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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[1] FREE

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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[1]

Shengli Xu, Siew-Cheng Wong, and Kong-Peng Lam[2]

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 $BLNK^{-/-}$ mice harboring a V_H12 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_H12-expressing B cells are $CD5^+$, the majority of the splenic V_H12-expressing $BLNK^{-/-}$ B cells are $CD5^-$. A small population of V_H12-expressing, $BLNK^{-/-}$ $CD5^+$ 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. ***The Journal of Immunology,*** **2000, 165: 4153–4157.**

In developing B lymphocytes, successful gene rearrangements at the IgH locus permits the formation of a pre-B cell receptor (pre-BCR)[3] complex that comprises the IgH chain, the surrogate light (L) chains λ5 and VpreB 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), λ5 (4), or the transmembrane portion of the μ H chain (5), all of which compromised 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). $BLNK^{-/-}$ B cells do not proliferate upon anti-IgM stimulation and fail to mount a T cell-independent immune response. In addition, $BLNK^{-/-}$ mice lack $CD5^+$ B cells (18–21). To further dissect the role of BLNK in B cell development, we now introduced a transgenic V_H12 H chain that is enriched in the normal B-1 cell population into $BLNK^{-/-}$ mice to analyze the role of BLNK in IgH allelic exclusion and in the development of $CD5^+$ B cells.

Materials and Methods

Mice

$BLNK^{-/-}$ (18) and V_H12f (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^a (DS-1), anti-μ^b (AF6-78.25), anti-B220 (RA3-6B2), anti-CD5 (53.7), and anti-CD43 (S7). The anti-V_H12 (5C5) mAb was obtained from Dr. G. Haughton (University of North Carolina, Chapel Hill, NC).

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[3] Abbreviations used in this paper: pre-BCR, pre-B cell receptor; BLNK, B cell linker; H, heavy; L, light; PerC, peritoneal cavity.

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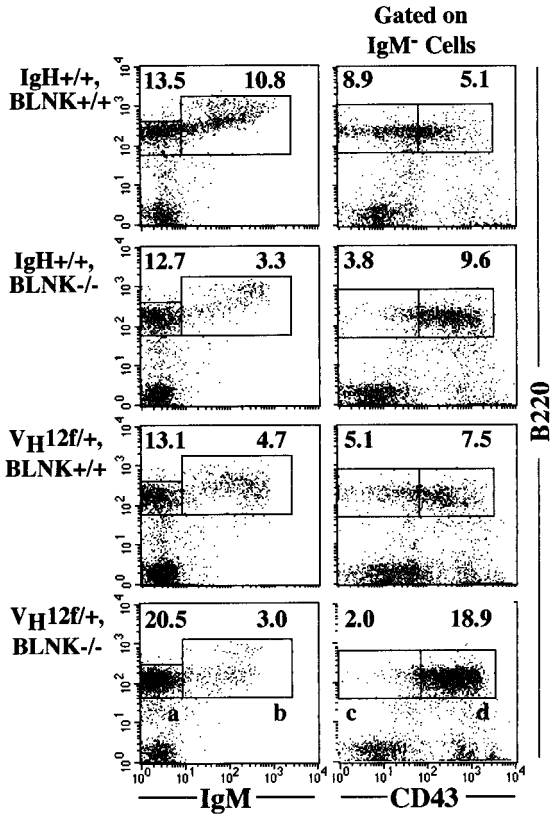

FIGURE 1. A V_H12 transgene did not rescue the B cell developmental block in $BLNK^{-/-}$ mice. Bone marrow cells from wild-type; $IgH^{+/+}$, $BLNK^{-/-}$; $V_H12f/+$, $BLNK^{+/+}$; and $V_H12f/+$, $BLNK^{-/-}$ mice were stained with anti-B220, anti-IgM, and anti-CD43 mAbs. The numbers indicate percent of total cells.

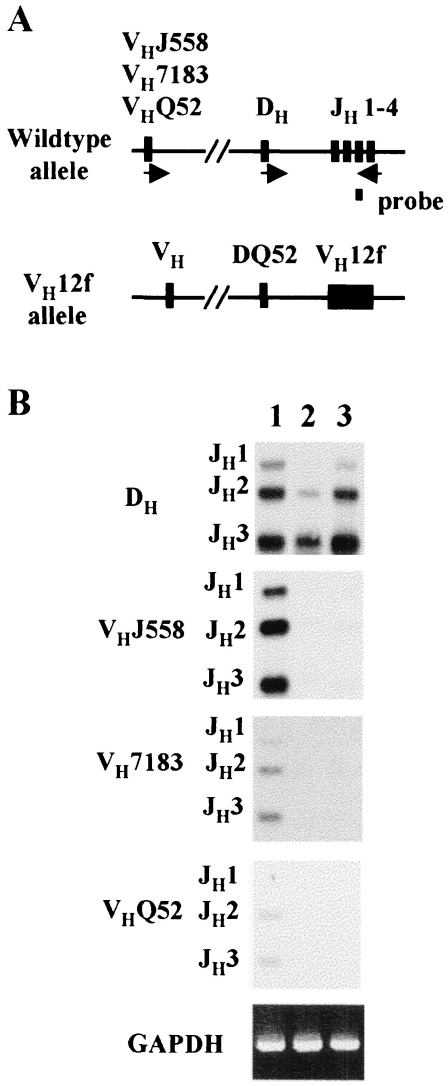

FIGURE 2. Intact IgH allelic exclusion in $BLNK^{-/-}$ mice. *A*, Schematic representation of the PCR strategy used to detect D_H to J_H and V_HJ558, V_H7183, or V_HQ52 to D_HJ_H gene rearrangements at the IgH loci. Arrowheads show the positions of the V_H family-, D_H-, and J_H4-specific primers used in the PCR. The J_H3-specific probe used in the Southern blot hybridization is indicated as a black rectangle. The configuration of the rearranged V_H12 insertion allele is also shown. *B*, Southern blot analyses of PCR products derived from the amplification of genomic DNA obtained from sorted $B220^+CD43^+IgM^-$ B cells (Fig. 1, *boxed*) of $IgH^{+/+}$, $BLNK^{+/+}$ (*lane 1*); $V_H12f/+$, $BLNK^{+/+}$ (*lane 2*); and $V_H12f/+$, $BLNK^{-/-}$ (*lane 3*) mice using J_H4 and D_H (top panel); J_H4 and V_HJ558, V_H7183, or V_HQ52 (*middle 3 panels*) primers. The positions corresponding to the rearrangements of D_H or V_H to either the J_H1, 2, or 3 gene segments are shown. The housekeeping gene GADPH was amplified as a control for the amount of genomic DNA used in the PCR (*bottom panel*).

Flow cytometry

Cells were obtained by injecting PBS containing 3% FCS and 0.1% NaN_3 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; $V_H12f/+$, $BLNK^{+/+}$ and $V_H12f/+$, $BLNK^{-/-}$ mice for a total of 35 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 10 min using the following primers (23): D_H, 5′-G(A/C)TT TTTGT(G/C)AAGGGATCTACTACTGTG-3′; V_HJ558, 5′-TCCA(A/G)CA CAGCCT(A/T)CATGCA(A/G)CTCA(A/G)C-3′; V_H7183, 5′-AAGAA(C/ G)A(A/G)CCTGT(A/T)CCTGCAAATGA(C/G)C-3′; V_HQ52, 5′-AGAC TGA(A/G)CATCA(C/G)CAAGGACAA(C/T)TCC-3′; and J_H4, 5′-TCCCT CAAATGAGCCTCCAAAGTCC-3′. The PCR products were subsequently analyzed by Southern blotting using the ^{32}P-end-labeled J_H3 probe, 5′-TTCTCACAAGAGTCCGATAGACCCTGG-3′.

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,

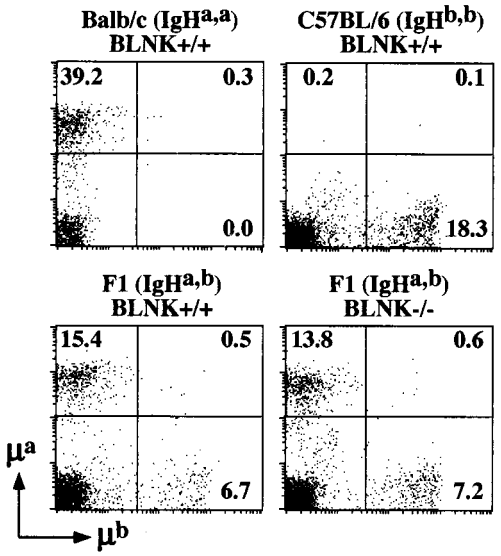

FIGURE 3. FACS analyses of PBL of wild-type and BLNK$^{-/-}$ mice bearing IgM^{a,b} alleles. Cells are stained with anti-μ^a and anti-μ^b mAbs. BALB/c (IgM^{a,a}) and C57BL/6 (IgM^{b,b}) mice are included as controls for the specificity of the reagents. The numbers indicate percent of total lymphocytes.

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$^{-/-}$ mice.

A V_H12 transgene inserted into one of the two IgH loci by gene-targeting (22) was introduced into BLNK$^{-/-}$ mice. As shown previously (18) and in Fig. 1, BLNK$^{-/-}$ B cells predominantly accumulate at the B220$^+$CD43$^+$ pro-B to large pre-B cell stage of differentiation. The introduction of a functional V_H12 transgene did not lead to further differentiation of BLNK$^{-/-}$ B cells into B220$^+$CD43$^-$ small pre-B cells. This is not surprising as BLNK$^{-/-}$ 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$^+$CD43$^+$IgM$^-$ bone marrow B cells (Fig. 1, *boxed*) of either wild-type; $V_H12f/+$, BLNK$^{+/+}$; or $V_H12f/+$, BLNK$^{-/-}$ 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_H to D_HJ_H rearrangement at the other endogenous H chain gene locus (23). As expected and shown in Fig. 2*B* (*upper panel*), $V_H12f/+$ mice bearing wild-type or mutant *blnk* alleles showed some levels of D_H to J_H gene rearrangements at the other IgH locus, similar to normal mice. However, in contrast to wild-type mice where the rearrangement of V_HJ558, V_H7183, and V_HQ52 gene family members to D_H and downstream J_H1, 2, or 3 gene segments can be detected, such gene rearrangement at the other IgH locus is severely suppressed in $V_H12f/+$ mice bearing either the normal or inactivated alleles of *blnk*. The lack of V_H to D_HJ_H gene rearrangements would suggest that the mechanism maintaining IgH allelic exclusion is intact in BLNK$^{-/-}$ mice bearing a V_H12 transgene.

To confirm that BLNK$^{-/-}$ B cells indeed maintain allelic exclusion at the IgH loci, we generated wild-type and BLNK$^{-/-}$ mice that possess both the IgM^a and IgM^b alleles. As shown in Fig. 3, FACS analysis of PBL in BLNK$^{-/-}$ mice indicates that the B cells present express either IgM^a or IgM^b 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^a and IgM^b in BLNK$^{-/-}$ 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$^{-/-}$ mice (26) and suggests that the intracellular signaling pathway controlling allelic exclusion in B and T cells may differ in

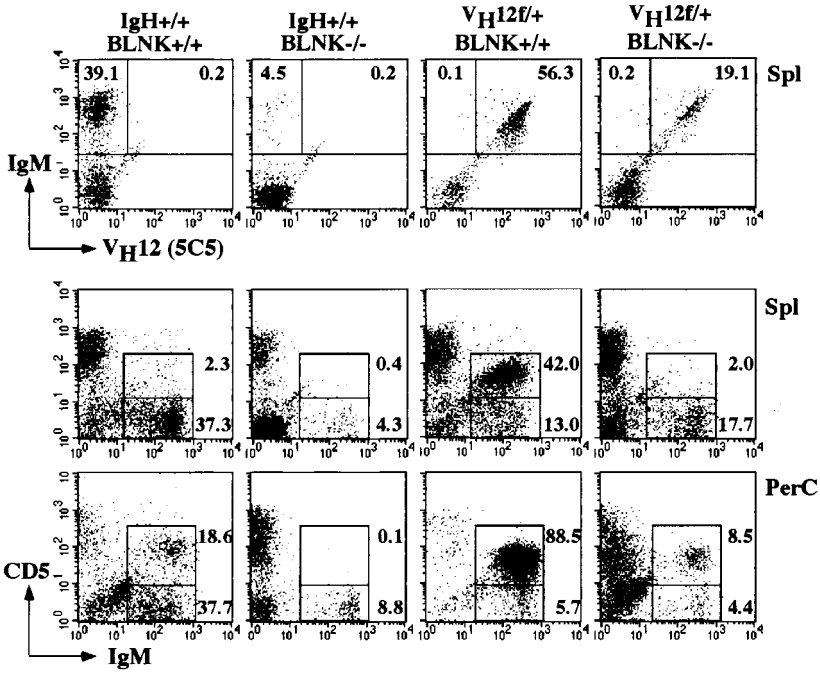

FIGURE 4. The majority of the V_H12-expressing BLNK$^{-/-}$ B cells are CD5$^-$. FACS analyses of splenic (*top two panels*) and PerC (*bottom panel*) cells obtained from 4-wk-old mice of various genotypes. Cells were stained with anti-IgM and anti-V_H12 (*top panel*) or anti-CD5 and anti-IgM (*middle and bottom panels*) mAbs. The numbers indicate percent of total lymphocytes.

Table I. *Number of B cells in the spleen and peritoneal cavity of mice of various genotypes*[a]

	$IgH^{+/+}$ $BLNK^{+/+}$	$IgH^{+/+}$ $BLNK^{-/-}$	$V_H12f/+$ $BLNK^{+/+}$	$V_H12f/+$ $BLNK^{-/-}$
Spleen ($\times 10^7$)				
(1) 4 wk	2.1 ± 0.5	0.1 ± 0.03	2.2 ± 0.3	0.4 ± 0.1
(2) 12 wk	4.0 ± 1.1	1.8 ± 0.5	2.7 ± 0.9	0.7 ± 0.2
PerC ($\times 10^5$)				
(1) 4 wk	6.9 ± 2.4	0.13 ± 0.01	43.4 ± 7.0	0.16 ± 0.02
(2) 12 wk	10.3 ± 3.9	0.18 ± 0.06	57.3 ± 2.7	0.41 ± 0.20

[a] 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.

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 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 generation and/or persistence of this B cell subset. To explore these possibilities, we examined the B cell populations in $BLNK^{-/-}$ mice bearing a V_H12 transgene. The V_H12 H chain is used preferentially in the recognition of phosphatidylcholine and B cells expressing this specificity predominantly differentiate into $CD5^+$ B cells and are heavily selected in vivo (28).

Splenic B cells obtained from wild-type or $BLNK^{-/-}$ mice carrying the V_H12 transgene stably expressed the H chain as shown by the anti-V_H12 specific mAb staining in FACS analysis (Fig. 4, *upper panel*). However, the introduction of the V_H12 transgene into $BLNK^{-/-}$ mice did not lead to a significant increase in the number of peripheral B cells in these mice (Table I). At 12 wk of age, there is respectively, a 4- and 25- to 100-fold reduction in the number of B cells in the spleen and PerC of $V_H12f/+$, $BLNK^{-/-}$ mice compared with wild-type or $V_H12f/+$, $BLNK^{+/+}$ mice of similar age.

$CD5^+$ B cells constitute a small subset of B cells in the spleen of normal mice. In $V_H12f/+$, $BLNK^{+/+}$ mice, a large fraction (>75%) of the splenic B cells are $CD5^+$, indicating that the enforced expression of V_H12 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 $V_H12f/+$ mice lacking BLNK, most of the splenic V_H12-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 $V_H12f/+$, $BLNK^{-/-}$ mice also indicates that a proportion (30% to 50%) of the V_H12-expressing B cells are $CD5^-$ (Fig. 4, *bottom panel* and Fig. 5, *top panel*), in contrast to the V_H12-expressing cells in the PerC of $V_H12f/+$, $BLNK^{+/+}$ mice in which >90% of the B cells present express the CD5 Ag. However, CD5-expressing V_H12-B cells can develop in the PerC of young 4-wk-old $V_H12f/+$, $BLNK^{-/-}$ mice although they are fewer in numbers compared with the cells in $V_H12f/+$, $BLNK^{+/+}$ 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_H12-expressing B cells persist and clonally expand in the absence of BLNK, we examined the PerC cells of older $V_H12f/+$, $BLNK^{-/-}$ 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 $V_H12f/+$, $BLNK^{-/-}$ mice and comprise up to ~50% of the B cells present, these cells are absent in the PerC of older $V_H12f/+$, $BLNK^{-/-}$ mice.

Our data show that most splenic V_H12-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

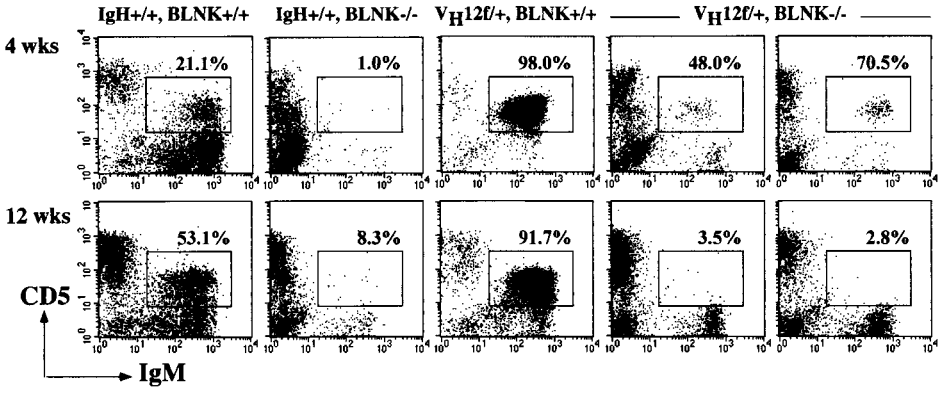

FIGURE 5. BLNK is required for the persistence of $CD5^+$ B cells. FACS analysis of PerC cells obtained from 4- and 12-wk-old mice of various genotypes. Cells were stained with FITC-anti-IgM and PE-anti-CD5 mAbs. The $CD5^+IgM^+$ B cells are boxed and expressed as a percentage of total IgM^+ B cells present. Figure shown is representative of >5 analyses.

deficiency affects the generation of B-1 cells. This observation is similar to that of V_H12-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_H12 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 B cells expressing these BCRs into the B-1 cell compartment. This could explain the presence of a small population of B-1 cells in the PerC of $V_H12f/+$, $BLNK^{-/-}$ mice. What is surprising is that these $CD5^+$ B cells are not found in the older mutant animals. B-1 cells are thought to be long-lived, undergo self-renewal, and clonally expand in older mice (11). Our data suggest that the persistence and/or self-renewal of B-1 cells would also require signaling from the BCR, and this is affected by the inactivation of BLNK. Future experiments involving the enforced expression of cell survival genes such as Bcl-2 and/or Bcl-xL in $V_H12f/+$, $BLNK^{-/-}$ mice may determine whether $CD5^+$ B cells indeed fail to survive in the absence of BLNK.

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