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Mechanism for Pre-B Cell Loss in $V_H$-Mutant Rabbits

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Pre-BCR signaling is a critical checkpoint in B cell development in which B-lineage cells expressing functional IgH $\mu$-chain are selectively expanded. B cell development is delayed in mutant ali/ali rabbits because the a-allotype encoding $V_{H1}$ gene, which is normally used in VDJ gene rearrangements in wt rabbits, is deleted, and instead, most B lineage cells use the a-allotype encoding $V_{H4}$ gene ($V_{H4}(a)$), which results in a severe developmental block at the pre-B cell stage. We found that $V_{H4}(a)$-utilizing pre-B cells exhibit reduced pre-BCR signaling and do not undergo normal expansion in vitro. Transduction of murine 38B9 pre-B cells with chimeric rabbit-VDJ mouse-C$\mu$1 encoding retroviruses showed $V_{H4}(a)$-encoded $\mu$-chains do not readily form signal-competent pre-BCR, thereby explaining the reduction in pre-BCR signaling and pre-B cell expansion. Development of $V_{H4}(a)$-utilizing B cells can be rescued in vivo by the expression of an Igk transgene, indicating that $V_{H4}(a)$-$\mu$ chains are not defective for conventional BCR formation and signaling. The ali/ali rabbit model system is unique because $V_{H4}(a)$-$\mu$ chains have the capacity to pair with a variety of conventional IgL chains and yet lack the capacity to form a signal-competent pre-BCR. This system could allow for identification of critical structural parameters that govern pre-BCR formation/signaling.

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uring B cell development in the bone marrow (BM), the pre-B cell checkpoint tests newly rearranged $\mu$-chains for their ability to fold properly and carry out signal transduction (1, 2). Signaling occurs through the pre-BCR consisting of Ig-$\alpha$/B, $\mu$-chain, and an IgL-like complex, designated surrogate L chain (SL) (3–5). Pre-BCR signaling presumably leads to expansion of B cells with $\mu$-chains that pair with conventional IgL (1, 6, 7). Studies in mouse have shown that 50% of $\mu$-chains from early pre-B cells do not pair with SL, and these cells are likely deleted in the BM environment (8). Developmental blocks at the pre-B cell stage are generally due to an inability of $\mu$-chains to form a signal-competent pre-BCR (6, 9). Instances in which restricted $\mu$-chains allow for normal pre-B cell development have been attributed to compensatory mechanisms of CDR3 that permit pre-BCR formation (6, 9, 10). We use a mutant rabbit model system to investigate a developmental bottleneck at the pre-B cell stage associated with utilization of a particular $V_{H1}$ gene.

The mutant ali/ali rabbit was discovered 25 y ago through the study of rabbit $V_{H1}$ allotypes (11). Serum analysis of young ali/ali rabbits indicated that $V_{H1}$-Ig; wt, wild-type. Surviving B-lineage cells are lost during the early stages of B cell development in the BM; however, the exact stage and mechanism for this selection remains unknown (15). One possibility is that $V_{H4}(a)$-utilizing pre-B cells are negatively selected due to BM environmental factors or to autoreactivity; we hypothesize, however, that $V_{H4}(a)$-utilizing B cells are lost due to a defect in pre-BCR formation/signaling. The purpose of this study was to define the stage at which these $V_{H4}(a)$-utilizing B-lineage cells are lost and also to identify the molecular mechanism(s) for this loss.

Materials and Methods

Rabbits

New Zealand White rabbits were from the colony maintained by K.L.K. at Loyola University Chicago. Mutant ali/ali rabbits were progeny of rabbits originally described by Kelsus and Weiss (11). Studies were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago. Peripheral blood samples from human-Igk ali/ali rabbits and age-matched nontransgenic controls were provided by J. Platerz (Roche Diagnostics, pRED, Pharma Research and Early Development, Biologics Research, Nonnenwald, Penzberg, Germany). The human-Igk transgenic rabbits were generated by modifying the bacterial artificial chromosome clone 179L1 (18) coding for parts of the rabbit Igkl locus by homologous recombination in Escherichia coli. Rabbit Ck was exchanged with human CkI and a rearranged human VJk with rabbit $\kappa$ promoter and was inserted into the Jk region. The linearized bacterial artificial chromosome was used for transfection in mouse 3T3 cells. The resulting clones were screened for the presence of the transgene and for expression of the transgene products in vitro by immunoblotting.
for transgenesis. Founder lines were backcrossed onto the Balb (19) background.

Abs and flow cytometry

Abs used: mouse anti-human CD79a-PE (catalog number 559395) or APC (catalog number 551134), rabbit anti-human caspase-3–FITC (catalog number 559341), mouse anti-human spleen tyrosine kinase (Syk)-P-YY348 (110-722), mouse anti-rabbit–μ (367.2-2), and rat anti-mouse pre-BCR (SL-156-bio) from Biolegend (San Diego, CA); rat anti-mouse PE and PE-Cy7 (Il4/1) from e Bioscience (San Diego, CA); goat Fab(1’2) anti-human IgH-FITC anti-goat Ig H+L, and allophycocyanin-conjugated streptavidin from Jackson ImmunoResearch Laboratories (West Grove, PA); and biotinylated rabbit anti-α2–allotype and FITC-rabbit anti-α2–allotype IgG (generated by K.L.K.’s laboratory). Viability was determined using Live/Dead Aqua from Invitrogen Molecular Probes (Carlsbad, CA) according to the manufacturer’s instructions. Fixation and permeabilization was performed using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and 3 h postirradiation. Cells were fixed using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Pro-B, pre-B, and immature B cells were distinguished by CD79a, cytoplasmic-μ, and surface-μ staining; pro-B (CD79a+cytoplasmic-μ+surface-μ-); pre-B (CD79a+c+μ−surface-μ+); and immature B (CD79a+c+μ−surface-μ−).

OP9 cocultures

Murine OP9-GFP stromal cells were seeded at 50–60% confluency, and 24 h later, 2.5 × 10⁹ total BM mononuclear cells prepared by ammonium chloride lysis from wt or ali/ali rabbits were seeded in a 100-mm dish (20) and cultured with 10 ng/ml recombinant human IL-7 (PreproTech, Rocky Hill, NJ). On day 4, cultures received an additional 3 ng/ml IL-7, and after 1 wk, nonadherent cells were harvested and phenotyped using flow cytometry. Equal numbers (7 × 10⁶) of ali/ali or wt pro-B cells were seeded into 24-well plates with fresh OP9-GFP stroma in the absence of IL-7. Cells were labeled with 0.25 μM CFSE using the CellTRACE CFSE Cell Proliferation Kit according to the manufacturer’s instructions (Intragen, Irvine, California). The ratio of pre-B to pro-B cells in these cultures was determined by adding an equal number (1.5 × 10⁶) of ali/ali or wt pro-B cells into each well. 3 × 10⁶ total OP9 cells were cultured in a 100-mm dish and transduced with polyethyleneimine at a ratio of 2.25 μg polyethyleneimine (Polysciences, Wadsworth, PA) to each sample. Total events were collected using a stopping gate of 3 × 10⁹ YG Microsphere beads (Polysciences), and the number of live pre-B and pro-B cells in the lymphocyte gate was used to generate the pre-B to pro-B ratio.

Induction of apoptosis in rabbit B-lineage cells

The rabbit B cell line 55D1 was irradiated by exposure to UV light (G365 tissue culture hood UV bulb) for 20 min and harvested immediately after (t₀) and 3 h postirradiation. Cells were fixed using BD Cytofix/Cytoperm (BD Pharmingen) and stained with anti-CD79a-PE and rabbit anti-caspase-3–FITC Abs or FITC rabbit anti-α2–allotype (isotype control) and analyzed by flow cytometry.

Retrovirus production and 38B9 cell transduction

The mouse SP6-Leader–VDJ-Cα expression cassette (8) was cloned into the Phoenix-ECO system, as described (21). Briefly, 5 × 10⁶ Phoenix-ECO cells were seeded in a 100-mm dish and transduced using polyethyleneimine at a ratio of 2.25 μg polyethyleneimine (Polysciences; Warrington, PA) per microgram DNA. Virus was harvested 48 h later, and 38B9 pre-B cells (a gift from Fritz Melchers) were transduced by spinoculation of 300 μl of virus buffer with 10 μM HEPES onto 7 × 10⁶ 38B9 cells/well (48-well plate) at 1200 × g for 1 h at 30°C. One half of the medium was replaced at 4 and 16 h following transduction. GFP⁺ cells were FAC-sorted and analyzed for surface pre-BCR and cytoplasmic-μ expression. 38B9 cells were tested pre- and posttransduction for the expression of endogenous-μ and Igκ expression and were not detected in any of the cultures. All cells were grown in DMEM with PenStrep (Invitrogen Life Technologies, Carlsbad, CA) and 10% FCS (Atlanta Biologicals, Lawrenceville, GA).

PCR amplification and sequencing of VDJ genes

Pro-B, pre-B, and immature B cells from ali/ali rabbit BM cultures were bulk sorted (1000 cells/well) between 0 and 7 d after transfer to fresh OP9 stroma, as described above. VDJ gene segments were PCR-amplified using the forward pan Vμ primer EcoRI, 5′-GGCTGAATTCGTCTCTTGCAGCTGTCGAC-3′, and reverse rabbit JH3-BamHI, 5′-GGATCCCGTGCACGGCTCTAC-3′. PCR products were cloned into pGEM-T-Easy Vector (Promega, Madison, WI), and the nucleotide sequences were determined in one orientation using T7 or SP6 promoter oligomers. VDJ sequences from single a2⁺ peripheral blood B cells of hu-Igκ transgenic ali/ali rabbits were determined as described (22).

Results

In vitro development of B-lineage cells from BM of ali/ali rabbits

To reproduce the in vivo selection against VμH(a)-utilizing B-lineage cells in BM of ali/ali rabbits, we cultured BM cells on OP9 stroma in the presence of IL-7. After 1 wk, most of the cells (~70–95%) were pro-B cells and were replated on fresh OP9 stroma and allowed to differentiate in the absence of IL-7. B-lineage cells of various stages from these cultures were sorted, and the VDJ genes were PCR-amplified and sequenced. We examined the nonfunctional VDJ gene rearrangements of pro-B cells to determine the frequency of VμH gene usage in the absence of selection. Consistent with previous in vivo observations (15, 23), the in vitro-derived ali/ali pro-B cells (CD79a⁺, cyto-μ−) used primarily a VμH-encoding gene, VμH(a), in their VDJ gene rearrangements (Fig. 1, left). In contrast, most of the productive rearrangements in immature B cells (CD79a⁺, cyto-μ−, surface-μ−) used Vμκ genes (Fig. 1, right), as was found in vivo (15, 23). The similarity of VμH gene utilization between the in vitro- and in vivo-derived pro-B and immature B cells demonstrates that the OP9 coculture system reproduces ali/ali B cell development and selection in vivo. Although VμH(a) was the predominantly used gene in pro-B cells both in vivo and in vitro, Vμ κ genes were used in most immature B cells, the Vμ κ gene usage in pro-B (CD79a⁺, cyto-μ−, surface-μ−) cells was different. In vitro-generated pre-B cells predominantly used VμH(a), whereas in vivo, Vμκ-genes predominated (Fig. 1, center). We hypothesize that the VμH(a)-
utilizing pre-B cells observed in vitro are early-stage pre-B cells that have not undergone selection.

Early pre-B cells differentiate to large pre-B cells, which then undergo proliferation (24, 25). To determine if the in vitro-derived ali/ali pre-B cells undergo proliferative expansion, we labeled pro-B cells with CFSE and transferred them to fresh OP9 stroma. Between days 3 and 6, we found no increase in proliferation of ali/ali pre-B cells relative to pro-B cells, whereas nearly all of the pre-B cells from wt rabbits proliferated more than the pro-B cells (gating of pro-B and pre-B in Fig. 2A; CFSE analysis in Fig. 2B). The differences in proliferation between ali/ali and wt pre-B cells were also reflected in pre-B to pro-B cell ratios found in these cultures. By day 6, the ratio of pre-B to pro-B cells in the wt cultures was 3:1, whereas in ali/ali cultures the ratio was <1:1 (Fig. 2C). We conclude that V_{H4(a)}-utilizing pre-B cells do not undergo normal proliferative expansion.

An alternative explanation for selection against developing V_{H4(a)}-utilizing B-lineage cells is increased apoptosis. We found no difference in the total number of live B-lineage cells between ali/ali and wt BM cultures (Fig. 3A). We also did not detect substantial increases in dead cells in ali/ali cultures compared with wt (Fig. 3B), except for an increased frequency of dead cells at day 2 in some, but not all, ali/ali cultures. At day 2, most cells in wt and ali/ali cultures are pro-B cells that do not express μ-chains and likely have not begun the selection process. Therefore, the small increase in dead cells in some ali/ali cultures is likely unrelated to negative selection associated with V_{H4(a)}-μ. To test for differences in apoptosis between wt and ali/ali BM cultures, we used a cross-reactive anti-human caspase-3 Ab that reacts with apoptotic rabbit B-lineage cells (Fig. 3C). We found no difference in the frequency of caspase-3^− B-lineage cells between wt and ali/ali cultures (Fig. 3D). The small population of CD79a^−/−/caspase-3^− cells (Fig. 3D) likely represents live non–B-lineage cells because a similar population is also present in the live lymphocyte gate (Fig. 2A). We conclude that V_{H4(a)}-utilizing B-lineage cells are not lost due to apoptosis and that the decreased V_{H4(a)}-utilizing pre-B cell population is due to reduced pre-B cell expansion.

Pre-BCR signaling in V_{H4(a)}-utilizing B-lineage cells

The pre-B cell stage is a checkpoint at which the newly expressed μ-chains are tested for their capacity to associate with SL and form a signal-competent pre-BCR. Pre-BCR signaling occurs through interactions of the unique region of A5 (SL) with adjacent pre-BCRs and/or with the stromal environment (26–29). Pre-BCR cross linking results in a signal cascade similar to conventional BCR signaling that includes phosphorylation of Igα/β and Syk kinase and leads to pre-B cell proliferation (30). We hypothesized that V_{H4(a)}-utilizing pre-B cells fail to proliferate due to insufficient pre-BCR signaling. We used a cross-reactive mAb that recognizes rabbit phospho-Syk (P-Syk) (Fig. 4A) to compare the phosphorylation status of Syk in ali/ali and wt pre-B cells obtained from OP9 cocultures. The P-Syk levels in pro-B cells served as a negative control because cells lacking μ-chains should not express P-Syk (31). We found consistently higher levels of P-Syk in wt pre-B cells than in ali/ali pre-B cells (Fig. 4B), indicating that pre-BCR signaling in ali/ali pre-B cells is decreased. These data are consistent with the decrease in ali/ali pre-B cell expansion shown in Fig. 2. From these data, we conclude that ali/ali V_{H4(a)}-utilizing B-lineage cells are lost during development due to insufficient pre-BCR signaling and reduced capacity to become proliferating pre-B cells.

Pairing of V_{H4(a)−μ} chains with SL

The reduction in ali/ali pre-BCR signaling and limited proliferation of ali/ali pre-B cells led us to hypothesize that V_{H4(a)}-μ chains do not form functional pre-BCRs. We tested this possibility by expressing chimeric rabbit V_{H4(a)}-DJ murine-Cμ chains in the SL-expressing murine 38B9 pre-B cell line (8). The μ-chains that pair with SL transit to the cell surface, resulting in surface pre-BCR^+ cells. We tested VDJ regions derived from OP9-cultured ali/ali pre-B cells and neonatal splenic B cells, and, with the exception of one V_{H4(a)}-μ chain derived from spleen, we were unable to detect any surface pre-BCR on 38B9 cells expressing chimeric V_{H4(a)}-μ chains, whereas V_{H1(a)}-μ chains were readily detected on the cell surface (Fig. 5A). Although one of the spleen-derived V_{H4(a)}-μ chains was detected at the cell surface in the context of pre-BCR, its expression level was lower than that of V_{H1(a)}-μ pre-BCR. The sequence of this V_{H4(a)}-μ chain (accession number JN408688 at http://www.ncbi.nlm.nih.gov/nuccore) encoded a shorter CDR3 (5 residues) compared with the average CDR3 size (11 residues) of V_{H4(a)}-μ chains that did not form detectable surface pre-BCR. We do not know if it is the size or sequence of this CDR3 that allows for surface V_{H4(a)}-μ pre-BCR detection, or if this low-level surface pre-BCR expression is sufficient for normal pre-B cell development.

The lack of surface pre-BCR formation by most V_{H4(a)}-μ chains was not due to limited μ-chain expression as V_{H4(a)}- and V_{H1(a)}-μ chains are expressed equivalently in the cytoplasm (Fig. 5B). In contrast to V_{H4(a)}-, V_{H3μ}-utilizing pre-BCR was detected at the cell surface with expression equivalent to that of V_{H1(a)}-μ (Fig. 5C). The inability to detect surface V_{H4(a)}-utilizing pre-BCR combined with decreased pre-BCR signaling and pre-B cell prolif-
eration by \(V_{\mu 4(a)}\)-utilizing cells indicates impaired pre-BCR function, which we suggest is most likely due to the inability of \(V_{\mu 4(a)}\)-\(\mu\) to pair with SL.

**Rescue of \(V_{\mu 4(a)}\)-utilizing B cells in Ig\(k\) transgenic rabbits**

Although most \(V_{\mu 4(a)}\)-utilizing B-lineage cells are lost at the pre-B cell stage, some cells survive this stage, differentiate to the immature B cell stage (Fig. 1) and enter into the periphery as immature B cells (15, 23). The capacity of some \(V_{\mu 4(a)}\)-utilizing cells to survive the pre-B stage may be due to select \(V_{\mu 4(a)}\)-\(\mu\) chains that can provide sufficient pre-BCR signaling; however, the inability of \(V_{\mu 4(a)}\)-\(\mu\) chains from the spleen to pair with SL suggests that there may be another mechanism by which \(V_{\mu 4(a)}\)-utilizing B cells progress beyond the pre-B cell stage. Studies in mouse indicate that a small percentage of developing B cells rearrange IgL genes prior to IgH genes (32), and we hypothesized that some \(V_{\mu 4(a)}\)-utilizing B-lineage cells may be rescued by expression of IgL in early B cell stages. We tested this possibility by examining the peripheral B cell compartment of \(ali/ali\) rabbits expressing a human Igk-chain transgene (Ig\(k\)-tg) (Fig. 6A). If the Ig\(k\)-tg rescued \(V_{\mu 4(a)}\)-utilizing pre-B cells, then we expected to find more \(V_{\mu 4(a)}\)-utilizing B-lineage cells in the blood of young (5 wk) Ig\(k\)-tg \(ali/ali\) rabbits than in nontransgenic \(ali/ali\) rabbits. As predicted, we found a doubling of peripheral B cells (as measured by the percentage of IgM\(^+\) cells; 42 versus 22\%) and also an ∼3-fold increase in the V\(\mu 4(a)\) B cell population compared with age-matched nontransgenic \(ali/ali\) rabbits (31 versus 12\%) (Fig. 6B); by nucleotide sequence analysis, 80% of the VDJ genes in V\(\mu 4(a)\) B cells from the Ig\(k\)-tg rabbit used \(V_{\mu 4(a)}\) (accession numbers JN131899–JN131908 at http://www.ncbi.nlm.nih.gov/nuccore). We conclude that developing B cells utilizing \(V_{\mu 4(a)}\) can be rescued by conventional Igk-chain expression, suggesting that L chain expression during development allows \(V_{\mu 4(a)}\)-utilizing B-lineage cells to bypass the pre-B stage.

**Discussion**

In wt rabbits, most B cells use the 3’-most V\(\mu\) gene, V\(\mu 1\)\(\mu\)\(\alpha\), which encodes V\(\mu 1\)\(\alpha\) allotype molecules (12, 33). These B cells readily exit the BM and expand in GALT, which in rabbit consists of the appendix, Peyer’s patches, and sacculus rotundus (34, 35). Most B-lineage cells in \(ali/ali\) rabbits also rearrange the 3’-most V\(\mu\) gene that encodes V\(\mu 9\alpha\) molecules, although in this case, the gene is \(V_{\mu 4(a)}\) because \(V_{\mu 1\alpha}\) is deleted (12, 13). In contrast to wt rabbits, most of the \(V_{\mu 4(a)}\)-utilizing B-lineage cells in \(ali/ali\) rabbits do not proceed beyond the pre-B cell stage, and they

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**FIGURE 3.** Flow cytometric analysis of \(ali/ali\) and wt B-lineage cell survival 2–6 d after transfer of pro-B cells to OP9 stroma in the absence of IL-7. A, Relative number of B-lineage (CD79a\(^+\)) cells normalized to \(ali/ali\) cultures using bead-based counting and gating strategy in Fig. 2A. Error bars show SD from three independent experiments. B, Live/Dead staining of total lymphocytes. C, Cross-reactivity of anti–caspase-3 Ab with anti-Ig–stimulated spleen cells. B cells (black solid line), non-B cells (black dashed line), and unstimulated (gray filled). D, Frequency of active caspase-3\(^+\) B-lineage cells (CD79a\(^+\)). Data for B and D are representative of three independent experiments.

**FIGURE 4.** Flow cytometric analysis of Syk phosphorylation in \(ali/ali\) pro-B and pre-B cells 5 d after transfer to OP9 stroma. A, Cross-reactivity of anti-P-Syk mAb with anti-Ig–stimulated spleen cells. B cells (black solid line), non-B cells (black dashed line), and unstimulated (gray filled). B, Pre-B cells (gray line) and pro-B cells (black line). \(\Delta\), fold change of P-Syk mean fluorescence intensity between pro-B and pre-B cells. P-Syk mean fluorescence intensity for pro-B in wt and \(ali/ali\) cultures were identical. Similar data were obtained in each of three independent experiments.
represent only a small proportion of the immature B cells that exit the BM. Most of the immature B cells that leave the BM use upstream V<sub>H</sub> genes encoding V<sub>H</sub>n allotypes (15, 23). Rhee et al. (36) showed that the V<sub>H</sub>n<sup>+</sup>B cells do not expand in GALT, and instead, the few VH4(a)-utilizing B cells that survive development in BM undergo extensive expansion through mechanisms that require the intestinal microbiota (Fig. 7). The ali/ali rabbit model presents an enigma for understanding how the B cell repertoire develops because m-chains that generally do not support early B cell development in BM in fact readily mediate expansion in the periphery. How can ali/ali rabbits negatively select VH4(a)-utilizing B-lineage cells in BM, only to later expand such cells in GALT?

Both selection processes, negative selection of VH4(a)-utilizing B-lineage cells in BM and positive selection of VH<sub>a</sub>-encoding cells in GALT are Ag independent (37, 38). The stimulation and expansion of V<sub>H</sub><sup>a</sup>-encoding cells in GALT is polyclonal, and we hypothesize that positive selection is due to V<sub>H</sub><sup>a</sup> allotype residues in framework regions (FR) that are not encoded by the V<sub>H</sub>n allotype (36). The negative selection of VH4(a)-utilizing B-lineage cells occurring at the pre-B cell stage could be due to autoreactivity or to a cell-intrinsic defect that does not allow signaling to occur. It is unlikely that the ali/ali VH4(a)-utilizing pre-B cells are eliminated as a result of autoreactivity because development of these cells is also impaired in vitro in the absence of autoantigens. Further, the ali/ali pre-B cells exhibit reduced pre-BCR signaling; if these cells were eliminated due to autoreactivity, then we would expect them to exhibit a high level of signaling and proliferation (39). Instead, we think that the level of pre-BCR signaling is insufficient to promote proliferation and that these cells eventually die in vivo.

Limited expansion of V<sub>H</sub><sup>a</sup> B-lineage cells in BM of ali/ali rabbits

VH4(a)-utilizing pre-B cells do not generate sufficient pre-BCR signals to support cell proliferation, thereby explaining the paucity of immature V<sub>H</sub><sup>a</sup> B lineage cells in the BM and in the periphery of young ali/ali rabbits (15, 23). The impaired pre-BCR signaling and pre-B cell expansion is likely due to the inability of VH4(a)-<i>m</i> chains to form pre-BCR. Large pre-B cells undergo two to five divisions before IgL genes rearrange (24), and, in the case of ali/ali rabbits, the pre-B cells expressing V<sub>H</sub><sup>a</sup>-<i>m</i>-chains can presumably undergo these divisions normally, whereas the VH<sub>a</sub>-V<sub>H</sub><sup>a</sup>-<i>m</i>-chains do not.
(a)-utilizing pre-B cells do not. Consequently, the V\text{H}4(a)-utilizing pre-B cells expand and become the predominant B-lineage cell, even though V\text{H}4(a) is the predominantly rearranged V\text{H} gene.

It was surprising that V\text{H}4(a)-DJ regions from ali/ali peripheral B cells failed to form pre-BCR because we assumed that being in the periphery meant that these cells had successfully passed through the pre-B cell stage. Melchers and Boekel et al. (1, 8) showed that most \(\mu\)-chains from peripheral B cells have the capacity to pair with SL, which led to the hypothesis that SL pre-selects B-lineage cells expressing \(\mu\)-chains that will pair with conventional IgL. The presence of V\text{H}4(a)-utilizing cells in the periphery suggests that some V\text{H}4(a)-utilizing cells must have survived development in the BM, presumably in an SL-independent manner. This survival could be due to select CDR3 regions that could stabilize the pre-BCR or render a signal-competent \(\mu\)-chain independent of SL (40–43). By analyzing CDR3 regions of these B cells, we did not find any unusual patterns in amino acid sequences (accession number JN131909–JN131918 at http://www.ncbi.nlm.nih.gov/nuccore). Alternatively, survival of V\text{H}4(a)-utilizing B cells could be due to rearrangement of IgL genes prior to IgH genes, thereby bypassing the need for pre-BCR signaling. In mice, IgL genes have been shown to rearrange prior to IgH genes, albeit at a low frequency (32). Although we have no evidence for rearrangement of IgL genes prior to IgH, the data from the \(\text{hu-lkg}\) transgenic rabbit shows that IgL expression can rescue V\text{H}4(a)-utilizing B cell development in ali/ali rabbits. This finding is consistent with studies in which Ig-transgene expression in \(\lambda^{S-}\) mice also rescued B cell development (44) and suggests that premature IgL gene rearrangement and expression is a plausible mechanism for survival of V\text{H}4(a)-utilizing B lineage cells.

Evolutionarily conserved FR2 tryptophan encoded by V\text{H}

SL is comprised of two proteins, Vpre-B (45) and \(\lambda^{S-}\) (46), that have canonical Ig-like folds and non-Ig tails designated unique regions (UR) (29), which are important for pre-BCR signaling (28). We searched for evidence of molecular interactions between V\text{H}4(a)-\(\mu\) and SL that could be deleterious to pre-BCR formation. V\text{H}4(a)-\(\mu\) has the capacity to form conventional BCR, suggesting that they do not have gross intrinsic structural or folding defects. V\text{H}4(a) is >90% identical to V\text{H}1(a), and we focused on residues encoded by V\text{H}4(a) that differed from those encoded by V\text{H}1(a) and were predicted to interact with SL. We found only a single amino acid residue encoded in FR2 of V\text{H}4(a) (Y47) that fit those criteria. V\text{H}1(a), and most V\text{H} genes across species, encode a tryptophan at this position (W47) (47) and is predicted to interact with the \(\lambda^{S-}\)UR (29). The interaction of W47 with \(\lambda^{S-}\)UR is unique to the pre-BCR structure because conventional IgL chains do not possess UR, and structural analysis of conventional BCR does not indicate a strong interaction between V\text{H}4 residue 47 and IgL (48–52). The difference in interactions between \(\mu\)-chain and SL versus conventional IgL may explain how the Y47 residue encoded by V\text{H}4(a) could be problematic for pre-BCR signaling and V\text{H}4(a)-utilizing pre-B cell development in the BM without having any deleterious effect on mature V\text{H}4(a)-utilizing B cell expansion in GALT. Smith et al. (35) reported that murine V\text{H}81x-utilizing pre-BCR surface expression increases upon mutation of L47 to the conserved W47, suggesting that W at this position is important for pre-BCR surface expression. Mutation of Y47 encoded by V\text{H}4(a) to W47 was not sufficient to restore pre-BCR formation using the 38B9 system. We also made the W47Y mutation in V\text{H}1(a) and observed little effect on surface pre-BCR expression (data not shown). These data indicate that Y47 is not solely responsible for the inability of V\text{H}4(a)-\(\mu\) to form pre-BCR; however, Y47 in conjunction with other residues in V\text{H}, or CDR3, could be responsible for decreased pre-BCR expression in vitro. More extensive mutagenesis of V\text{H}4(a) will be required to determine the exact combination of residues that negate pre-BCR formation.

Development of the B cell repertoire: GALT versus non-GALT species

V\text{H} genes identified in mouse studies [e.g., V\text{H}81× (7) and V\text{H}12 (9)] that encode \(\mu\)-chains that fail to pair with SL generally exhibit defects in conventional IgL pairing, which differs from our ali/ali rabbit model in which the defect is in the pre-BCR, but not in the BCR. V\text{H} gene usage during B cell development in mouse and humans includes multiple gene families, whereas rabbits use a single V\text{H} gene in most VDJ gene rearrangements; V\text{H} genes, such as V\text{H}4(a), that bottleneck early B cell development could severely limit the early B cell repertoire. The block associated with V\text{H}4(a)-utilizing pre-B cells results in reduced peripheral B cell populations through adolescence in ali/ali rabbits (Fig. 6) (G.R. Robbins and K.L. Knight, unpublished observations). As ali/ali rabbits age, their peripheral B cell populations increase and achieve wt levels through expansion and selection of V\text{H}4(a)+ cells in GALT (36).

Because nearly all pro-B cells in ali/ali rabbits use the V\text{H}4(a) gene that does not allow for normal pre-BCR signaling/expansion, we wondered what advantage is provided by limited V\text{H}4 gene rearrangement? For rabbits, and other GALT species, the limited diversity of the Ab repertoire due to restricted V\text{H} gene rearrangement is overcome by mechanisms of B cell diversification in GALT (54–56). Gene conversion and somatic hypermutation in GALT continually transform an initially narrow B cell repertoire into one that recognizes a broad range of Ags with extremely high affinity (57, 58). The mechanisms that provide GALT species with high-affinity Abs require an initial B cell population emanating from the BM (35). If the single V\text{H}4 gene used during development is deleterious at the pre-B cell stage, then the initial seeding of GALT may be lagging or absent and could lead to an immunocompromised state early in life. The ali/ali rabbit is a spontaneous mutant discovered in a laboratory setting, and it is possible that the decreased peripheral B cell population early in life could increase ali/ali disease susceptibility if these rabbits were in the wild. Perhaps the ideal situation for generating the primary B cell repertoire would be a combination of development schemes used by GALT and non-GALT species. Utilizing multiple V\text{H} genes during early B cell development would prevent major blocks in the BM (as observed in ali/ali rabbits) and allow for efficient seeding of the periphery even if utilization of certain V\text{H} genes was deleterious. GALT would then serve as a site for continued B cell expansion and BCR diversification through somatic hypermutation and gene conversion. The bias toward V\text{H}4(a) utilization during wt rabbit B cell development likely relieves the selective pressure on V\text{H}4(a)-\(\mu\) chains to form pre-BCR, and we hypothesize that V\text{H}4(a) is maintained in the rabbit Ig locus to serve as a nucleotide sequence donor during gene conversion in GALT. Current studies in our laboratory are focused on understanding the mechanisms by which the intestinal microbiota drives the expansion and diversification of V\text{H}4(a)+ B cells in GALT.

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Disclosures

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