Distinct Signal Thresholds for the Unique Antigen Receptor-linked Gene Expression Programs in Mature and Immature B Cells

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Summary

Although it is well established that immature B lymphocytes are exquisitely sensitive to tolerance induction compared with their mature counterparts, the molecular basis for this difference is unknown. We demonstrate that signaling by B cell antigen receptors leads to distinct and mutually exclusive biologic responses in mature and immature B cells: upregulation of CD86, CD69, and MHC class II in mature cells and receptor editing in immature cells. These responses can be induced simply by elevation of intracellular free calcium levels, as occurs after receptor aggregation. Importantly, induction of immature B cell responses requires much smaller increases in intracellular free calcium than does induction of mature B cell responses. These differences in biologic response and sensitivity to intracellular free calcium likely contribute to selective elimination at the immature stage of even those B cells that express low affinity for self-antigens.

Key words: signal transduction • B cells • receptor editing • B cell antigen receptor • development

A n essential feature of the immune system is its ability to develop protective immunity to foreign pathogens while remaining unresponsive to self-antigens. In the B lymphocyte compartment, self-tolerance can be achieved by antigen stimulation of immature B cells (1–4). Findings that immature B cells are exquisitely sensitive to tolerance induction compared with mature B cells (5–7) suggest that antigen–receptor signaling may be qualitatively or quantitatively distinct in these populations. Although developmentally programmed differences in B cells have been addressed in a number of studies (8–10), underlying differences in signal transduction and transcriptional regulation after antigen stimulation are unknown. Using antireceptor antibodies, it was shown that aggregation of the B cell antigen receptor (BCR) induces proliferation of mature B cells (11, 12), immature cells fail to progress beyond the early G1 phase of the cell cycle (13) and in some cases initiate apoptosis (14). Low to absent expression of bcl-2 may predispose immature B cells to the latter response (15). Unlike the situation in their mature counterparts, aggregation of the BCR on immature cells activates receptor editing via induced expression of rag genes (16, 17) but fails to initiate expression of egr-1, c-fos, and c-myc (12, 13).

Differential susceptibility to tolerance induction may reflect differences in proximal events in antigen–receptor signal transduction. In mature and immature B cells, the BCR is composed of membrane (m)Ig noncovalently associated with disulfide-linked heterodimers of immunoreceptor tyrosine-based activation motif (ITAM)-containing CD79a and CD79b (18–20). BCR aggregation by antigen or antireceptor antibody mediates clustering of associated Src family kinases, leading to phosphorylation of ITAM tyrosines. ITAM tyrosines are required for receptor-mediated activation of gene expression (21), and their phosphorylation leads to the recruitment of additional Src family kinases as well as the tyrosine kinase Syk (22–24). Upon recruitment, Syk becomes tyrosyl phosphorylated and activated, which is essential for activation of Btk and the phospholipase C (PLC) pathway (23, 25). Recent studies have defined a B cell–unique linker molecule, BLNK or Slp-65, which serves as a substrate for Syk and is essential for the activation of PLCγ2 (26–28). Subsequent tyrosyl phosphorylation of CD19 leads to phosphorylation of 3 kinase and generation of phosphatidylinositol 3-4,5-P3 (PtdIns3,4,5P3) (29, 30). Phosphorylation of Btk and PLCγ1 and 2 and translocation of these enzymes to the

A bbreviations used in this paper: BCR, B cell antigen receptor; BM, bone marrow; HEL, hen egg lysozyme; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; RAG, recombinase activator gene; Tg, transgenic.
plasma membrane, where they bind PtdIns3,4,5P$_3$, leads to PLC-$\gamma$-mediated hydrolysis of phosphoinositides, generation of inositol-1,4,5-triphosphate (Ins1,4,5P$_3$), and mobilization of calcium (31–33). Comparative analysis of signal transduction has revealed that immature B cells mobilize intracellular calcium in response to BCR aggregation by anti-receptor antibodies but fail to produce levels of Ins1,4,5P$_3$ found in mature B cells (12). These findings suggest that (a) certain intermediatory events required for antigen receptor-mediated phosphoinositide hydrolysis are impaired in immature cells and (b) immature B cell calcium stores are more sensitive to Ins1,4,5P$_3$. Neither the ability of cognate antigen to induce distinct signal transduction responses in these cells nor the role of these responses in determining the unique biologic outcome of BCR signaling in immature cells has been assessed.

Here we report a comparative analysis of events after antigen-induced signal transduction in mature and immature B cells. Results indicate that antigen stimulates distinct and mutually exclusive biologic responses in immature and mature B cells, receptor editing and upregulation of activation markers (CD86, CD69, and MHC class II), respectively. Both responses can be attributed to receptor-mediated elevation of intracellular free calcium levels ([Ca$^{2+}$]), as elevation of [Ca$^{2+}$], using agents such as ionomycin, is sufficient to trigger them. Importantly, induction of receptor editing in immature B cells requires much lower increase in [Ca$^{2+}$] than does the induction of activation marker expression in mature B cells. These results demonstrate differential activation of gene expression in immature and mature B cells after BCR aggregation and that immature B cells display increased sensitivity in the induction of these genetic programs.

Materials and Methods

Animals and Tissue Culture. B10.D2N/SnJ and two Ig transgenic (Tg) mouse models were used in the experiments: the 3-83 Ig Tg mice express IgM and IgD specific for H-2K$^k$ (1), and Ig Tg anti-hen egg lysozyme (α-HEL) mice express IgM and IgD specific for HEL (2). Bone marrow (BM) was obtained from 3–4-mo-old mice; a single-cell suspension was prepared by flushing femurs with IMDM to dislodge cells, followed by gentle deaggregation using a 5-ml syringe. BM was depleted of erythrocytes using Gey's solution, washed twice in IMDM, and cultured at 5 x 10$^5$ cells/ml in 10-cm petri dish (7% CO$_2$, 37°C) in IMDM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, 10% FCS (HyClone), and 50–100 U IL-7 (34) (derived from culture supernatant of J558L cells transfected with mlIL-7 cDNA, a gift from Dr. A. R. Oldfield, Basel Institute for Immunology, Switzerland). Typically, cells were harvested after 6–7 d in culture, washed twice with IMDM, and used in subsequent experiments. Resting splenic B cells (p > 1.066) were obtained from 3–4-mo-old adult mice and prepared as previously described (35). Cell viability was assessed using trypan blue dye exclusion.

Reagents. Antigens: an H-2K$^k$ mimetic peptide (CSGFGG-FQHLCCGAAGA) that binds specifically to the 3-83 receptor (35, 36) was synthesized and multimerized by coupling to N-ethylmaleimide-activated dextran (a gift from CorTech, Inc.) at a 100:1 peptide/dextran molar ratio; HEL was purchased from Sigma Chemical Co. Antibodies directed against the following molecules were used: MHC class II (Ia$^{d}$; D3.137); CD69 (H.12F3, Pharmingen); CD86 (GL-1, Pharmingen); CD21/35 (CR2/CR1); 7G6, Pharmingen; CD19 (1D3, Pharmingen); IgM (b-7-6); IgD (JA12.5); CD22 (CY34.1.2); CD23 (IgE Fc receptor; B3B4, Pharmingen); CD45 (13/12.5); and CD45R (anti-B220; RA 3-3A1 and RA 3-6B2). Ionomycin was purchased from Calbiochem Corp.; propidium iodide was from Sigma Chemical Co.

Phenotypic Analysis. Cells were washed once, resuspended in PBS containing 1% BSA and 0.1% sodium azide, and incubated with an optimal amount of biotinylated or directly fluorescinated antibody. Cells were incubated for 30 min at 4°C and washed twice in PBS/BSA/azide. In the case of biotinylated antibodies, cells were incubated as before with avidin–FITC or avidin–PE (Becton Dickinson). After washing, cells were analyzed on a flow cytometer. Histograms were constructed based on analysis of 10,000 cells.

Calcium Mobilization. For measurements of [Ca$^{2+}$], cells were loaded with Indo-1AM (Molecular Probes, Inc.), suspended at 10$^6$ cells/ml in IMDM, and stimulated with either antigen or anti-IgM antibody (b-7-6). Mean [Ca$^{2+}$] was evaluated over time using a flow cytometer (Model 50H; Ortho Diagnostic Systems Inc.) with appended data acquisition system and MultiTime software (Phoenix Flow Systems) as previously described (35).

Analysis of Rho Kinase A1/2 and B2 Expression. Levels of recombinant a-adaptor gene (RAG)-2 and CD19 mRNA were determined by RT-PCR assay as described in detail elsewhere (34). To obtain a semiquantitative estimate of gene expression, the signal for RAG-2 was normalized to the CD19 signal.

Results and Discussion

Analysis of molecular mechanisms underlying the responses of immature and mature B cells to antigen required generation of homogeneous populations of antigen-specific cells. To generate antigen-specific immature B cells, cells were isolated from BM of 3-83 μd (specific for H-2K$^k$) Ig Tg (1) or α-HEL Ig Tg (2) mice and cultured with IL-7 in IL-7–containing medium. Over this period, the total number of cells per dish increased from 5 x 10$^5$ to 30–40 x 10$^5$ (data not shown). The percentage of B cells (CD45R$^+$) increased from 12–18% at the onset of the culture to >95% after 5 d, demonstrating the selective outgrowth of IL-7-responsive B cell precursors (34, 37). Phenotypic analysis further indicated that few cells from non-Tg BM grown in IL-7 progressed to the IgM$^+$IgD$^+$ immature B cell stage compared with the Ig Tg animals (Fig 1). Cells from Tg BM grown under these conditions were >90% IgM$^+$IgD$^-$; upon removal of IL-7, cells progressed through IgM$^+$IgD$^-$, the immature stage, to IgM$^+$IgD$^+$ (data not shown and reference 34). Elative expression of mlIGM and mlIGD transgenes as well as other markers was consistent with development of a homogeneous population of immature cells in these cultures (Fig 1); cells were CD45$^+$ (also CD45R$^-$, not shown), CD22$^+$, and CD19$^+$ (all com-
pared with mature B cells), whereas they were negative for CD21/35 and CD23, which are only expressed on mature B cells (38–40). Equivalent phenotypes were seen when BM cells from α-HEL Tg animals were grown in IL-7 (data not shown). These data confirm a previous report (34) that the presence of mIgM transgenes is sufficient to accelerate B cell maturation through the pro- and pre-B cell stages to an immature phenotype. Although it is difficult to exclude the possibility that the cells grown in this system are developmentally pre-B cells that simply express a Tg BCR on their surfaces, these cells express all known markers associated with the immature B cell stage (41, 42).

We then compared early biologic responses of the respective B cell populations to antigen, analyzing induction of rag gene expression, indicative of clonal elimination by receptor editing (3), and CD69, CD86, and MHC class II expression, indicative of BCR signal transduction leading to initiation of an immune response. Immature B cells from BM cultures and mature splenic B cells from 3-83 mice or α-HEL Tg animals were incubated with antigen, and the expression of activation markers was assessed (Fig. 2 A). Mature B cells upregulated CD69, CD86, and MHC class II molecules within 12 h of stimulation, as previously shown (43–45). In immature 3-83 Tg cells, however, only slight upregulation of CD69 and CD86 was detected, and this was seen only at 12 h after stimulation. HEL stimulation of immature, α-HEL Tg cells also led to upregulation of these markers at 12 h. The responses were more pronounced than those of immature 3-83 Tg cells but much less than those of mature α-HEL Tg cells (Fig. 2 A). No antigen induction of MHC class II was observed in immature cells. The difference in the degree of upregulation between the 3-83 Tg and α-HEL Tg cells is probably due to the affinity of the transgene-encoded receptor for its ligand (z2 × 10^-5 M for 3-83 Tg [reference 46] and 2 × 10^-9 M for α-HEL [reference 2]). These results demonstrate that immature B cells are unable to effectively upregulate molecules essential for T–B cell collaboration; this probably prevents their participation in T cell-dependent immune responses.

Recent studies demonstrate that binding of antigen to mIgM on immature B cells induces recombinase gene expression and L chain receptor rearrangements (16, 17), a process that results in alteration of receptor specificity, termed receptor editing (3). This process leads to clonal elimination of immature cells but may also contribute to increasing diversity in a germinal center reaction (47). To compare the ability of immature and mature B cells to undergo a receptor editing response to antigen, we tested for antigen-induced reactivation of rag genes using RT-PCR. These analyses demonstrated a greater than twofold (normalized to CD19 mRNA levels) increase in RAG-2 expression upon antigen stimulation in immature cells (Fig. 2 B), resembling responses observed in vivo (3). No RAG-2 mRNA was detectable in mature B cells before or after 18-h antigen stimulation. Finally, no antigen-induced cell death
was observed over the 18-h period in mature or immature cells (data not shown). Thus, as previously shown (16), antigen-induced cross-linking of the BCR in immature cells results in the induction of receptor editing, and this response is not seen in mature, naïve B cells. Together, the data demonstrate two mutually exclusive response patterns in mature and immature cells. These distinct patterns may ensure that autoreactive, immature B cells do not mature into autoreactive, mature B cells and that only B cells that have been properly vetted to eliminate those that are autoreactive can effectively upregulate ligands for CD28 in immature and mature B cells: RAG-2 expression was increased 1.7–2.3-fold after 80–120-nM rises in \([\text{Ca}^{2+}]_i\), 3.1–5-fold after a 50-nM rise in \([\text{Ca}^{2+}]_i\) (Fig. 3 C). High doses of ionomycin (inducing \(>600 \text{nM} \text{ increase in } [\text{Ca}^{2+}]_i\)) only raised RAG-2 marginally and were accompanied by an increased apoptotic response (two- to threefold increase over unstimulated control), whereas little to no apoptosis was induced at 100 and 10 nM ionomycin (data not shown and reference 49). This suggests that extreme increases in \([\text{Ca}^{2+}]_i\) in immature B cells (possibly reflecting a signal through a high-avidity interaction with self-antigen) leads to an apoptotic rather than a receptor editing response. No RAG-2 induction was detected in mature B cells. These data demonstrate that increases in \([\text{Ca}^{2+}]_i\) can mediate the unique, antigen-induced changes in CD86 and RAG-2 seen in mature and immature B cells, respectively. These distinct biologic responses could be generated via two mechanisms. First, BCR stimulation could lead to activation of different transcription factors that are developmental stage specific in their expression. Second, different genetic loci could be more accessible in immature and mature B cells by regulated demethylation and chromatin remodeling; it has been demonstrated that these processes are involved in differential induction of c-myc in immature versus mature B cells (9), allelic exclusion during B cell development (10), and regulation of cytokine gene expression in Th1 and Th2 cells (50). According to this model, BCR stimulation would activate calcium-dependent transcription factors (e.g., NFAT [nuclear factor of activated T lymphocytes]), leading to rapid transcription of the accessible loci in each cell type, these would include rag in immature cells and CD86 and MHC class II in mature cells. The ability of HEL to induce transient upregulation...
of CD86 in immature cells appears inconsistent with both of these hypotheses. The high frequency of cells displaying this response excludes the possibility that it is a consequence of contaminating mature cells. It is possible that the accessibility of the loci is not absolute. Perhaps a sufficiently strong antigen receptor signal can lead to some expression of a relatively inaccessible locus. More studies are required to resolve this question.

The observations presented here are consistent with the behavior of the 3-83 μδ Ig Tg model of tolerance induction wherein immature B cells encounter autoantigen in the BM and are induced to edit their receptors (1, 3). However, they appear inconsistent with the HEL/α-HEL model. Specifically, findings that HEL induces RAG in immature B cells from α-HEL mice predict that B cells in HEL/α-HEL double-Tg mice should undergo editing in vivo. Evidence for receptor editing is observed when HEL is expressed as a membrane-associated form (51) but is less obvious when HEL is present as a secreted protein (2). In the latter double-Tg animals, however, a decline in the number and frequency of HEL-specific B cells is observed, suggesting that a portion of B cells may have been deleted (e.g., the IgMhigh cells) (2). It is possible that soluble HEL may not reach sufficient concentrations in the BM milieu to generate a strong enough signal to induce RAG in most developing B cells. Upon exiting the BM, B cells would encounter concentrations of soluble HEL in the periphery sufficient to render them anergic. Considering that the soluble HEL Tg is under the control of a metallothionein promoter, highest secretion of soluble HEL would be expected in the liver, kidney, or pancreas (52, 53). Thus, it is conceivable that immature B cells would only encounter low concentrations of soluble HEL in the BM. In addition, it could be hypothesized that only a limited number of B cells (e.g., only IgMhigh B cells) receive a signal strong enough to induce receptor editing; these cells are then either deleted or successfully edited away from their specificity for HEL but are not seen in vivo because of the size of the anergic B cell population. Mice expressing Ig Tg receptors for another soluble but multivalent ligand, DNA, demonstrate that B cells are rendered anergic when recognizing single-stranded DNA (low affinity), whereas B cells binding double-stranded DNA (high affinity) are deleted (4, 54). This supports the hypothesis that antigen valency in combination with receptor affinity determines the mechanism of tolerance induction in immature B cells. Resolution of these and other alternatives awaits further study.

The data presented also demonstrate a clear difference in sensitivity of the responses of mature and immature B cell to rises in intracellular calcium. Differences in these responses may be linked to the observed difference in tolerance sensitivity (5–7), where low signals in immature B
cells (induced by a low antigen concentration) result in clonal elimination, presumably by receptor editing. Similarly, the higher signal threshold for activation of mature B cells in the periphery protects against autoimmunity by preventing activation of mature B cells with low affinity for self. The question remains whether BCR-proximal signal transduction events differ between immature and mature B cells. It will be especially important to assess differences pertaining to the generation of Ca\(^{2+}\) mobilization, as this leads to the activation of specific genes. The increased sensitivity to antigen imposed by these mechanisms in mature B cells is likely to play a very important role in repertoire development, purging the repertoire of cells with even low affinities for self-antigens.

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References

1. Russell, D.M., Z. Dembic, G. Morahan, J.F. Miller, K. Burki, and D. Nemazee. 1991. Peripheral deletion of self-reactive B cells. Nature 354:308–311.
2. Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature 334:676–682.
3. Tiegs, S.L., D.M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. J. Exp. Med. 177:1009–1020.
4. Erikson, J., M.Z. Radic, S.A. Camper, R.R. Hardy, C. Carmack, and M. Wiegert. 1991. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. Nature 349:331–334.
5. Cambier, J.C., J.R. Kettman, E.S. Vitetta, and J.W. Uhr. 1976. Differential susceptibility of neonatal and adult murine spleen cells to in vitro induction of B cell tolerance. J. Exp. Med. 144:293–297.
6. Metcalfe, E.S., and N.R. Kleinman. 1976. In vitro tolerance induction of neonatal murine B cells. J. Exp. Med. 143:1327–1340.
7. Nossal, G.J. 1983. Cellular mechanisms of immunologic tolerance. Annu. Rev. Immunol. 1:33–62.
8. Kelley, D.E., B.A. Pollok, M.L. Atchison, and R.P. Perry. 1988. The coupling between enhancer activity and hypomethylation of kappa immunoglobulin genes is developmentally regulated. Mol. Cell. Biol. 8:920–937.
9. Seyfert, V.L., S.B. McAlonan, W.D. Glenn, A.J. Yellen, V.P. Sukhatme, X.M. Cao, and J.G. Monroe. 1990. Methylation of an immediate-early inducible gene as a mechanism for B cell tolerance induction. Science. 250:797–800.
10. Mostoslavsky, R., N. Singh, A. Kirillov, R. Pelanda, H. Cedar, A. Chess, and Y. Bergman. 1998. Kappa chain monoallelic demethylation and the establishment of allelic exclusion. Genes Dev. 12:1801–1811.
11. Weiner, H.L., J.W. Moorhead, and H.N. Claman. 1976. Anti-immunoglobulin stimulation of murine lymphocytes I. Age dependency of the proliferative response. J. Immunol. 116:1656–1661.
12. Yellen, A.J., W. Glenn, V.P. Sukhatme, X.M. Cao, and J.G. Monroe. 1991. Signaling through surface IgM in tolerance-sensitive immature murine B lymphocytes. Developmentally regulated differences in transmembrane signaling in splenic B cells from adult and neonatal mice. J. Immunol. 146:1446–1454.
13. Carman, J.A., R.J. Wechsler-Reya, and J.G. Monroe. 1996. Immature stage B cells enter but do not progress beyond the early GI phase of the cell cycle in response to antigen receptor signaling. J. Immunol. 156:4562–4569.
14. Norvell, A., L. Mandik, and J.G. Monroe. 1995. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. J. Immunol. 154:4404–4413.
15. Li, Y.S., K. Hayakawa, and R.R. Hardy. 1993. The regulated expression of B lineage-associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. 178:951–960.
16. Melamed, D., and D. Nemazee. 1997. Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest. Proc. Natl. Acad. Sci. USA. 94:9267–9272.
17. Hertz, M., and D. Nemazee. 1997. BCR ligation induces receptor editing in IgM+IgD− bone marrow B cells in vitro. Immunity. 6:429–436.
18. Cambier, J.C., C.M. Pleiman, and M.R. Clark. 1994. Signal

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transduction by the B cell antigen receptor and its coreceptors. Ann. Rev. Immunol. 12:457–486.

19. Cambier, J.C., W. Bedzyk, K. Campbell, N. Chien, J. Friedrich, A. Harwood, W. Jensen, C. Pleiman, and M.R. Clark. 1993. The B-cell antigen receptor: structure and function of primary, secondary, tertiary and quaternary components. Immunol. Rev. 132:85–106.

20. Tamir, I., and J.C. Cambier. 1998. Antigen receptor signaling: integration of protein tyrosine kinase functions. Onco-gene. 17:1353–1646.

21. Letourneur, F., and R.D. Klausner. 1992. Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 epsilon. Science. 255:79–82.

22. Clark, M.R., K.S. Campbell, A. Kazlauskas, S.A. Johnson, M. Hertz, T.A. Potter, C. Pleiman, and J.C. Cambier. 1992. The B cell antigen receptor complex: association of Ig-alpha and Ig-beta with distinct cytoplasmic effectors. Science. 258:123–126.

23. Kurosaki, T., S.A. Johnson, L. Pao, K. Sada, H. Yamamura, and J.C. Cambier. 1995. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. J. Exp. Med. 182:1815–1823.

24. Pao, L.I., S.J. Famiglietti, and J.C. Cambier. 1998. Asymmetrical phosphorylation and function of immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. J. Immunol. 160:3305–3314.

25. Kolanus, W., C. Romeo, and B. Seed. 1993. T cell activation by clustered tyrosine kinases. Cell. 74:171–183.

26. Fu, C., C.W. Turk, T. Kurosaki, and A.C. Chan. 1998. BLNK: a central linker protein in B cell activation. Immunity. 9:93–103.

27. Wienands, J., J. Schwickert, B. Wollscheid, H. Jumaa, P.J. Nielsen, and M. Reth. 1998. SLPI-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. J. Exp. Med. 188:791–795.

28. Ishiai, M., M. Kurosaki, R. Pappu, K. Okaawa, I. Ronkon, C. Fu, M. Shibata, A. Iwamatsu, A.C. Chan, and T. Kurosaki. 1999. BLNK required for coupling Syk to PLC gamma 2 and Ralpha1-JNK in B cells. Immunol. 10:117–125.

29. Buhl, A.M., C.M. Pleiman, R.C. Rickert, and J.C. Cambier. 1997. Qualitative regulation of B cell antigen receptor signaling by CD19: selective requirement for PI3-kinase activation, inositol-1,4,5-trisphosphate production, and Ca2+ mobilization. J. Exp. Med. 186:1897–1910.

30. Buhl, A.M., and J.C. Cambier. 1999. Phosphorylation of CD19 Y484 and Y515, and linked activation of phosphatidylinositol 3-kinase, are required for B cell antigen receptor-mediated activation of Bruton's tyrosine kinase. J. Immunol. 162:4438–4446.

31. Ransom, J.T., L.K. Harris, and J.C. Cambier. 1986. Anti-lg induces release of inositol 1,4,5-trisphosphate, which mediates mobilization of intracellular Ca2+ stores in B lymphocytes. J. Immunol. 137:708–714.

32. Bolland, S., R.N. Pearse, T. Kurosaki, and J.V. Ravetch. 1998. SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunology. 8:509–516.

33. Fluckiger, A.C., Z. Li, R.M. Kato, M.I. Wahl, H.D. Ochs, R. Longnecker, J.P. Kinet, O.N. Witte, A.M. Scharenberg, and D.J. Rawlings. 1998. Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation. EMBO J. (Eur. Mol. Biol. Org.) 17:1973–1985.

34. Melamed, D., J.A. Kench, K. Grabstein, A. Rolink, and D. Nemazee. 1997. A functional B cell receptor transgene allows efficient IL-7-independent maturation of B cell precursors. J. Immunol. 159:1233–1239.

35. Vilien, B.J., S.J. Famiglietti, A.M. Carbone, B.K. Kay, and J.C. Cambier. 1997. B cell antigen receptor desensitization: disruption of receptor coupling to tyrosine kinase activation. J. Immunol. 159:231–243.

36. Kouskoff, V., S. Famiglietti, G. Laudau, P. Lang, J.E. Rider, B.K. Kay, J.C. Cambier, and D. Nemazee. 1998. Antigens varying in affinity for the B cell receptor induce differential B lymphocyte responses. J. Exp. Med. 186:4153–4166.

37. Röllin, A., A. Kudo, H. Karasuyama, Y. Kikuchi, and F. Mönch. 1991. Long-term proliferating early B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J. (Eur. Mol. Biol. Org.) 10:327–336.

38. Waldschmidt, T.J., D.H. Conrad, and R.G. Lynch. 1988. The expression of B cell surface receptors. I. The ontology and distribution of the murine B cell IgE Fc receptor. J. Immunol. 140:2148–2154.

39. Takahashi, K., Y. Kozono, T.J. Waldschmidt, D. Berthiaume, R.J. Quigg, A. Barou, and V.M. Holmes. 1997. Mouse complement receptors type 1 (CR1;CD35) and type 2 (CR2;CD21): expression on normal B cell subpopulations and decreased levels during the development of autoimmunity in MRL/lpr mice. J. Immunol. 159:1557–1569.

40. Erickson, L.D., L.T. Tygrett, S.K. Bhatia, K.H. Grabstein, and T.J. Waldschmidt. 1996. Differential expression of CD22 (lyb8) on murine B cells. Int. Immunol. 8:1121–1129.

41. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med. 173:1213–1225.

42. Benschop, R.J., and J.C. Cambier. 1999. B cell development: signal transduction by antigen receptors and their surrogates. Curr. Opin. Immunol. 11:143–151.

43. Moran, J.G., and J.C. Cambier. 1983. B cell activation. III. B cell plasma membrane depolarization and hyper-Ia antigen expression induced by receptor immunoglobulin cross-linking are coupled. J. Exp. Med. 158:1589–1599.

44. Risso, A., M.E. Cosulich, A. Urbartelli, M.R. Azza, and A. Bargellesi. 1989. M LR3 molecule is an activation antigen shared by human B, T lymphocytes and T cell precursors. Eur. J. Immunol. 19:323–328.

45. Halhouch, K.S., G. Laslo, C. Pucillo, P. Linsey, and R.J. Hodes. 1994. Comparative analysis of B7-1 and B7-2 co-stimulatory ligands: expression and function. J. Exp. Med. 180:631–640.

46. Lang, J., M. Jackson, L. Teyton, A. Brunmark, K. Kanne, and D. Nemazee. 1996. B cells are exquisitely sensitive to central tolerance and receptor editing induced by ultralow affinity, membrane-bound antigen. J. Exp. Med. 184:1685–1697.

47. Han, S., B. Zheng, D.G. Schatz, E. Spanopoulou, and G. Kelsoe. 1996. Nef in lymphocytes R gag1 and R gag2 expression in germinal center B cells. Science. 274:2094–2097.

48. Bootman, M.D., and M.J. Berridge. 1995. The elemental principles of calcium signaling. Cell. 83:675–678.

49. Melamed, D., R.J. Benschop, J.C. Cambier, and D. Nemazee. 1998. Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. Celn. 92:173–182.

50. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell
differentiation. Immunity. 9:765–775.
51. Hartley, S.B., J. Crosbie, R. Brink, A.B. Kantor, A. Basten, and C.C. Goodnow. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature. 353:765–769.
52. Palmiter, R. D., and R. L. Brinster. 1986. Germ-line transformation of mice. Annu. Rev. Genet. 20:465–499.
53. Morahan, G., F.E. Brennan, P.S. Bhathal, J. Allison, K.O. Cox, and J.F. Miller. 1989. Expression in transgenic mice of class I histocompatibility antigens controlled by the metallothionein promoter. Proc. Natl. Acad. Sci. USA. 86:3782–3786.
54. Chen, C., M.Z. Radic, J. Erikson, S.A. Camper, S. Litwin, R.R. Hardy, and M. Weigert. 1994. Deletion and editing of B cells that express antibodies to DNA. J. Immunol. 152:1970–1982.