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This information is current as of July 8, 2015.

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J Immunol 2007; 178:1981-1985; ;
doi: 10.4049/jimmunol.178.4.1981
http://www.jimmunol.org/content/178/4/1981

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: The PI3K p110δ Is Required for Down-Regulation of RAG Expression in Immature B Cells[1]

Miriam Llorian, Zania Stamataki, Susan Hill, Martin Turner, and Inga-Lill Mårtensson[2]

***At the immature B cell stage the BCR signals the down-regulation of the RAG genes and Ig L chain (LC) allelic and isotype exclusion. The signaling pathway that regulates these events is poorly characterized. We demonstrate that immature B cells from mice deficient in the PI3K catalytic subunit p110δ fail to suppress RAG expression and inappropriately recombine κ and λ LC loci. In addition, in the presence of the autoantigen, clonal deletion and receptor editing still takes place, demonstrating that these processes are independent of p110δ. These results demonstrate a role for p110δ in the regulation of RAG gene expression and thereby LC allelic/isotype exclusion.** The Journal of Immunology,* **2007, 178: 1981–1985.**

The hallmark of B lymphocytes is the cell surface expression of a BCR for Ag, which is composed of an Ig H (HC)[3] and L chain (LC) (1, 2). BCR repertoire diversity is achieved by the combinatorial recombination of V(D)J gene segments in the Ig HC and LC loci. Each B cell expresses a unique BCR encoded by single HC and LC genes as a consequence of allelic exclusion. Successful pairing of HC and LC in immature B cells mediates signals that result in allelic and isotype exclusion, survival, and progression to the mature B cell stage. These signals are mediated by the BCR in the absence of high-affinity ligand and have been termed "tonic" signals (3). In contrast, B cells expressing autoreactive BCRs engage ligand and are negatively selected but may escape deletion through receptor editing (4). An essential feature of receptor editing is continued RAG expression and recombination of the LC loci. If receptor editing yields a non-autoreactive BCR, subsequent delivery of a tonic signal permits B cell maturation.

The process of allelic exclusion can be mimicked in mouse models when expression of a recombined HC and LC transgene (TG), allows B cell development. Under such circumstances, the TG BCR prevents rearrangement of endogenous HC and LC genes and inhibits expression of the RAG-1 and RAG-2 genes, which are essential for HC and LC recombination. In the 3-83 model, the TG BCR, composed of a μδ HC and κ LC, recognizes MHC class I $K^{k,b}$. In the absence of autoantigen B cells develop that express the 3-83 BCR (5); however, autoantigen induces receptor editing. This results in RAG gene expression, recombination of the endogenous LC loci, and the development of B cells expressing an innocuous BCR comprised of the TG HC and endogenous κ or λ LC.

Signals from the BCR and CD19 are required for LC isotype exclusion. In the 3-83 TG model the tyrosine kinase Syk is required to shut down rearrangements of endogenous LC genes (6). In the absence of CD19 there is an inability to down-regulate RAG genes and B cells expressing endogenous λ LC develop (7). The signaling pathway that regulates RAG gene expression and leads to LC allelic/isotype exclusion, as well as receptor editing, is largely unknown. However, enhanced RAG gene expression mediated by the inhibitor wortmannin (8, 9) suggests a role for PI3K a downstream effector of both BCR and CD19. There are three class IA PI3K catalytic subunits, encoded by the p110α, β, and δ genes, all of which are expressed in B cells (10). In $p110\delta^{-/-}$ mice, bone marrow (BM) B cell development is relatively normal, whereas there are profound effects on the peripheral B cell compartment and immune responses (11). In this study, we demonstrate a role for p110δ in LC allelic/isotype exclusion and show that p110δ is required for effective silencing of RAG expression. In addition, in the presence of the autoantigen, receptor editing still takes place, thus this process is independent of p110δ.

Materials and Methods

Mice

$p110\delta^{-/-}$, 3-83 (Tol 1), Bcl-2 (Bcl-2; 36), and $CD19^{-/-}$ mice have been previously described (5, 11–13). Mice were backcrossed to B10.D2 and B10.BR background to obtain the genotypes used herein keeping the 3-83 and Bcl-2 TGs hemizygous. Mice were used at 10–16 wk of age.

Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, Cambridge, United Kingdom

Received for publication August 16, 2006. Accepted for publication December 1, 2006.

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[1] This work was supported by Medical Research Council and the Biotechnology and Biological Sciences Research Council.

[2] Address correspondence and reprint requests to Dr. Inga-Lill Mårtensson, Laboratory of Lymphocyte Signalling and Development, The Babraham Institute, The Babraham Campus, Cambridge CB2 4AT, U.K. E-mail address: lill.martensson@bbsrc.ac.uk

[3] Abbreviations used in this paper: HC, H chain; LC, L chain; TG, transgene; BM, bone marrow; LN, lymph node.

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Flow Cytometry and Cell Sorting

Cells from BM and spleen were stained with Abs as previously described (14). The anti-3-83 Id mAb 54.1 (15) was purified in the laboratory and detected as described (6). Cells were acquired on a FACSCalibur or FACSAria (BD Biosciences) and analyzed with FlowJo software (Tree Star).

(RT)-PCR analysis

Total RNA was prepared from sorted cells using RNABee (Tel Test), cDNA synthesized, and serial 5-fold dilutions analyzed by PCR using primers specific for hypoxanthine phosphoribosyltransferase (16), RAG-1 and RAG-2, or by quantitative PCR using previously described primers (17). Genomic DNA was prepared and analyzed by PCR using a degenerate Vκ (18) together with a Jκ 1 IVS primer as previously described (15). RAG-1 s 5′-TGCAGACATTCTAG CACTCTG-3′; as 5′-ACATCTGCCTTCACGTCGAT-3′; RAG-2 s 5′-ATGTCCCTGCAGATGGTAACA-3′; as 5′-GCCTTTGTATGAGCAAG TAGC-3′.

Western blot

Lysates were prepared from 5×10^5 cells in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.5% (v/v) Triton X-100, 1 mM DTT, protease inhibitors cocktail, Sigma-Aldrich). Samples were incubated for 15 min on ice, centrifuged for 10 min (13,000 rpm, 4°C), supernatants harvested and resolved on NuPage 4–12% Bis-Tris gels (Invitrogen Life Technologies). Membranes were probed with affinity-purified rabbit-anti-RAG-2 Abs (19) and peroxidase-labeled anti-rabbit Abs (Dako Cytomation), thereafter stripped and re-probed with biotinylated-anti-IgM (Sigma-Aldrich) and peroxidase-labeled streptavidin and visualized using Supersignal West Dura (Perbio). Statistical analyses in Fig. 2*C*: Because the variable displayed a very large range, there was every reason to expect heterogeneous random error. Consequently, and to stabilize the errors, a logarithmic transformation of the data was carried before analysis.

Results

p110δ is important for LC isotype exclusion

Previous studies have noted that B cells from CD19$^{-/-}$ mice, which have a defect in LC isotype exclusion, display an increased number of λ positive cells yielding a decrease in the κλ ratio (7). To assess the possible participation of p110δ in LC isotype exclusion, we analyzed lymph node (LN) B cells from mice lacking p110δ by FACS and calculated the κλ ratio. Lymph node B cells were chosen for this measurement as lymph nodes lack the transitional and MZ B cell subsets found in the spleen which may confound this analysis. The κ:λ ratio was significantly decreased ($p = 0.002$) in cells from p110δ$^{-/-}$ mice (Fig. 1*A*), and as expected, a decrease was also observed in cells from CD19$^{-/-}$ mice (7).

To further investigate the role of p110δ in LC isotype exclusion we used the 3-83TG in the absence of the autoantigen. As PI3K deficiency may affect cell survival (11) mice coexpressing a Bcl-2TG were also analyzed. In the BM of both 3-83$^+$ and p110δ$^{-/-}$3-83$^+$ mice 20–30% of the cells were IgM$^+$; two thirds of these coexpressed IgD (Fig. 1*B*). Overall, there were similar numbers of IgM$^+$ cells in the two genotypes, and the vast majority expressed the 3-83 Id (Table I). Although there were no major differences in the BM, there was a clear disparity in the spleen where the number of IgM$^+$ cells was reduced around 5-fold in the absence of p110δ (Table I). The Bcl-2TG increased the number of IgM$^+$ cells in the BM and spleen 2- to 4-fold, irrespective of the presence or absence of p110δ (Table I). The λ-expressing cells were not detected in the BM or spleen of 3-83$^+$ or p110δ$^{-/-}$3-83$^+$ mice. However, a distinct population of B220$^+$λ$^+$ cells that coexpressed κ LC was observed in BM and spleen of p110δ$^{-/-}$3-83$^+$Bcl-2$^+$ that was absent in 3-83$^+$Bcl-2$^+$ mice (Fig. 1, *B* and *C*) resulting in an increase in λ$^+$ cells in the former (Table I). These data suggest that p110δ is involved in LC isotype exclusion and that the Bcl-2TG may permit κ$^+$λ$^+$ B cells to survive.

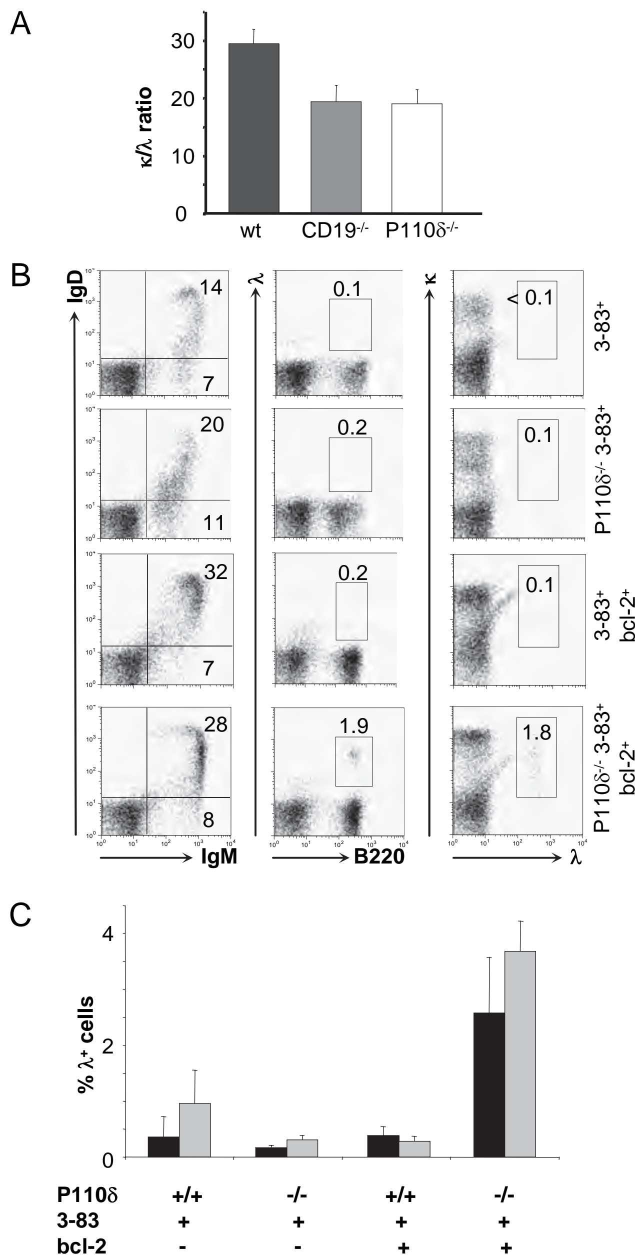

FIGURE 1. p110δ$^{-/-}$ B cells fail to impose LC allelic/isotype exclusion. *A*, LN cells were stained for surface expression of B220, IgM, κ and λ. Cells were gated as B220$^+$IgM$^+$ and the ratio of κ$^+$ vs λ$^+$ was calculated. Mean ± SD; wild type ($n = 7$), CD19$^{-/-}$ ($n = 7$), and p110δ$^{-/-}$ ($n = 6$). *B*, Flow cytometry analysis of BM cells from mice of indicated genotypes stained for surface expression markers as shown. Representative of three to four mice of each genotype. *C*, Summary data (mean ± SD) showing percentage of λ$^+$ cells in BM (black) and spleen (gray) from mice of the indicated genotypes.

p110δ regulates RAG expression

The RAG genes mediate V(D)J recombination (20, 21), and RAG expression is extinguished at the mature B cell stage in normal and 3-83 TG mice (5, 22). The presence of κ$^+$λ$^+$ B cells in p110δ$^{-/-}$3-83$^+$Bcl-2$^+$ mice suggested that the RAG-1 and -2 genes may be inappropriately active. Therefore, we analyzed B220$^+$IgM$^+$ cells for RAG gene expression. BM and splenic B cells from p110δ$^{-/-}$3-83$^+$ mice contained high levels

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Table I. *BM and splenic B cell numbers in 3-83 TG mice in the absence of p110δ*[a]

	$3\text{-}83^+$	$p110\delta^{-/-}3\text{-}83^+$	$3\text{-}83^+Bcl\text{-}2^+$	$p110\delta^{-/-}3\text{-}83^+Bcl\text{-}2^+$
BM				
Total cells	40.5 ± 4.61	58.62 ± 6.8	56.3 ± 10.7	42.6 ± 5.21
Lymphocytes	16.6 ± 3.61	16.22 ± 5.52	21.3 ± 2.27	19.68 ± 5.73
IgM	3.9 ± 2.07	3.34 ± 0.78	9.4 ± 1.54	7.29 ± 1.26
κ	3.95±2.1	2.97 ± 0.67	7.19 ± 1.16	7.28 ± 0.95
Id	4.2 ± 2.68	3.96 ± 1.22	ND	8.9 ± 0.056
λ	0.064 ± 0.031	0.022 ± 0.0048	0.082 ± 0.039	0.473 ± 0.06
Spleen				
Total cells	41.8 ± 17.9	27.5 ± 4.0	81 ± 3.92	56.62 ± 4.7
Lymphocytes	29.6 ± 14.8	10.22 ± 6.05	51.4 ± 2.19	33.45 ± 4.5
IgM	14.9 ± 6.47	3.15 ± 1.36	33.7 ± 2.95	12.4 ± 12.8
κ	13.6 ± 8.46	2.72 ± 1.23	29.4 ± 3.33	13.1 ± 0.92
Id	17.1 ± 3.58	2.95 ± 1.99	ND	10.7 ± 2.38
λ	0.31 ± 0.2	0.047 ± 0.018	0.15 ± 0.055	1.15 ± 0.0537

[a] Absolute numbers ($\times 10^6$) of indicated cell populations in BM (2 femurs) and spleen from mice of indicated genotypes. The results are means of four mice from each group ± SD, except $3\text{-}83^+Bcl\text{-}2^+$ which were three mice. ND, Not done.

of RAG-1 and -2 mRNA when compared with $3\text{-}83^+$ mice, irrespective of the presence of the Bcl-2TG (Fig. 2*A*, data not shown). In addition, this was accompanied by a 4-fold increase in RAG-2 protein levels in the BM and aberrant expression in splenic B cells that did not normally express detectable RAG-2 (Fig. 2, *B* and *C*). Thus, the ability to down-regulate the RAG genes at both the mRNA and protein level requires p110δ.

Although the number of BM IgM^+ cells was similar, the levels of IgD were lower in the absence of p110δ (Fig. 1*B*); this was also evident in the spleen (data not shown). To investigate whether this reflected a difference in the proportions of immature and mature B cells, we analyzed expression of CD93 (23, 24). In the BM, the majority of IgM^+ cells were $CD93^+$, irrespective of p110δ genotype, whereas in the spleen around 40–50% were IgM^+CD93^+ (Fig. 2*D*). RAG-1 and -2 mRNA levels were higher in IgM^+CD93^+ cells from BM and spleen of $p110\delta^{-/-}3\text{-}83^+$ mice, and in addition, the RAG-2 protein levels were significantly higher in the BM (Fig. 2*C*, data not shown). Even though λ-expressing cells were only observed in $p110\delta^{-/-}3\text{-}83^+Bcl\text{-}2^+$ mice (Fig. 1, *B* and *C*), the presence of RAG mRNA and protein in immature B cells of mice lacking the Bcl-2TG indicated that recombination may be ongoing. In agreement with such a hypothesis, high levels of endogenous Vκ-Jκ 1 DNA rearrangements were detected in IgM^+CD93^+ cells from BM and spleen of $p110\delta^{-/-}3\text{-}83^+$ mice (Fig. 2*E*). Furthermore, the κ:λ ratio in BM B cells from non-TG $p110\delta^{-/-}$ mice showed an increase in λ^+ cells that was similar to that of LNs (Fig. 1*A*, data not shown) and RAG-1 and -2 mRNA levels were increased (3-fold) in BM IgM^+CD93^+ cells from $p110\delta^{-/-}$ mice (Fig. 2*F*). Taken together, these data

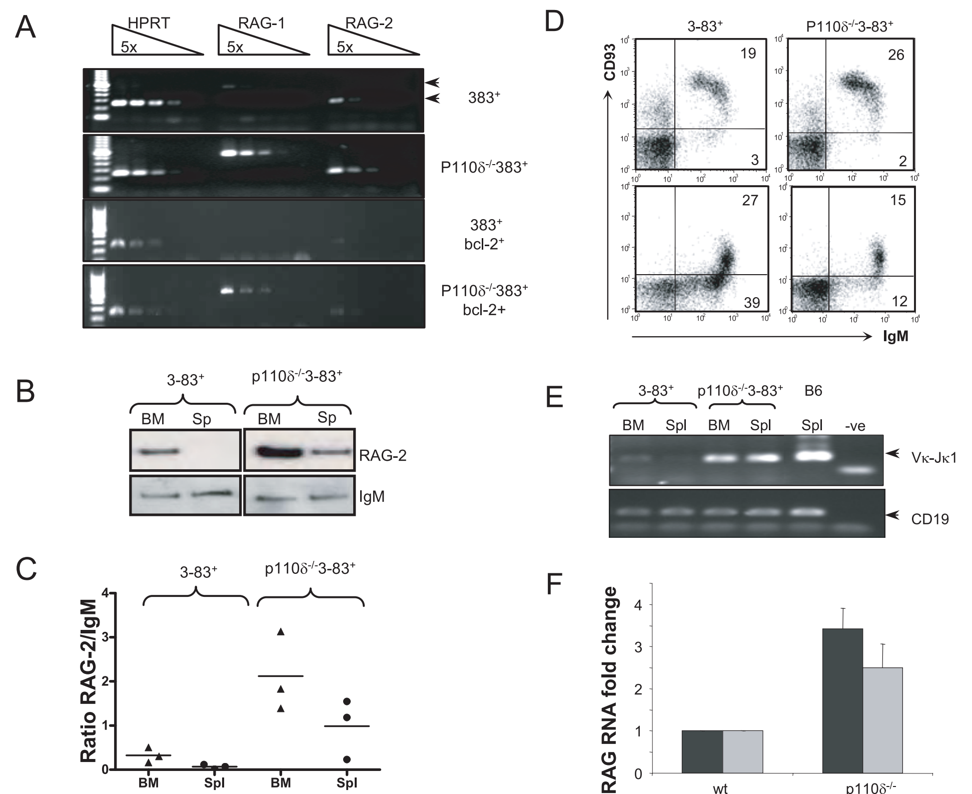

FIGURE 2. Elevated RAG expression and VJκ recombination in B cells lacking $p110\delta^{-/-}$. *A*, Semiquantitative RT-PCR analysis of RAG-1 and -2 expression in BM $B220^+IgM^+$ cells. *B*, Western blot of cell lysates from $B220^+IgM^+$ cells. Membranes were probed with anti-RAG-2 and reprobed with anti-IgM Abs. *C*, Ratio of RAG-2 and IgM protein levels in BM (p = 0.009) and spleen (p = 0.118) $B220^+IgM^+$ and IgM^+CD93^+ cells. *D*, Flow cytometry analysis of BM (*top*) and spleen (*bottom*) cells stained for immature B cells (IgM vs CD93). *E*, PCR analysis of endogenous VJκ1 recombination in BM and spleen IgM^+CD93^+ cells. CD19 was used as a control. *F*, Quantitative RT-PCR analysis of RAG-1 (black) and -2 (gray) levels in BM IgM^+CD93^+ cells. Genotypes are indicated in the figure. Representative results from ≥2 to 3 mice from each genotype performed as separate experiments.

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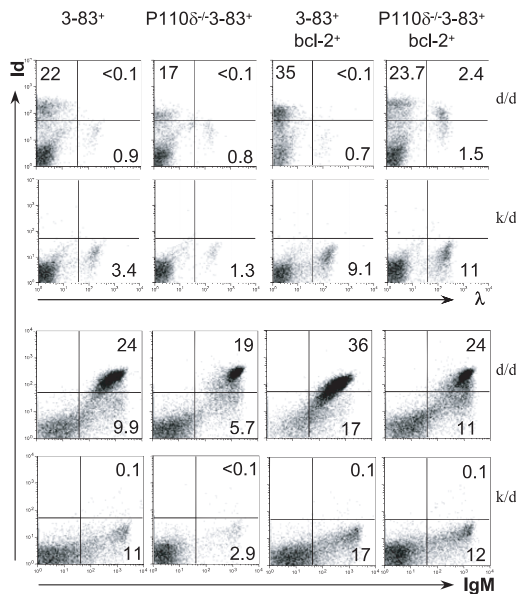

FIGURE 3. Receptor editing in the absence of p110δ$^{-/-}$. Flow cytometry analysis of splenic B cells from mice of the indicated genotypes bred in the absence (H-2^{d}) or presence (H-2^{k}) of the autoantigen. Cells were stained for surface markers as indicated. The figure is representative of three mice of each genotype, except 3-83^{+}Bcl-2^{+} on the H-2^{d} background, which was one mouse.

demonstrate that p110δ is required to extinguish RAG gene expression and impose isotype and allelic exclusion of the LC loci.

Receptor editing is independent of p110δ

We next examined whether p110δ is important for signals mediating negative selection. In the 3-83 model H-2^{k} or H-2^{b} triggers clonal deletion and receptor editing (25). Autoantigen led to a reduction in IgM^{+} B cells independently of the presence of p110δ (Fig. 3 and Table II). In addition, independent of genotype, cells expressing the Id were greatly reduced whereas the proportion of λ-expressing cells increased. Thus, clonal deletion and receptor editing takes place in mutant mice, demonstrating that these processes are independent of p110δ.

Discussion

The present work demonstrates that p110δ is required for silencing RAG expression and LC allelic/isotype exclusion. In addition, in the presence of the autoantigen, the processes of receptor editing and clonal deletion are intact.

We have previously reported that the absence of either Syk (6) or Lyn (26) result in impaired LC isotype exclusion, whereas others have shown that the coreceptor CD19 plays an essential role in this process (7). Previous work using wortmannin, a nonselective inhibitor of PI3K, suggested this pathway inhibits RAG gene expression (8, 9). In this study, we provide genetic evidence to demonstrate that the PI3K catalytic subunit p110δ is required for the repression of the RAG genes and further LC recombination.

Recent work suggests receptor editing is the main mechanism of B cell tolerance and in its failure B cells undergo deletion (4). We find that in the presence of H-2K^{k} both wild-type and p110δ-deficient cells undergo receptor editing as well as negative selection; these processes do not display a requirement for p110δ. As the process of receptor editing requires RAG expression, this would be in line with the inability of p110δ-deficient B cells to silence the RAG genes.

Our data show direct involvement of PI3K in regulating RAG gene expression. However, the molecular targets of PI3K remain to be defined. NF-κB (8) and E2A (27) have been implicated in allelic/isotype exclusion; it is not known if either of these are regulated by PI3K in immature B cells. This work provides genetic proof for a role of p110δ in regulating RAG gene expression, and it will be interesting to determine whether other catalytic subunits of PI3K participate in this process.

Acknowledgments

We thank S. Desiderio for the anti-RAG-2 antiserum, S. License for assistance, A. Segonds-Pichon for statistical analyses, and A. Corcoran and D. Bolland for discussions.

Disclosures

The authors have no financial conflict of interest.

Table II. *Absolute numbers of splenic B cell subsets on H-2$^{d/d}$ and H-2$^{k/d}$ background*[a]

	3-83^{+}	p110δ$^{-/-}$3-83^{+}	3-83^{+}bcl-2^{+}	p110δ$^{-/-}$3-83^{+}bcl-2^{+}
H-2$^{k/d}$				
Total cells	5.73 ± 1.14	14.13 ± 1.79	36.13 ± 13.3	23.067 ± 6.81
Lymphocytes	1.94 ± 0.36	5.65 ± 0.62	16.76 ± 6.4	9.32 ± 2.27
IgM	0.17 ± 0.037	0.16 ± 0.016	2.88 ± 0.83	1.06 ± 0.11
IgD	0.11 ± 0.027	0.14 ± 0.016	2.38 ± 0.70	1.01 ± 0.13
Id^{+}IgM^{+}	0.0025 ± 0.001	0.00286 ± 0.00032	0.022 ± 0.0075	0.0128 ± 0.0012
IgM^{+}λ^{+}	0.066 ± 0.015	0.08 ± 0.0053	1.19 ± 0.31	0.68 ± 0.13
Id^{+}λ^{+}	0.00088 ± 0.0003	0.0013 ± 0.0002	0.0081 ± 0.0031	0.0069 ± 0.0008
H-2$^{d/d}$				
Total cells	26.93 ± 0.93	12.93 ± 1.47	28.4	42.1 ± 7.57
Lymphocytes	12.21 ± 0.82	5.37 ± 0.64	13.43	17.73 ± 4.2
IgM	5.43 ± 0.98	1.19 ± 0.26	5.95	5.04 ± 1.50
IgD	4.98 ± 0.94	1.04 ± 0.22	5.17	4.53 ± 1.38
Id^{+}IgM^{+}	4.5 ± 0.9	0.92 ± 0.20	4.92	4.56 ± 1.43
IgM^{+}λ^{+}	0.122 ± 0.011	0.0538 ± 0.0088	0.087	0.72 ± 0.31
Id^{+}λ^{+}	0.0097 ± 0.0008	0.017 ± 0.0069	0.0056	0.37 ± 0.15

[a] Absolute numbers (×10^{6}) of cell populations in the spleen from mice of indicated genotype on H-2$^{d/d}$ (non-deleting) and H-2$^{k/d}$ (deleting) background. The results are means ± SEM of three mice from each group, except 3-83^{+}bcl-2^{+} on the H-2$^{d/d}$ background which was from one mouse.

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