products were cloned into pGEM-T Easy (Promega), and the nucleotide sequences were determined using an automated ABI Prism 310 sequence analyzer with Big Dye–labeled terminators (PerkinElmer and Applied Biosystems). The \( V_{H} \) gene segments used in the VDJ genes were identically by comparing the nucleotide sequences to those of known germline \( V_{H} \) gene segments. The germline \( V_{H} \) gene segment sequences most similar to those of the VDJ genes were designated as the used genes. All \( V_{H} \) gene sequences were submitted to GenBank/EMBL/DDJB and are available under the following accession nos.: rabbit, no. 32P2 (AY676759–AY676781); no. 144T (AY676782–AY676802); no. 948 (12 wk) (AY676803–AY676823); no. 199T1 (AY676824–AY676838); no. 948 (8 mo) (AY676895–AY676710); no. 329W2 (AY676711–AY676729); no. 127W1 (AY676726–AY676736); no. 199T3 (AY676737–AY676748); and no. 127W2 (AY676749–AY676758).

#### Ca\(^{2+}\) mobilization.

PBLs isolated with LSM® (ICN Biomedicals) were stained with anti-rabbit T cell mAb (clone KEN5; reference 43) and biotinylated anti-\( V_{H4} \) or anti-\( V_{H4} \) 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 Ca²⁺ and Mg²⁺ (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 μM 20% pluronic F-127 (Molecular Probes). VHa or VHn B cells were electronically gated as follows: VHn B cells were those cells in the lymphocyte gate that did not react with anti-VHa or anti-V-T cell antibodies, and the VHn B cells were cells that did not react with anti-VH or anti-V-T cell antibodies. The electronically gated VHn and VHa B cells were FACs sorted and, upon reanalysis by FACSCalibur, were shown to be at least 90% pure. The calcium flux of the VHn and VHa 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 VHn domain. The crystal structure of a Fab fragment of a human IgM antibody-encoding IgM rheumatoid factor (V(λ)3-30/1.9III; reference 35) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) and used as a modeling template for the rabbit VHn region. Modeling was performed using DeepView/Swiss-PdbViewer v3.7 (http://www.expasy.org/spdbv), and images were rendered using POV-Ray for Windows v3.5 (http://www.povray.org).

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