# 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 contributes 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

An 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 whereas aggregation of the B cell antigen receptor (BCR)[1] 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 kinase molecules, 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)γ 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 phosphatidylinositol 3 kinase activation and generation of phosphatidylinositol-3,4,5-$P_3$ (PtdIns3,4,5$P_3$) (29, 30). Phosphorylation of Btk and PLCγ1 and 2 and translocation of these enzymes to the

[1]*Abbreviations 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.

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plasma membrane, where they bind PtdIns3,4,5$P_3$, leads to PLC$\gamma$1/2-mediated hydrolysis of phosphoinositides, generation of inositol-1,4,5-triphosphate (Ins1,4,5$P_3$), and mobilization of calcium (31–33). Comparative analysis of signal transduction has revealed that immature B cells mobilize intracellular calcium in response to BCR aggregation by anti-receptor antibodies but fail to produce levels of Ins1,4,5$P_3$ found in mature B cells (12). These findings suggest that (a) certain intermediary events required for antigen receptor–mediated phosphoinositide hydrolysis are impaired in immature cells and (b) immature B cell calcium stores are more sensitive to Ins1,4,5$P_3$. 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 ($[Ca^{2+}]_i$), as elevation of $[Ca^{2+}]_i$, using agents such as ionomycin, is sufficient to trigger them. Importantly, induction of receptor editing in immature B cells requires much lower increases in $[Ca^{2+}]_i$ 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.D2nSn/J and two Ig transgenic (Tg) mouse models were used in the experiments: the 3-83 $\mu\delta$ Ig Tg mice express IgM and IgD specific for H-2K$^k$ (1), and Ig Tg anti–hen egg lysozyme ($\alpha$-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 $5 \times 10^5$ cells/ml per 10-cm petri dish (7% $CO_2$, 37°C) in IMDM containing 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 50 $\mu$g/ml gentamycin, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 $\mu$M 2-ME, 10% FCS (Hyclone), and 50–100 U IL-7 (34) (derived from culture supernatant of J558L cells transfected with mIL-7 cDNA, a gift from Dr. A. Rolink, 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 ($\rho > 1.066$) 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 (CSGFGGFQHLCCGAAGA) that binds specifically to the 3-83 receptor (35, 36) was synthesized and multimerized by coupling to *N*-ethylmaleamide–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$^{b/d}$; D3.137); CD69 (H1.2F3, 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 (I3/12.5); and CD45R (anti-B220; RA3-3A1 and RA3-6B2). 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 $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ 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 for Recombinase Activator Gene 2 Expression.* Levels of recombinase activator 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 $\mu\delta$ (specific for H-2K$^k$) Ig Tg (1) or $\alpha$-HEL Ig Tg (2) mice and cultured for 6–7 d in IL-7–containing medium. Over this period, the total number of cells per dish increased from $5 \times 10^6$ to $30–40 \times 10^6$ (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$^{low}$ IgD$^-$; upon removal of IL-7, cells progressed through IgM$^{high}$IgD$^-$, the immature stage, to IgM$^{high}$IgD$^{low}$, the transitional stage, and finally to mature B cells (data not shown and reference 34). Relative expression of mIgM and mIgD 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$^{low}$ (also CD45R$^{low}$, not shown), CD22$^{low}$, and CD19$^{low}$ (all com-

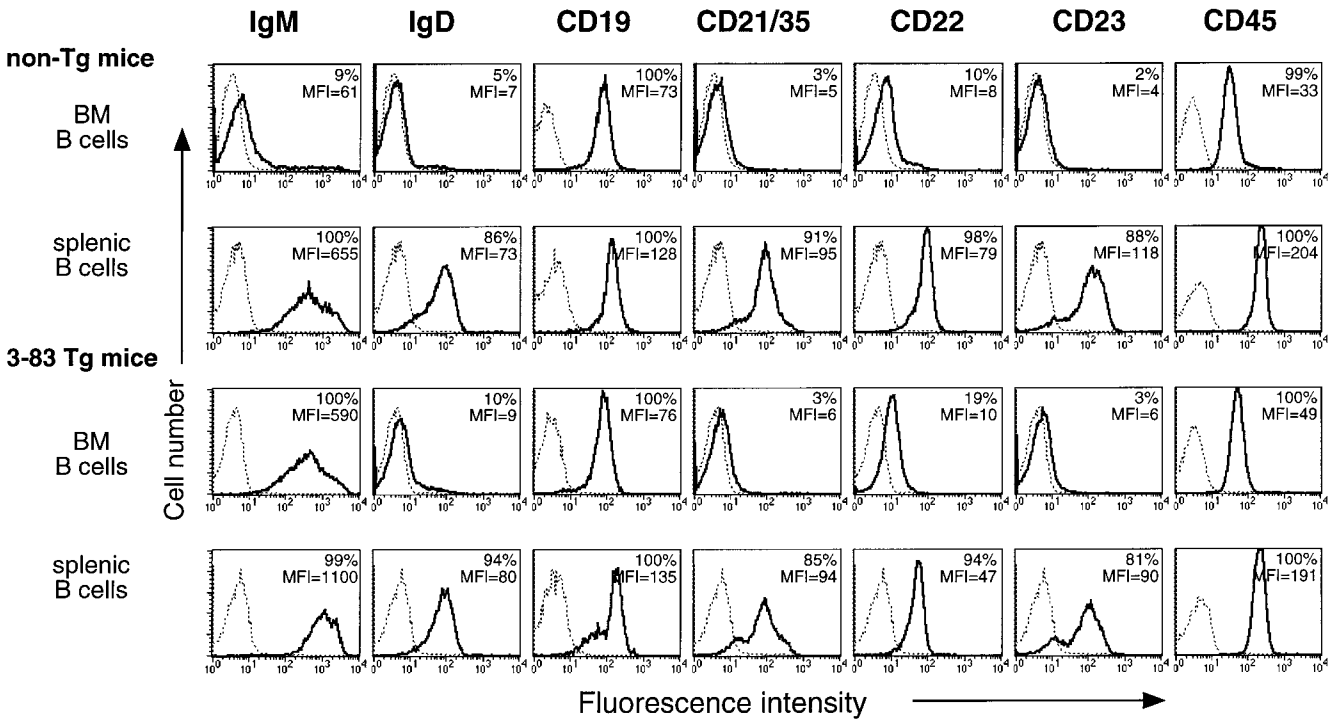


**Figure 1.** Generation of immature B cells in BM cultures containing IL-7. B cells isolated from BM from 3-83 μδ Tg and non-Tg littermate mice (B10.D2) were cultured for 7 d in the presence of IL-7. Cells, as well as mature splenic B cells, were analyzed by flow cytometry. Negative control histograms are shown as dashed lines. Shown in the upper right corner of each panel is the percentage of cells positive for the respective marker along with the relative mean fluorescence intensity (MFI).

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 μδ 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 μδ 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 μδ cells but much less than those of mature α-HEL Tg B 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 μδ and α-HEL Tg cells is probably due to the affinity of the transgene-encoded receptor for its ligand ($\sim 2 \times 10^{-5}$ M for 3-83 μδ [reference 46] and $2 \times 10^{-9}$ 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

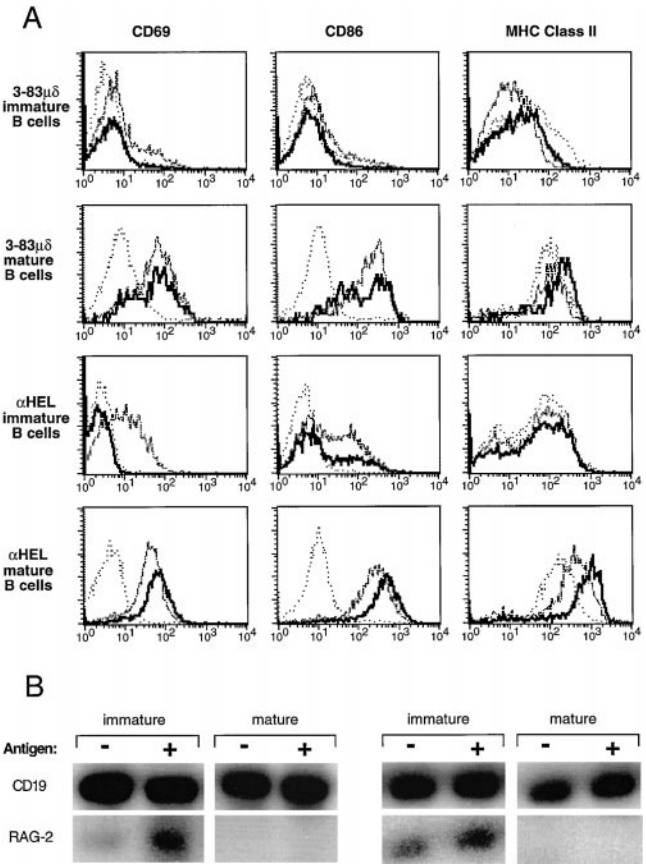


**Figure 2.** Antigen induces qualitatively distinct biologic responses in immature and mature B cells. (A) Expression of CD69, CD86, and MHC class II was analyzed in immature and mature B cells from 3-83 μδ and α-HEL Tg animals after stimulation with peptidic 3-83 antigen–dextran conjugates (500 ng/$10^6$ cells) or HEL (1,000 ng/$10^6$ cells), respectively for 0 (dotted lines), 12 (dashed lines), or 24 h (bold lines); surface expression of markers was monitored by immunofluorescence with analysis by flow cytometry. (B) RT-PCR analysis of RAG-2 and CD19 mRNA in immature and mature B cells from 3-83 μδ and α-HEL Tg animals before and after 18-h stimulation with their respective antigens.

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, naive 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 (CD86) and T cell antigen receptor (MHC class II) and thus participate in a productive immune response.

The observed differences in the biologic responses of mature and immature cells may result from differences in BCR coupling to proximal signal transduction pathways. Alternatively and/or additionally, these B cells may be programmed to respond differently to the same constellation of "second" messengers. Calcium mobilization plays an important role in triggering a wide array of cellular processes (48), including RAG induction (49), and could drive the BCR-mediated expression for CD69, MHC class II, and CD86. To determine if calcium mobilization is sufficient to initiate the biologic responses of immature and mature B cells, ionomycin was used to induce graded rises in the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Ionomycin induced comparable increases in $[Ca^{2+}]_i$ in immature and mature B cells at each concentration used (Fig. 3 A). Analysis of marker induction after 18 h of ionomycin stimulation demonstrated profound upregulation of CD86 in mature B cells but only at doses of ionomycin that induced increases of >600 nM $[Ca^{2+}]_i$; small increases were observed at doses that raised $[Ca^{2+}]_i$ by 80–120 nM, and no upregulation was seen when $[Ca^{2+}]_i$ was raised by 50 nM (Fig. 3 B). Consistent with the effects of antigen (Fig. 2), ionomycin did not induce CD86 in immature B cells. Similar observations were made for MHC class II and CD69 (data not shown). This demonstrates the sufficiency of calcium mobilization in antigen activation of CD86 and MHC class II expression in mature B cells.

As previously reported (49), elevation of $[Ca^{2+}]_i$ induced RAG-2 expression in immature B cells. However, as shown in Fig. 3, this response followed much smaller increases in $[Ca^{2+}]_i$ than required to induce CD86 in mature cells: RAG-2 expression was increased 1.7–2.3-fold after 80–120-nM rises in $[Ca^{2+}]_i$ and 3.1–5-fold after a 50-nM rise in $[Ca^{2+}]_i$ (Fig. 3 C). High doses of ionomycin (inducing >600 nM increase in $[Ca^{2+}]_i$) 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 *egr-1* 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

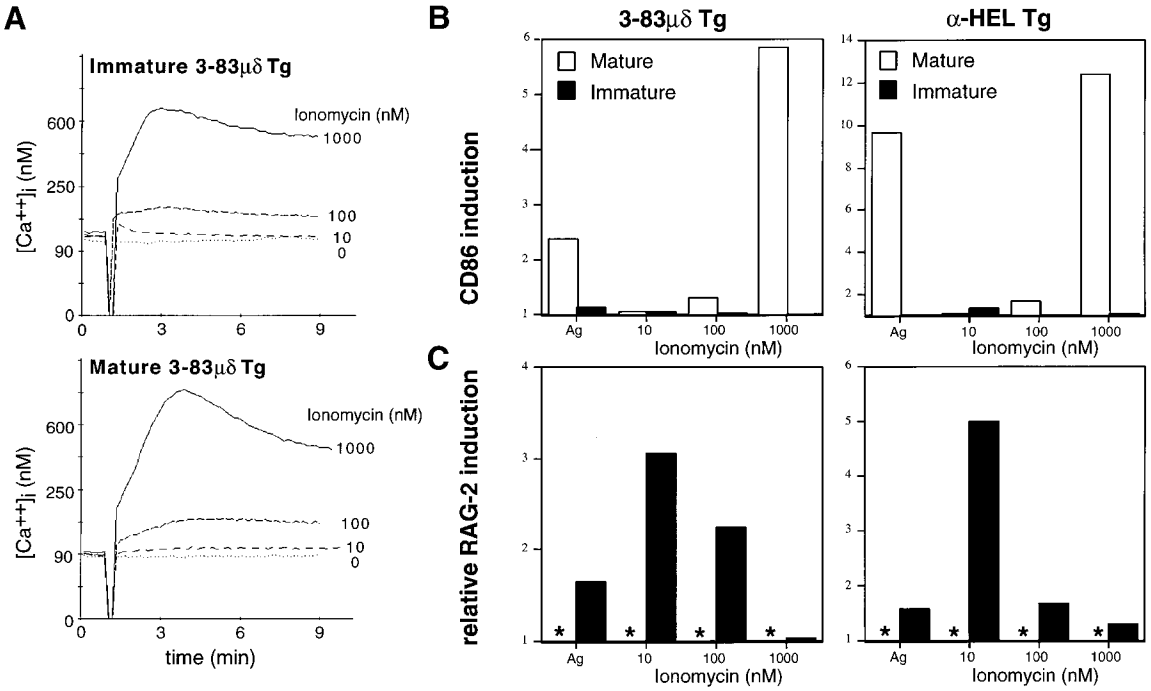


**Figure 3.** Calcium elevation induces RAG-2 expression in immature B cells and CD86 in mature B cells, and these responses exhibit different sensitivities to $[Ca^{2+}]_i$. (A) $[Ca^{2+}]_i$ was measured in immature and mature B cells from 3-83 μδ Tg animals after stimulation with ionomycin. (B) Immature (filled bars) and mature (open bars) B cells from 3-83 μδ and α-HEL Tg animals were stimulated with varying doses of ionomycin (10–1,000 nM) or their respective antigens (500 ng peptidic 3-83 antigen–dextran/$10^6$ cells and 1,000 ng/ml HEL) for 18 h; induction of CD86 is expressed as fold increase in mean fluorescence intensity over unstimulated control. (C) RT-PCR analysis of mRNA expression of RAG-2 was normalized to that of CD19; induction of RAG-2 is expressed as fold increase over unstimulated control. Asterisks indicate absence of data because RAG-2 could not be detected in mature B cells before or after ionomycin. Results are representative of three experiments.

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 in 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 $IgM^{high}$ 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 $IgM^{high}$ 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 immature 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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