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_Hn allotype immunoglobulin (Ig)M. Within weeks, the number of V_Hn B cells decreases, whereas V_Ha allotype B cells increase in number and become predominant. We hypothesized that the repertoire shift from V_Hn to V_Ha 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_Hn to V_Ha repertoire shift did not occur, demonstrating the requirement for interactions between GALT and intestinal microflora in the selective expansion of V_Ha B cells. By comparing amino acid sequences of V_Hn and V_Ha Ig, we identified a putative V_H ligand binding site for a bacterial or endogenous B cell superantigen. We propose that interaction of such a superantigen with V_Ha B cells results in their selective expansion.

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Abbreviations used: BCR, B cell receptor; FR, framework region; GALT, gut-associated lymphoid tissue; LigApx, ligated appendix.

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 gene, *V_H1*, 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_Ha allotypic markers that are encoded by the predominantly rearranged gene, *V_H1* (1). The following three alleles of *V_H1* are found in laboratory rabbits: *V_H1-a^1*, *V_H1-a^2*, and *V_H1-a^3*; they encode the V_Ha1, V_Ha2, and V_Ha3 allotypes, respectively. These V_Ha 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_Ha1, anti-V_Ha2, or anti-V_Ha3 allotypic antibodies and is referred to as V_Hn (V_Ha-negative) Ig.

Kelus and Weiss (11) identified rabbits with a variant V_Ha2 allotype-encoding allele, *ali*, which has a 10-kb deletion of DNA encompassing *V_H1* (Fig. 1 and reference 1). In contrast with wild-type rabbits, nearly all Ig in young *ali/ali* rabbits (designated Alicia) is V_Hn. V_Hn Ig is encoded predominantly by *V_Hx*, *$V_H\gamma$*, and *$V_H\zeta$* (12, 13), which reside >50 kb upstream of *V_H1* (1). In adult Alicia rabbits, high levels of serum Ig with the V_Ha (a2) allotype are found (11). This increase in V_Ha Ig is a result of increased numbers of V_Ha B cells that use *V_H4*, *V_H7*, and *V_H9*, gene segments that encode several of the V_Ha (a2) allotype-associated amino acids (14, 15). Pospisil et al. (16) found that, in the appendix, more V_Ha B cells were proliferating and fewer were dying compared with V_Hn B cells.

K.-J. Rhee and P.J. Jasper contributed equally to this work.

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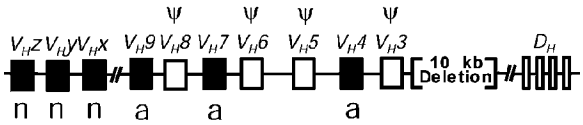

Figure 1. Organization of the 3′-most V_H gene segments in Alicia (ali/ali) rabbits. *V_H1* and *V_H2* are absent in Alicia rabbits because of a 10-kb deletion (brackets). Functional V_H gene segments that encode either the V_Ha allotype (a) or the V_Hn allotype (n) are indicated. The V_Hn allotype-encoding gene segments *V_Hx*, *V_Hy*, and *V_Hz* reside >50 kb upstream of *V_H1*. Their location respective to each other is unknown. ψ, nonfunctional V_H gene segments. D_H, D gene segments.

The molecular basis underlying the repertoire shift from V_Hn to V_Ha is unknown and is the subject of the current paper.

A shift in the B cell repertoire could arise from V_H 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 V_H gene replacement and secondary Ig gene rearrangement occur in peripheral tissues (20, 21). Another possible explanation for the B cell repertoire shift is that V_Ha 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 increased proliferation of V_Ha B cells compared with V_Hn B cells and lead to the repertoire shift from V_Hn to V_Ha B cells. To test this possibility, we surgically disrupted the GALT–bacterial interaction in Alicia rabbits and tested whether the repertoire shift from V_Hn to V_Ha B cells was abrogated.

RESULTS

Kinetics of the B cell repertoire shift

The repertoire shift in Alicia rabbits, from the predominant expression of V_Hn allotype early in life to the predominant expression of V_Ha 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_Hn and V_Ha allotypes, we found that, in 9-wk-old rabbits, V_Ha 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_Ha and V_Hn B cells, respectively, throughout life. We found that, although 10–25% of B cells at birth are V_Ha, at 3 wk of age essentially all B cells (~95%) in spleen, appendix, and PBLs were V_Hn (Fig. 3 and not depicted). Subsequently, the percentage of V_Hn cells steadily declined, so that by 2 yr of age, <20% of B cells were V_Hn and >75% were V_Ha. These data demonstrate that V_Ha B cells accumulate throughout life, with a sharp increase between 4 and 10 wk of age. The V_Ha B cells accumulate faster in the appendix than in spleen, suggesting that the B cell repertoire shift from V_Hn to V_Ha 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 sacculus 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_Hn to V_Ha B cells, we expected that the peripheral blood B cells in LigApx rabbits would be predominantly V_Hn. 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_Hn (Fig. 4). As expected, the percentage of V_Hn B cells in unmanipu-

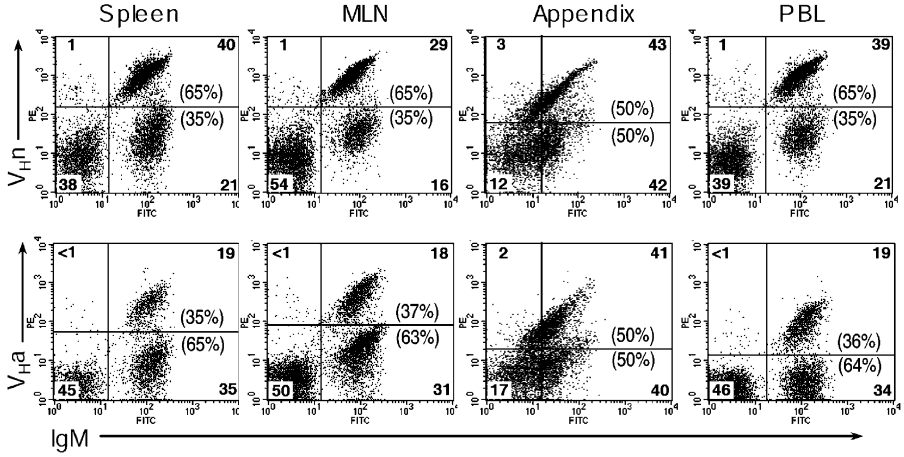

Figure 2. Flow cytometric analysis of B cells from a 9-wk-old Alicia rabbit. Cells from the spleen, mesenteric lymph node (MLN), appendix, and PBLs were double stained with anti-IgM (FITC) and either anti-V_Hn (PE; top) or anti-V_Ha (PE; bottom) antibodies. The numbers in parentheses indicate percentages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

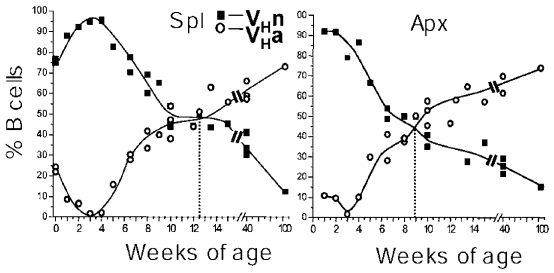

Figure 3. Kinetics of the change in percentage of V_Ha and V_Hn B cells from birth to 2 yr of age in spleen and appendix of Alicia rabbits. Cells were stained with anti-IgM (FITC) and either anti-V_Hn (PE) or anti-V_Ha (PE) antibodies (see Materials and Methods), and the percentages of V_Hn (■) and V_Ha (○) B cells for each rabbit are shown. The dotted line indicates the age at which V_Ha and V_Hn B cells each comprise 50% of the total number of B cells.

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 V_Hn, showing that the B cells remained predominantly V_Hn for many months. These results indicate that, without interactions between GALT and intestinal flora, the shift from V_Hn to V_Ha did not occur.

To confirm that the repertoire shift from V_Hn to V_Ha 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 V_H 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 V_Hn, whereas only 4% of the PCR-amplified VDJ genes were V_Hn.

Table I. V_Hn and V_Ha genes used in VDJ genes of LigApx and unmanipulated (control) Alicia rabbits[a]

	No. of VDJ sequences	
Rabbit no.	**V_Hn (%)**	**V_Ha**
LigApx, 12 wk		
32P2	16 (70)	7
144T	18 (86)	3
94S	17 (81)[b]	4
199T1	10 (67)	5
LigApx, 8 mo		
94S	13 (81)	3
Control, 12 wk		
320W2	0 (0)	15
127W1	0 (0)[b]	11
199T3	0 (0)[b]	12
127W2	2 (20)[b]	8

[a]VDJ genes were PCR amplified from PBLs.
[b]The data were obtained by using V_HRPS as the 5′ primer. Similar results were obtained in four additional experiments utilizing the $V_H$ldr primer as the 5′ primer: for rabbit 94S (12 wk), 5 out of 22 (23%) sequences were derived from V_Ha genes; for rabbit 127W1, 8 out of 9 (89%) were derived from V_Ha genes; for rabbit 199T3, 8 out of 8 (100%) sequences were derived from V_Ha genes; and for rabbit 127W2, 8 of 11 (73%) were derived from V_Ha genes.

To determine whether the low percentage of V_Hn-encoding genes (Table I) resulted from preferential amplification of V_Ha 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_Ha 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_Ha gene segments. The reduced number of V_Hn PCR products was also observed with another 5′ V_H primer, $V_H$ldr (5′-GGCTTCTCCTGGTCGCTG-3′), which anneals to a different target site. The preferential amplification of V_Ha cDNA with independent primers suggests that, even though V_Ha and V_Hn B cells appear to express equivalent amounts of surface IgM (Fig. 4), V_Ha 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_Ha-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_Ha. We conclude that the repertoire shift from V_Hn to V_Ha B cells re-

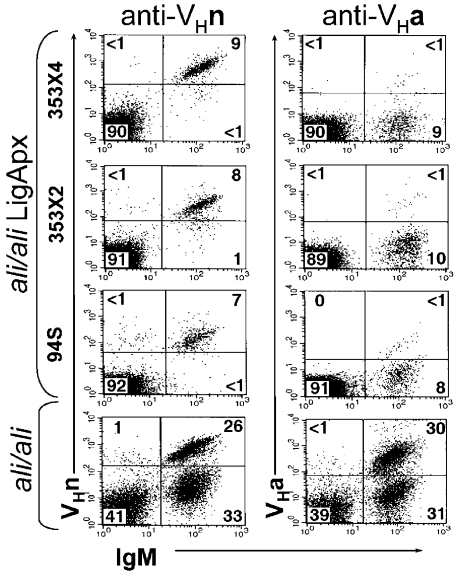

Figure 4. Flow cytometric analysis of PBLs of LigApx and unmanipulated Alicia rabbits. PBLs from 12-wk-old LigApx Alicia rabbits (rabbit nos. 94S, 353X2, and 353X4) and an unmanipulated rabbit were stained with anti-IgM (FITC) and either anti-V_Hn (PE; left) or anti-V_Ha (PE; right) antibodies. Percentage of cells in each quadrant is shown. The mean fluorescence intensity of surface IgM in V_Ha and V_Hn B cells in unmanipulated Alicia rabbits is 87.8 and 87.6, respectively.

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_Ha 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 on both IgH alleles in V_Ha B cells. To test this possibility, we sorted V_Ha B cells from an adult Alicia rabbit and assessed the status of 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.

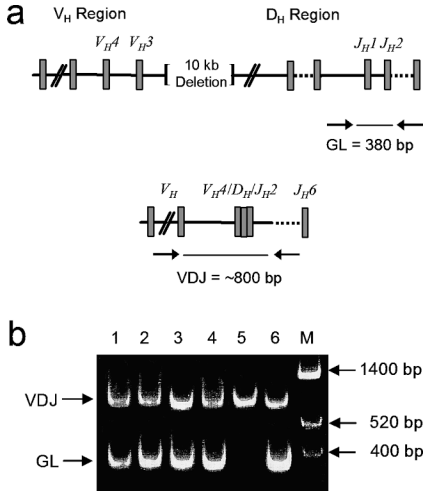

Figure 5. Single cell PCR of V_Ha B cells from PBLs of unmanipulated adult Alicia rabbits. (a) Location of PCR primer binding sites. Sense primer 5′ of *J_H1* and antisense primer 3′ of *J_H2* region results in a band of ~380 bp for germline configuration; sense primer from conserved region 5′ of *V_H* promoters and antisense primer 3′ of the *J_H* 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.

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 κ 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 κ 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_Hn 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

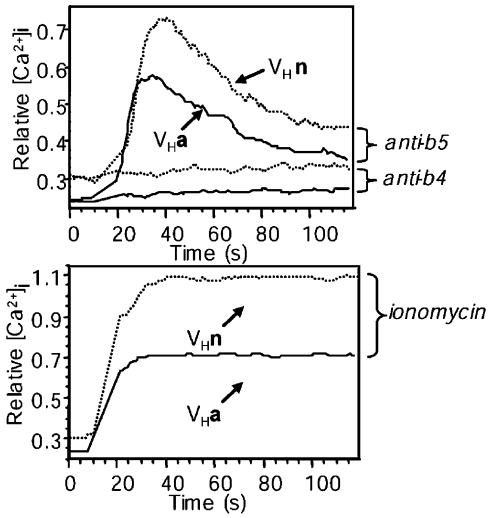

Figure 6. Intracellular calcium mobilization in V_Ha and V_Hn B cells after stimulation with anti–κ chain allotype antibodies, anti-b4 and anti-b5 (top). (solid line) V_Ha B cells; (dashed line) V_Hn B cells. PBLs from 12-wk-old control Alicia rabbits (b^5/b^5) were loaded with Fluo-3 and Fura-red, and calcium mobilization in electronically gated V_Hn and V_Ha B cells (see Material and Methods) was determined. Relative concentrations of cytoplasmic calcium $[Ca^{2+}]_i$ are shown as the mean of the ratio of Fluo-3 to Fura-red fluorescence. The release of $[Ca^{2+}]_i$ 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_Ha, and these B cells subsequently declined to nearly undetectable levels by week 3. Although these V_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_Hn B cells with maternal V_Ha Ig bound through Fc receptors because, in this case, we would expect all B cells, rather than a subset, to be V_Ha. Instead, we think the decline in the percentage of V_Ha B cells may be due to a dramatic increase in V_Hn 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_Hn (30).

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

When we compared amino acid sequences encoded by V_Ha and V_Hn gene segments, we found many differences in FR1 and FR3. These differences include V_Ha2 allotype-associated amino acids, which Pospisil et al. (16, 34) proposed may interact with a ligand, leading to expansion of V_Ha2 B cells. However, because the V_Ha2 allotype-associated amino acids are not present in allelically encoded V_Ha1 and V_Ha3 allotypes (10), and because V_Ha1 and V_Ha3 B cells in a^1/a^1 and a^3/a^3 rabbits, respectively, also proliferate in GALT, we suggest that the a2 allotype-associated amino acids are not critical for preferential expansion of V_Ha B cells. Instead, we suggest that the nonallotype-associated amino acids present in V_Ha molecules, but absent in V_Hn molecules, are responsible for preferential expansion of V_Ha B cells.

We examined the amino acid sequences encoded by V_Ha and V_Hn 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_H gene segments known to encode V_Ha molecules, but not by the three V_H gene segments known to encode V_Hn 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_Ha gene segments, but not by the V_Hn gene segments (Fig. 7 a). If selective expansion of V_Ha B cells results from interaction of a ligand with V_H molecules, the contacting amino acids are likely to be present on the exterior surface of the V_H 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_H 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_Ha 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_Ha and V_Hn molecules. We propose that a combination of seven V_H amino acids at positions 19, 21, 23,

a

	FR1					FR3				
Amino acid Position →	*3*	**19**	**21**	**23**	**77**	*78*	**79**	**81**	*82*	**82A**
Consensus	*S*	**T**	**T**	**T**	**T**	*V*	**T**	**K**	*M*	**T**
V_H1-a1	.	.	.	.	.	.	**D**	.	*I*	.
V_H1-a2	.	.	.	.	.	.	.	.	.	.
V_H1-a3	.	.	.	.	.	.	.	**Q**	.	.
V_H4-a2	.	.	.	.	.	.	.	.	.	.
V_H7-a2	.	.	.	.	.	.	.	.	.	.
V_H9-a2	.	.	.	.	.	.	.	.	.	.
V_HX	*Q*	**K**	**S**	**K**	.	*L*	**Y**	**Q**	*L*	**N**
V_HY	*Q*	**E**	**C**	**K**	.	*G*	**C**	**Q**	*L*	**N**
V_HZ	*Q*	**K**	**C**	**K**	.	*G*	**C**	**Q**	*L*	**N**

b

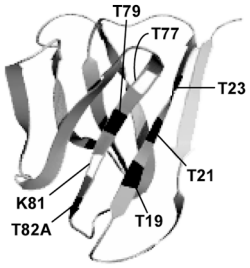

Figure 7. Proposed ligand binding sites in the V_H region of V_Ha B cells. (a) Comparison of select amino acids in FR1/FR3 of V_Ha and V_Hn molecules encoded by V_Ha (*V_H1a1*, *V_H1a2*, *V_H1a3*, *V_H4*, *V_H7*, *V_H9*) and V_Hn (*V_HX*, *V_HY*, *V_HZ*) gene segments, respectively. (dots) Identity to consensus V_Ha amino acids; (bold text) residues proposed to contact a potential ligand. (b) Three-dimensional model of rabbit V_H region depicting proposed contact residues (dark and light) for a B cell superantigen. White residues (77, 81) are those that do not distinguish between V_Ha and V_Hn molecules but by three-dimensional modeling appear to be part of the putative ligand-binding site. Numbers indicate amino acid position based on the Kabat numbering system (reference 48).

77, 79, 81, and 82A constitutes a ligand binding site and, furthermore, that the ligand interacts more strongly with V_Ha than with V_Hn molecules, leading to the differential stimulation and subsequent expansion of V_Ha 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 B cell superantigen (37) or a bacterially induced GALT-derived superantigen (38) preferentially binds to and stimulates V_Ha B cells. If a B cell superantigen promotes positive selection of V_Ha 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 peritoneal 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 (b^5/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_Hn antibody directed against V_Hx and V_Hy allotypes was produced by immunizing a homozygous $a^1x^-y^-$ (IgH haplotype A/A) rabbit (L76-3) with IgG from a homozygous a2-suppressed $a^2x^{32}y^{33}$ rabbit (42). Ig fractions of the anti-V_Hn and anti-V_Ha2 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_Hn antibody reacted with <5% of peripheral B cells in adult homozygous $a^2x^{32}y^{33}$ rabbits, as expected (unpublished data).

To confirm that the anti-V_Hn allotype antibodies reacted with V_Hx and V_Hy Ig, we analyzed PCR-amplified VDJ genes from FACS-sorted splenic V_Hn B cells from Alicia rabbits, using a 5′ conserved V_H leader primer and a 3′ primer specific for J_H. Nearly all of the VDJ genes (32 out of 34) encoded amino acids characteristic of the V_Hn molecules encoded by the V_Hx and V_Hy 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_Hn antibodies and found that, as expected, all 12 genes encoded amino acids characteristic of those encoded by the V_Ha gene segments V_H4, V_H7, and V_H9 (10, 12).

Immunofluorescence and flow cytometry. 10^6 PBLs were prepared from buffy coat and stained with biotinylated rabbit anti-V_Hn or biotinylated rabbit anti-V_Ha2 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-IgL 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_Ha 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′-T[G/C]-GATAT[T/G]AAGGG[T/C]ACACA-3′ (sense-outside primer) and 5′-CATAAAAATTCA[T/C]ATGATC-3′ (sense-inside primer), taken from conserved sequences 5′ of V_H promoter regions; the 3′ primers were 5′-AGTTGAGTAGGAGAGAGA-3′ (antisense-outside primer) and 5′-GAG-TTGGCAAGGACTCAC-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_H2. To determine whether rearrangements in the J_H region had occurred, nested PCR amplification was performed by using the 5′ primers 5′-TGAGTGCTGTTGGACTGGCT-3′ (sense-outside primer) and 5′-CAGAGCTGGAGCTGTGCTAT-3′ (sense-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_HRPS; reference 45) and a 3′ primer specific for exon 1 of Cμ (primer C_H1-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 gene segments used in the VDJ genes were identified 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/DDBJ and are available under the following accession nos.: rabbit, no. 32P2 (AY676759-AY676781); no. 144T (AY676782-AY676802); no. 94S (12 wk) (AY676803-AY676823); no. 199T1 (AY676824-AY676838); no. 94S (8 mo) (AY676695-AY676710); no. 320W2 (AY676711-AY676725); no. 127W1 (AY676726-AY676736); 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 with biotinylated anti-V_Hn or anti-V_Ha 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 Ca^{2+} and Mg^{2+} (GIBCO BRL) and were incubated with rotation for 45 min at room temperature in 10 μM Fura-red, 5 μM Fluo-3 (prepared as 1 mM stocks in 100% DMSO; Molecular Probes), and 2.8 μl 20% pluronic F-127 (Molecular Probes). V_Ha or V_Hn B cells were electronically gated as follows: V_Hn B cells were those cells in the lymphocyte gate that did not react with anti-V_Ha or anti–T cell antibodies, and the V_Ha B cells were cells that did not react with anti-V_Hn or anti–T cell antibodies. The electronically gated V_Hn and V_Ha B cells were FACS sorted and, upon reanalysis by FACSCalibur, were shown to be at least 90% pure. The calcium flux of the V_Hn and V_Ha 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 ($[Ca^{2+}]i$) levels were calculated and analyzed using FlowJo software (Tree Star, Inc.).

Three-dimensional modeling of rabbit V_H domain. The crystal structure of a Fab fragment of a human IgM antibody-encoding IgM rheumatoid factor (V_H3-30/1.9III; reference 35) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) and used as a modeling template for the rabbit V_H 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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REFERENCES

1. Knight, K.L., and R.S. Becker. 1990. Molecular basis of the allelic inheritance of rabbit immunoglobulin VH allotypes: implications for the generation of antibody diversity. *Cell.* 60:963–970.
2. Weinstein, P.D., A.O. Anderson, and R.G. Mage. 1994. Rabbit IgH sequences in appendix germinal centers: VH diversification by gene conversion-like and hypermutation mechanisms. *Immunity.* 1:647–659.
3. Vajdy, M., P. Sethupathi, and K.L. Knight. 1998. Dependence of antibody somatic diversification on gut-associated lymphoid tissue in rabbits. *J. Immunol.* 160:2725–2729.
4. Reynaud, C.A., V. Anquez, H. Grimal, and J.-C. Weill. 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell.* 48:379–388.
5. Thompson, C.B., and P.E. Neiman. 1987. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell.* 48:369–378.
6. Reynaud, C.A., C.R. Mackay, R.G. Muller, and J.-C. Weill. 1991. Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches. *Cell.* 64:995–1005.
7. Jenne, C.N., L.J. Kennedy, P. McCullagh, and J.D. Reynolds. 2003. A new model of sheep Ig diversification: shifting the emphasis toward combinatorial mechanisms and away from hypermutation. *J. Immunol.* 170:3739–3750.
8. Tunyaplin, C., and K.L. Knight. 1995. Fetal VDJ gene repertoire in rabbit: evidence for preferential rearrangement of *V_H1*. *Eur. J. Immunol.* 25:2783–2787.
9. Lanning, D., P. Sethupathi, K.-J. Rhee, S.-K. Zhai, and K.L. Knight. 2000. Intestinal microflora and diversification of the rabbit antibody repertoire. *J. Immunol.* 165:2012–2019.
10. Mage, R.G., K.E. Bernstein, N. McCartney-Francis, C.B. Alexander, G.O. Young-Cooper, E.A. Padlan, and G.H. Cohen. 1984. The structural and genetic basis for expression of normal and latent V_Ha allotypes of the rabbit. *Mol. Immunol.* 21:1067–1081.
11. Kelus, A.S., and S. Weiss. 1986. Mutation affecting the expression of immunoglobulin variable regions in the rabbit. *Proc. Natl. Acad. Sci. USA.* 83:4883–4886.
12. Short, J.A., P. Sethupathi, S.-K. Zhai, and K.L. Knight. 1991. VDJ genes in V_Ha2 allotype-suppressed rabbits. Limited germline V_H gene usage and accumulation of somatic mutations in D regions. *J. Immunol.* 147:4014–4018.
13. Friedman, M.L., C. Tunyaplin, S.-K. Zhai, and K.L. Knight. 1994. Neonatal V_H, D, and J_H gene usage in rabbit B lineage cells. *J. Immunol.* 152:632–641.
14. Sehgal, D., R.G. Mage, and E. Schiaffella. 1998. V_H mutant rabbits lacking the V_H1a2 gene develop $a2^+$ B cells in the appendix by gene conversion-like alteration of a rearranged V_H4 gene. *J. Immunol.* 160:1246–1255.
15. Zhu, X., A. Boonthum, S.-K. Zhai, and K.L. Knight. 1999. B lymphocyte selection and age-related changes in V_H gene usage in mutant Alicia rabbits. *J. Immunol.* 163:3313–3320. (published erratum appears in *J. Immunol.* 2000. 164:2838)
16. Pospisil, R., G.O. Young-Cooper, and R.G. Mage. 1995. Preferential expansion and survival of B lymphocytes based on V_H framework 1 and framework 3 expression: "positive" selection in appendix of normal and V_H-mutant rabbits. *Proc. Natl. Acad. Sci. USA.* 92:6961–6965.
17. Han, S., S.R. Dillon, B. Zheng, M. Shimoda, M.S. Schlissel, and G. Kelsoe. 1997. V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science.* 278:301–305.
18. Qin, X.-F., S. Schwers, W. Yu, F. Papavasiliou, H. Suh, A. Nussenzweig, K. Rajewsky, and M.C. Nussenzweig. 1999. Secondary *V(D)J* recombination in B-1 cells. *Nature.* 397:355–359.
19. Yu, W., H. Nagaoka, M. Jankovic, Z. Misulovin, H. Suh, A. Rolink, F. Melchers, E. Meffre, and M.C. Nussenzweig. 1999. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature.* 400:682–687.
20. Madan, M.K., R. Golub, M. Wabl, and G.E. Wu. 2000. Specific antibody production by V_H-gene replacement. *Eur. J. Immunol.* 30:2404–2411.
21. Sekiguchi, D.R., R.A. Eisenberg, and M. Weigert. 2003. Secondary heavy chain rearrangement: a mechanism for generating anti–double-stranded DNA B cells. *J. Exp. Med.* 197:27–39.
22. Cyster, J.G., J.I. Healy, K. Kishihara, T.W. Mak, M.L. Thomas, and C.C. Goodnow. 1996. Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. *Nature.* 381:325–328.
23. Hayakawa, K., M. Asano, S.A. Shinton, M. Gui, D. Allman, C.L. Stewart, J. Silver, and R.R. Hardy. 1999. Positive selection of natural autoreactive B cells. *Science.* 285:113–116.
24. Levine, M.H., A.M. Haberman, D.B. Sant'Angelo, L.G. Hannum, M.P. Cancro, C.A. Janeway Jr., and M.J. Shlomchik. 2000. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. *Proc. Natl. Acad. Sci. USA.* 97:2743–2748.
25. Hayakawa, K., M. Asano, S.A. Shinton, M. Gui, L.-J. Wen, J. Dashoff, and R.R. Hardy. 2003. Positive selection of anti–thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. *J. Exp. Med.* 197:87–99.
26. Julien, S., P. Soulas, J.-C. Garaud, T. Martin, and J.-L. Pasquali. 2002. B cell positive selection by soluble self-antigen. *J. Immunol.* 169:4198–4204.
27. Štěpánková, R., F. Kovářů, and J. Kruml. 1980. Lymphatic tissue of the intestinal tract of germfree and conventional rabbits. *Folia Microbiol.* 25:491–495.
28. Sudo, N., S. Sawamura, K. Tanaka, Y. Aiba, C. Kubo, and Y. Koga. 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J. Immunol.* 159:1739–1745.
29. Talham, G.L., H.Q. Jiang, N.A. Bos, and J.J. Cebra. 1999. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect. Immun.* 67:1992–2000.
30. Jasper, P.J., S.-K. Zhai, S.L. Kalis, M. Kingzette, and K.L. Knight. 2003. B lymphocyte development in rabbit: progenitor B cells and waning of B lymphopoiesis. *J. Immunol.* 171:6372–6380.
31. Sanchez, P., A.-M. Crain-Denoyelle, P. Daras, M.-C. Gendron, and

C. Kanellopoulos-Langevin. 2000. The level of expression of μ heavy chain modifies the composition of peripheral B cell subpopulations. *Int. Immunol.* 12:1459–1466.
32. Heltemes, L.M., and T. Manser. 2002. Level of B cell antigen receptor surface expression influences both positive and negative selection of B cells during primary development. *J. Immunol.* 169:1283–1292.
33. Cheng, P.C., M.L. Dykstra, R.N. Mitchell, and S.K. Pierce. 1999. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* 190:1549–1560.
34. Pospisil, R., and R.G. Mage. 1998. B-cell superantigens may play a role in B-cell development and selection in the young rabbit appendix. *Cell. Immunol.* 185:93–100.
35. Graille, M., E.A. Stura, A.L. Corper, B.J. Sutton, M.J. Taussig, J.B. Charbonnier, and G.J. Silverman. 2000. Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. *Proc. Natl. Acad. Sci. USA.* 97:5399–5404.
36. Silverman, G.J., and C.S. Goodyear. 2002. A model B-cell superantigen and the immunobiology of B lymphocytes. *Clin. Immunol.* 102: 117–734.
37. Rhee, K.-J., P. Sethupathi, A. Driks, D.K. Lanning, and K.L. Knight. 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J. Immunol.* 172: 1118–1124.
38. Pospisil, R., M.G. Fitts, and R.G. Mage. 1996. CD5 is a potential selecting ligand for B cell surface immunoglobulin framework region sequences. *J. Exp. Med.* 184:1279–1284.
39. Sehgal, D., H. Obiakor, and R.G. Mage. 2002. Distinct clonal Ig diversification patterns in young appendix compared to antigen-specific splenic clones. *J. Immunol.* 168:5424–5433.
40. Casola, S., K.L. Otipoby, M. Alimzhanov, S. Humme, N. Uyttersprot, J.L. Kutok, M.C. Carroll, and K. Rajewsky. 2004. B cell receptor signal strength determines B cell fate. *Nat. Immunol.* 5:317–327.
41. Dray, S., G.O. Young, and L. Gerald. 1963. Immunochemical identification and genetics of rabbit γ-globulin allotypes. *J. Immunol.* 91:403–415.
42. Knight, K.L., A. Gilman-Sachs, R. Fields, and S. Dray. 1971. Allotypic determinants on the Fab fragment of rabbit *Aa* locus negative IgG-immunoglobulin. *J. Immunol.* 106:761–767.
43. Kotani, M., Y. Yamamura, T. Tamatani, F. Kitamura, and M. Miyasaka. 1993. Generation and characterization of monoclonal antibodies against rabbit CD4, CD5 and CD11a antigens. *J. Immunol. Methods.* 157:241–252.
44. Krug, M.S., and S.L. Berger. 1987. First-strand cDNA synthesis primed with oligo(dT). *Methods Enzymol.* 152:316–325.
45. Winstead, C.R., S.-K. Zhai, P. Sethupathi, and K.L. Knight. 1999. Antigen-induced somatic diversification of rabbit IgH genes: gene conversion and point mutation. *J. Immunol.* 162:6602–6612.
46. Crane, M.A., M. Kingzette, and K.L. Knight. 1996. Evidence for limited B-lymphopoiesis in adult rabbits. *J. Exp. Med.* 183:2119–2127.
47. Sun, T., M.R. Clark, and U. Storb. 2002. A point mutation in the constant region of Ig lambda1 prevents normal B cell development due to defective BCR signaling. *Immunity.* 16:245–255.
48. Kabat, E.A., T.T. Wu, H.M. Perry, K.S. Gottesman, and C. Foeller. 1991. Sequences of Proteins of Immunological Interest, fifth edition. United States Department of Health and Human Services, Washington D.C. 2597 pp.