Cutting Edge: B Cell Linker Protein Is Dispensable for the Allelic Exclusion of Immunoglobulin Heavy Chain Locus But Required for the Persistence of CD5⁺ B Cells

Shengli Xu, Siew-Cheng Wong and Kong-Peng Lam

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The pre-B cell receptor (pre-BCR) and the BCR are required for B lymphopoiesis and for the allelic exclusion of Ig genes. Mice lacking B cell linker (BLNK) protein that is a component of the BCR signaling pathway have impaired B cell development. In this report, we show that allelic exclusion is intact in BLNK<sup>−/−</sup> mice harboring a V<sub>γ</sub>12 transgene. This differs from mice lacking the tyrosine kinase Syk that is upstream of BLNK in BCR signaling and contrasts with mice lacking SLP-76 that is the equivalent adaptor molecule in TCR-signal transduction. We also show that, whereas most wild-type V<sub>γ</sub>12-expressing B cells are CD5<sup>+</sup>, the majority of the splenic V<sub>γ</sub>12-expressing BLNK<sup>−/−</sup> B cells are CD5<sup>+</sup>. A small population of V<sub>γ</sub>12-expressing, BLNK<sup>−/−</sup> CD5<sup>+</sup> B cells is detectable in the peritoneal cavity of younger but not older mice. This suggests that BLNK deficiency affects not only the generation but also the persistence of B-1 cells.


In developing B lymphocytes, successful gene rearrangements at the IgH locus permits the formation of a pre-B cell receptor (pre-BCR) complex that comprises the IgH chain, the surrogate light (L) chains α<sub>5</sub> and V<sub>preB</sub> and the signal-transducing subunits Igα and Igβ (1). The pre-BCR plays a central role in B cell development (2). It signals the pro-B to pre-B cell transition during B lymphopoiesis and mediates the allelic exclusion of the IgH locus in which V(D)J recombination and subsequent heavy (H) chain expression on the second allele is inhibited. This is evident by the targeted disruption of Igβ (3), α<sub>5</sub> (4), or the transmembrane portion of the μ H chain (5), all of which compromise the surface expression of the pre-BCR and lead to a block in B lymphopoiesis and an impairment in IgH allelic exclusion (6, 7).

The BCR replaces the pre-BCR as the central molecule regulating the fate of mature B lymphocytes. Continuous expression of the BCR is required for the persistence of peripheral B cells (8). Engagement of the BCR by self-Ag leads to receptor editing (9) or clonal deletion (10) of autoreactive B lymphocytes, whereas triggering of BCR by foreign Ag leads to the activation, proliferation, and differentiation of Ag-specific B cells (2). Furthermore, the specificity of the BCR may also determine the development of B-1 and B-2 cell that are distinguishable from each other by their cell surface phenotype (11).

Although past studies have indicated the importance of the pre-BCR and BCR in the differentiation and activation of B cells, the signaling cascades that mediate the different cellular responses remain partially elucidated. It is known that cross-linking of the BCR activates cytoplasmic tyrosine kinases such as Syk, Lyn, Blk, and Bruton’s tyrosine kinase (12). Recently, adaptor proteins have been shown to interface tyrosine kinase activation with selective downstream molecules (13) and, therefore, could channel BCR signaling to elicit specific cellular responses. One such adaptors in B cells is the B cell linker (BLNK) (14), otherwise known as SLP-65 (15) or BASH (16), which couples activated Syk to PLC-γ, Vav, Grb2, and Nck (17).

We and others have generated mice lacking BLNK (18–21). BLNK<sup>−/−</sup> B cells do not proliferate upon anti-IgM stimulation and fail to mount a T cell-independent immune response. In addition, BLNK<sup>−/−</sup> mice lack CD5<sup>+</sup> B cells (18–21). To further dissect the role of BLNK in B cell development, we now introduced a transgenic V<sub>H</sub>12 H chain that is enriched in the normal B-1 cell population into BLNK<sup>−/−</sup> mice to analyze the role of BLNK in IgH allelic exclusion and in the development of CD5<sup>+</sup> B cells.

Materials and Methods

**Mice**

BLNK<sup>−/−</sup> (18) and V<sub>H</sub>12f (22) mice had been described previously. BALB/c and C57BL/6 mice were obtained from the Animal Resource Center in Australia.

**Antibodies**

The following mAbs were purchased from PharMingen (San Diego, CA): anti-IgM (R6-60.2), anti-μ<sub>5</sub> (DS-1), anti-μ<sub>κ</sub> (AF6-78.25), anti-B220 (RA3-6B2), anti-CD5 (53.7), and anti-CD43 (S7). The anti-V<sub>H</sub>12 (5C5) mAb was obtained from Dr. G. Haughton (University of North Carolina, Chapel Hill, NC).
Flow cytometry

Cells were obtained by injecting PBS containing 3% FCS and 0.1% NaN₃ into the femurs and tibia or peritoneal cavity (PerC) of mice. PBL were obtained from mice by tail bleed and isolated on a density gradient of Lymphoprep (Nycomed, France). For FACS analyses, cells were stained with FITC-, PE-, and biotin-conjugated mAbs for 10 min on ice and washed twice with PBS. Biotin-conjugated mAbs were revealed with streptavidin-Cychrome. FACS analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA) and cell sorting was done on a FACSort.

Analyses of IgH gene rearrangements

PCR was performed on genomic DNA obtained from sorted B220⁺CD43⁻ IgM⁻ bone marrow B cells of wild-type; IgH⁺/⁺, BLNK⁻/⁻; V₅₂₁⁺/⁺, BLNK⁺/⁺; and V₅₂₁⁺/⁺, BLNK⁻/⁻ mice were stained with anti-B220, anti-IgM, and anti-CD43 mAbs. The numbers indicate percent of total cells.

Results and Discussion

Intact allelic exclusion of IgH locus in BLNK⁻/⁻ B cells

Each B cell expresses an Ab of a unique specificity comprising of one H and L chain pair even though it possesses two H and four L chain (κ and λ) alleles. Similarly, each T cell expresses a TCR of a single specificity even though it possesses two alleles each for α and β as well as γ and δ genes. This phenomenon is known as allelic exclusion (2). The exact mechanism regulating the allelic exclusion of Ag receptor genes is poorly understood although a signal mediated by the pre-BCR or pre-TCR is necessary for the process to occur (7, 24). In developing thymocytes, signaling via the pre-TCR activates the tyrosine kinases Lck, ZAP-70, and Syk, and adaptor proteins such as SLP-76, Vav, and Cbl further propagate the signals downstream (13). Studies have indicated that some of these molecules are involved in signaling allelic exclusion in T cells, e.g., an activated lck transgene is sufficient to inhibit TCR β-chain gene rearrangements in thymocytes (25). Recently,
the adaptor protein SLP-76 that is involved in TCR signaling is shown to be essential for the allelic exclusion of TCR β locus (26).

Given the similarities in BCR and TCR signaling pathways, namely the activation of cytoplasmic tyrosine kinases and propagation of signals by adaptor proteins, and that BLNK is the adaptor protein equivalent to SLP-76, we examined IgH allelic exclusion in BLNK<sup>−/−</sup> mice.

A V<sub>H</sub>12 transgene inserted into one of the two IgH loci by gene-targeting (22) was introduced into BLNK<sup>−/−</sup> mice. As shown previously (18) and in Fig. 1, BLNK<sup>−/−</sup> B cells predominantly accumulate at the B220<sup>+</sup>CD43<sup>1</sup> pro-B to large pre-B cell stage of differentiation. The introduction of a functional V<sub>H</sub>12 transgene did not lead to further differentiation of BLNK<sup>−/−</sup> B cells into B220<sup>+</sup>CD43<sup>−</sup> small pre-B cells. This is not surprising as BLNK<sup>−/−</sup> B cells are arrested at a developmental stage whereby surface expression of the pre-BCR already occurred (21).

To determine whether BLNK is essential for IgH allelic exclusion, we performed PCR on genomic DNA isolated from FACS-sorted B220<sup>+</sup>CD43<sup>+</sup> bone marrow B cells (Fig. 1, boxed) of either wild-type; V<sub>H</sub>12<sup>+/+</sup>, BLNK<sup>−/−</sup>; or V<sub>H</sub>12<sup>+/−</sup>, BLNK<sup>−/−</sup> mice. The expression of an IgH transgene in pro-B cells leads to the early assembly of a pre-BCR that signals allelic exclusion by suppressing V<sub>H</sub> to D<sub>H</sub> J<sub>H</sub> rearrangement at the other endogenous H chain gene locus (23). As expected and shown in Fig. 2B (upper panel), V<sub>H</sub>12<sup>+/−</sup> mice bearing wild-type or mutant blnk alleles showed some levels of D<sub>H</sub> to J<sub>H</sub> gene rearrangements at the other IgH locus, similar to normal mice. However, in contrast to wild-type mice where the rearrangement of V<sub>H</sub> J558, V<sub>H</sub>7183, and V<sub>H</sub>Q52 gene family members to D<sub>H</sub> and downstream J<sub>H</sub>1, 2, or 3 gene segments can be detected, such gene rearrangement at the other IgH locus is severely suppressed in V<sub>H</sub>12<sup>+/−</sup> mice bearing either the normal or inactivated alleles of blnk. The lack of V<sub>H</sub> to D<sub>H</sub> J<sub>H</sub> gene rearrangements would suggest that the mechanism maintaining IgH allelic exclusion is intact in BLNK<sup>−/−</sup> mice bearing a V<sub>H</sub>12 transgene.

To confirm that BLNK<sup>−/−</sup> B cells indeed maintain allelic exclusion at the IgH loci, we generated wild-type and BLNK<sup>−/−</sup> mice that possess both the IgM<sub>a</sub> and IgM<sub>b</sub> alleles. As shown in Fig. 3, FACS analysis of PBL in BLNK<sup>−/−</sup> mice indicates that the B cells present express either IgM<sub>a</sub> or IgM<sub>b</sub> but not both molecules on their cell surfaces. This is similar to the B cells found in wild-type mice bearing the two different IgM alleles. The lack of a sizeable population of B cells that coexpress IgM<sub>a</sub> and IgM<sub>b</sub> in BLNK<sup>−/−</sup> mice would again suggest that signal(s) transduced by BLNK is not required for the allelic exclusion of the IgH loci.

Thus, our data presented in this report indicate that BLNK is not essential for the allelic exclusion of Ig genes. This contrasts significantly with the impairment of TCR β-chain allelic exclusion in SLP-76<sup>−/−</sup> mice (26) and suggests that the intracellular signaling pathway controlling allelic exclusion in B and T cells may differ in

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**FIGURE 3.** FACS analyses of PBL of wild-type and BLNK<sup>−/−</sup> mice bearing IgM<sub>a</sub>,b alleles. Cells are stained with anti-μ<sub>a</sub> and anti-μ<sub>b</sub> mAbs. BALB/c (IgM<sup>a</sup>) and C57BL/6 (IgM<sup>b</sup>) mice are included as controls for the specificity of the reagents. The numbers indicate percent of total lymphocytes.

**FIGURE 4.** The majority of the V<sub>H</sub>12-expressing BLNK<sup>−/−</sup> B cells are CD5<sup>−</sup>. FACS analyses of splenic (top two panels) and PerC (bottom panel) cells obtained from 4-wk-old mice of various genotypes. Cells were stained with anti-IgM and anti-V<sub>H</sub>12 (top panel) or anti-CD5 and anti-IgM (middle and bottom panels) mAbs. The numbers indicate percent of total lymphocytes.
some aspects. The current finding is also interesting as BLNK is a direct downstream substrate of Syk in BCR signaling (14, 17). Previous analysis of Syk~−/− mice had indicated that this tyrosine kinase is involved in mediating allelic exclusion of Ig genes in B cells (27). Our data would suggest that bifurcation of signal transduction occurs downstream of Syk such that perhaps another signaling molecule but not BLNK is responsible for transducing the signal for IgH allelic exclusion in B cells.

Role of BLNK in the generation and persistence of CD5+ B cells

CD5+ B cells are distinguishable from the majority of the conventional B cells by their unique cell surface phenotype and anatomical localization (11). The origins of these cells remain controversial (11) although certain BCR specificities seem to bias their anatomical localization (11). The origins of these cells remain controversial (11) although certain BCR specificities seem to bias their generation in the mouse (29). We and others previously showed that BLNK~−/− mice lack CD5+ B cells (18–21). The absence of CD5+ B cells in BLNK~−/− mice could be due to impairment in the self-renewal process (11). To determine whether the small population of normal mice. In V(H)12f/+ mice, the majority of the splenic B cells are CD5+, indicating that the enforced expression of V(H)12 leads to the preferential development of B-1 cells. These cells express low levels of B220 and the majority of them are also CD43+ and CD23− (data not shown), in agreement with previous reports (22, 28). However, in V(H)12f/+ mice, the majority of the splenic CD5-expressing B cells are now CD5− (Fig. 3, middle panel) and they express high levels of B220 and do not express CD23 (data not shown).

Examination of the PerC of V(H)12f/+, BLNK~−/− mice also indicates that a proportion (30% to 50%) of the V(H)12-expressing B cells are CD5− (Fig. 4, bottom panel and Fig. 5, top panel), in contrast to the V(H)12-expressing cells in the PerC of V(H)12f/+ BLNK~−/− mice in which >90% of the B cells present express the CD5 Ag. However, CD5-expressing V(H)12-B cells can develop in the PerC of young 4-wk-old V(H)12f/+, BLNK~−/− mice although they are fewer in numbers compared with the cells in V(H)12f/+, BLNK~−/− mice (Fig. 4, bottom panel and Table I).

CD5+ B cells are thought to be long-lived and to undergo a self-renewal process (11). To determine whether the small population of CD5+ V(H)12-expressing B cells persist and clonally expand in the absence of BLNK, we examined the PerC cells of older V(H)12f/+, BLNK~−/− mice. In normal mice, the population of CD5+ B cells expanded as the mice aged (Fig. 5). In BLNK~−/− mice, CD5+ B cells were largely absent in both the younger and older animals. Interestingly, whereas CD5+ B cells can be found in younger V(H)12f/+, BLNK~−/− mice and comprise up to ~50% of the B cells present, these cells are absent in the PerC of older V(H)12f/+, BLNK~−/− mice.

Our data show that most splenic V(H)12-expressing BLNK~−/− B cells assume a B-2 cell phenotype even though they are constrained by transgenesis to express a BCR that is preferentially enriched in the normal B-1 cell population. Therefore, BLNK

### Table I. Number of B cells in the spleen and peritoneal cavity of mice of various genotypes

<table>
<thead>
<tr>
<th></th>
<th>IgH+/− BLNK+/+</th>
<th>IgH+/− BLNK−/−</th>
<th>V(H)12f/+ BLNK+/+</th>
<th>V(H)12f/+ BLNK−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen (×10⁷)</td>
<td></td>
<td></td>
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<tr>
<td>(1) 4 wk</td>
<td>2.1 ± 0.5</td>
<td>0.1 ± 0.03</td>
<td>2.2 ± 0.3</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>(2) 12 wk</td>
<td>4.0 ± 1.1</td>
<td>1.8 ± 0.5</td>
<td>2.7 ± 0.9</td>
<td>0.7 ± 0.2</td>
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<tr>
<td>PerC (×10⁷)</td>
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<td></td>
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<tr>
<td>(1) 4 wk</td>
<td>6.9 ± 2.4</td>
<td>0.13 ± 0.01</td>
<td>43.4 ± 7.0</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>(2) 12 wk</td>
<td>10.3 ± 3.9</td>
<td>0.18 ± 0.06</td>
<td>57.3 ± 2.7</td>
<td>0.41 ± 0.20</td>
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*The number of B cells is estimated on the basis of total cell count and FACS analyses using anti-B220 and anti-IgM mAbs. More than five mice were analyzed for each age group.

**FIGURE 5.** BLNK is required for the persistence of CD5+ B cells. FACS analysis of PerC cells obtained from 4- and 12-wk-old mice of various genotypes. Cells were stained with FITC-anti-IgM and PE-anti-CD5 mAbs. The CD5+ IgM+ B cells are boxed and expressed as a percentage of total IgM+ B cells present. Figure shown is representative of >5 analyses.
deficiency affects the generation of B-1 cells. This observation is similar to that of V\textsubscript{H}12-expressing B cells lacking the tyrosine kinase Bruton’s tyrosine kinase (29) and is consistent with the idea that BCR-signaling may be required for the generation of B-1 cells (30).

Because our mice are polyclonal with respect to Ig L chain usage, it is likely that V\textsubscript{H}12 in combination with certain L chains generate some BCR whose signaling are strong enough to compensate for BLNK deficiency and, therefore, drive the differentiation of these cells. BLNK deletion affects the generation of B-1 cells. This observation is consistent with the idea that BCR-signaling may be required for the generation of B-1 cells. This observation is consistent with the idea that BCR-signaling may be required for the generation of B-1 cells.

References