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Developmental Regulation of B Lymphocyte Immune Tolerance Compartmentalizes Clonal Selection from Receptor Selection

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Summary

B lymphocyte development is a highly ordered process that involves immunoglobulin gene rearrangements, antigen receptor expression, and a learning process that minimizes the development of cells with reactivity to self tissue. Two distinct mechanisms for immune tolerance have been defined that operate during early bone marrow stages of B cell development: apoptosis, which eliminates clones of cells, and receptor editing, which spares the cells but genetically reprograms their autoreactive antigen receptors through nested immunoglobulin L chain gene rearrangements. We show here that sensitivity to antigen-induced apoptosis arises relatively late in B cell development and is preceded by a functionally distinct developmental stage capable of receptor editing. This regulation compartmentalizes clonal selection from receptor selection.

Introduction

B lymphocyte development in the bone marrow (BM) is characterized by essential and often irreversible changes in gene expression that are guided in large part by signaling through the antigen (Ag)-specific receptors (reviewed in Rajewsky, 1996). Immunoglobulin (Ig) genes are rearranged and assembled in a quasiordered cascade that is regulated both by the expression of the recombinase activator genes *RAG1* and *RAG2* (Grawunder et al., 1995) and by receptor gene accessibility to recombination (Sleckman et al., 1996). Immunoglobulin gene assembly starts at the heavy (H) chain loci with ligation of D_H to J_H gene segments, followed by V_H-to-DJ assembly (Tonegawa, 1983; Hardy et al., 1991; Boekel et al., 1995). As this process is error prone, often leading to out-of-frame or incomplete rearrangements (Tonegawa, 1983), expression of productive V-D-J genes is monitored by the cell through the ability of H chain protein to assemble in a preB cell receptor (BCR) complex together with λ5 and VpreB proteins ("surrogate L chain") (Melchers et al., 1995; Burrows and Cooper, 1997), which appear to have a chaperone function (Shaffer and Schlissel, 1997) allowing plasma membrane deposition. Signaling through the preBCR is transduced through associated Ig-α and Ig-β molecules (Melchers et al., 1994; Reth, 1995) resulting in down-regulation of V(D)J recombination that prevents further H chain rearrangements and insures "allelic exclusion," i.e., that the B cell expresses only one of its two potential H chain gene alleles (Storb et al., 1994). PreBCR signaling also results in a burst of interleukin 7 (IL-7)-dependent proliferative expansion (Rolink et al., 1994). Rapidly proliferating preB cells then exit cell cycle and redirect V(D)J recombinase activity to initiate immunoglobulin light (L) chain gene rearrangements (Constantinescu and Schlissel, 1997) that fuse V_L elements to J_L elements (Boekel et al., 1995). Like H chain gene assembly, this process is both tightly regulated and error prone. Inability to complete functional L chain gene rearrangement at this stage results in developmental arrest and subsequent cell death (Spanopoulou et al., 1994; Young et al., 1994). Successful L chain gene expression permits surface IgM (sIgM) expression and differentiation to maturity, a process that involves feedback suppression of further V(D)J recombination (Hardy et al., 1991; Storb et al., 1994; Boekel et al., 1995), and is followed by the appearance on the cell surface of a host of differentiation markers such as IgD, CD21, and CD23 (Hartley et al., 1993; Hardy and Hayakawa, 1995).

With the expression of sIgM and resultant acquisition of antigen specificity, maturing B cells become susceptible to immune tolerance (Nossal, 1994; Goodnow, 1996; Klinman, 1996; Monroe, 1996), which is probably important in limiting autoantibody production that can lead to disease (Radic and Weigert, 1995). Two concepts have dominated the thinking about immune tolerance in B lymphocytes. One postulates that developing lymphocytes pass through a tolerance-susceptible stage in which antigens encounter leads to deletion or functional inactivation; cells progressing through this stage in the absence of antigens then lose tolerance sensitivity and acquire antigen-inducible effector function (Lederberg, 1959). The second idea is that B cells require two inductive signals to become activated, one provided by antigen-binding to the BCR and a second provided by a helper T cell (Bretscher and Cohn, 1970). Experimental data generated over the years have suggested that both of these models are partly valid, as immature B cells are particularly tolerance susceptible but can be stimulated to effector function with strong costimulus (Metcalf and Klinman, 1977; Pike et al., 1980; Nossal, 1983; Klinman, 1996), whereas mature B cells can be eliminated by a strong BCR signal that occurs in the absence of an appropriate costimulus (Russell et al., 1991; Parry et al, 1994; Tsubata et al., 1994; Finkelman et al., 1995). Furthermore, mature and immature B cells differ biochemically in BCR-signaling responses (Monroe, 1996). Early in vitro studies analyzing the mechanisms for the increased tolerance sensitivity of immature B cells found that BM or fetal B cells treated with anti-IgM did not rapidly die but lost sIgM expression that was never completely restored upon reculture without anti-IgM, whereas similarly treated splenic B cells completely recovered sIgM expression (Raff et al., 1975; Sidman and Unanue, 1975). Analysis of tolerance in mice bearing autoantigen

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transgenes showed that in the presence of membrane-bound autoantigens mature B cells were eliminated, while immature B cells bearing a low level of sIgM were abundant in the BM (Nemazee and Burki, 1989; Hartley et al., 1991). In these transgenic (Tg) models, autoantigen binding to sIgM on developing B cells blocks their further development but fails to induce rapid cell loss and is reversible upon antigen removal (Hartley et al., 1993; Melamed and Nemazee, 1997). Recent studies show that the tolerance-induced developmental block results in continued L chain gene rearrangements in sIgM$^+$ cells (Hertz and Nemazee, 1997; Melamed and Nemazee, 1997), violating a strict feedback suppression model for L chain allelic exclusion. Importantly, this so-called "receptor editing" process can reprogram BCR specificity through nested L chain gene rearrangements that are capable of substituting one functional L chain for another, allowing cells whose receptors are successfully altered and that no longer bind to autoantigen to resume their maturation program and to survive long term (Gay et al., 1993; Tiegs et al., 1993; Radic and Zouali, 1996; Chen et al., 1997).

Importantly, contrasting studies have shown that immature IgM$^+$/IgD$^-$ B cells have heightened sensitivity to antigen-induced apoptosis (Nossal, 1994; Norvell et al., 1995; Klinman, 1996; Monroe, 1996), a finding that is difficult to reconcile both with the ability of autoantigen to induce receptor editing and with the inability of Bcl-2 overexpression to block immune tolerance at this stage of development in BCR Tg mouse models (Hartley et al., 1993; Lang et al., 1997). To resolve the apparent contradiction, we hypothesized that the mechanism of central tolerance in B cells might be under developmental regulation. In the present study, we have tested the notion that newly generated IgM$^+$/IgD$^-$ B cells include two functionally distinct subpopulations that are divisible based on BCR expression level into populations that respond to antigen stimulation by undergoing receptor editing or accelerated apoptosis. To perform this analysis, we have relied on an IL-7-driven BM culture system that was well described previously (Cumano et al., 1990; Rolink et al., 1991; Billips et al., 1992) and further modified by us (Melamed and Nemazee, 1997; Melamed et al., 1997). In these cultures, B-lineage cells grow robustly and can undergo some differentiation, but V(D)J recombination is suppressed (Melamed et al., 1997) probably because of the nature of the cell cycle regulation of the *RAG* gene products (Li et al., 1996). Subsequent removal of IL-7 from these cultures promotes exit from cell cycle, sIgM expression, differentiation (which is correlated to the level of BCR expression [Hardy et al., 1991]) and acquisition of maturation markers such as IgD, CD23, CD21, and L-selectin (Hartley et al., 1993; Melamed et al., 1997). Although the cells' survival in the absence of IL-7 is limited, under normal circumstances newly generated IgM$^+$ cells can be followed for 2–3 days (Rolink et al., 1991, 1993; Melamed et al., 1997), permitting analysis of central B cell tolerance in vitro. In these cultures, B-lineage cells from BM of 3–83 $\mu\delta$ immunoglobulin H + L Tg mice grow well, and their differentiation and response to antigen-specific stimulation can be controlled (Melamed and Nemazee, 1997; Melamed et al., 1997). Using this system

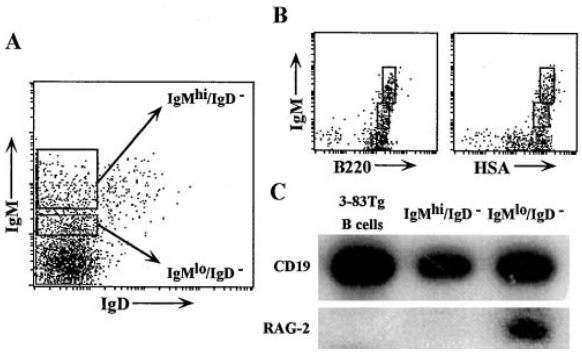

Figure 1. Expression of *RAG2* Gene in IgM^{lo}/IgD$^-$ but Not in IgM^{hi}/IgD$^-$ BM B Cells

(A) Normal, non-Tg B cell precursors were grown for 5 days in IL-7 culture, stained for surface expression of IgM and IgD, and sorted as shown. Sort purity was >95% (not shown).

(B) B220 and HSA levels increase with sIgM density in IgM$^+$IgD$^-$ B cells as determined by flow cytometry.

(C) RT–PCR analysis of mRNA expression of *RAG2* in IgM^{lo}/IgD$^-$ and IgM^{hi}/IgD$^-$ B cells. CD19 signal served as internal control. Data shown is representative of four different experiments.

to analyze antigen-specific tolerance mechanisms, we have been able to define two distinct functional responses to autoantigen challenge among the IgM$^+$/IgD$^-$ B cells: those that expressed low levels of BCR responded with robust receptor editing but little or no apoptosis, while those that expressed high levels of BCR underwent rapid antigen-induced apoptosis.

Results

Expression of *RAG2* Gene in IgM^{lo}/IgD$^-$, but Not in IgM^{hi}/IgD$^-$ B Cells

To better define the developmental stages at which cells can undergo receptor editing, we studied B cell development in IL-7-containing BM cultures of normal, non-Tg mice. In non-Tg BM cultures most cells lacked sIgM, but some B cells matured to the IgM$^+$/D$^-$ and IgM$^+$/D$^+$ stages (Figure 1A). Among the IgM$^+$/D$^-$ cells there was a broad distribution of IgM levels that was correlated with the expression of a number of other important surface markers including CD45R(B220) and CD24 (heat stable antigen [HSA]) (Figure 1B). These characteristics were consistent with those of ex vivo cells (Hardy et al., 1991). To study whether *RAG2* expression, which is required for V(D)J recombination, is correlated to the mIgM levels, IgM^{hi}/IgD$^-$ and IgM^{lo}/IgD$^-$ B cell precursors were sorted as shown in Figure 1A, and *RAG2* mRNA levels were determined by RT–PCR (Figure 1C). As shown in Figure 1C, *RAG2* mRNA was detectable in IgM^{lo}/IgD$^-$ cells but not in IgM^{hi}/IgD$^-$ cells, consistent with the prediction that high-level, cell-surface IgM expression is a either a marker of or a signal for developmental progression and recombinase down-regulation (Hardy et al., 1991; Storb et al., 1994; Boekel et al., 1995).

These results suggested to us that the ability to undergo receptor editing in response to autoantigen stimulation might be lost upon high-level expression of sIgM; however, the correlation between sIgM density and recombinase expression was difficult to interpret because

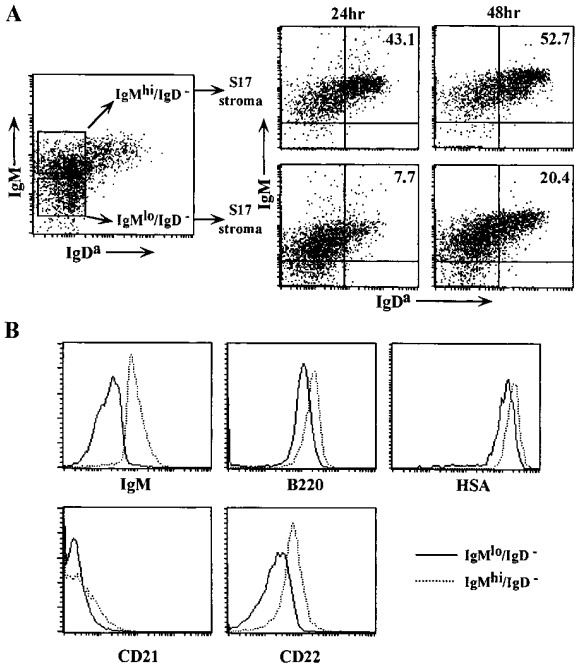

Figure 2. Developmental Progression of 3–83 Tg IgM$^+$IgD$^-$ B Cells upon Reculture on S17 Stromal Cells in the Absence of IL-7

(A) 3–83 Tg BM cells were grown in IL-7, stained for surface expression of IgM and IgD^a, and sorted as shown (left). Sorted cells were cultured on S17 stroma in complete medium lacking IL-7 for 24–48 hr, collected, and restained for sIgM and sIgD.

(B) Flow cytometry analysis of cell surface marker expression on sorted IgM^{lo}/IgD$^-$ and IgM^{hi}/IgD$^-$ populations.

B cells in these normal, non-Tg cultures must rearrange immunoglobulin genes to generate receptors and their receptor specificities are unknown and heterogeneous, generating an unknown frequency of autoreactive cells that might interact with ligands present in the cultures. Differences in *RAG* mRNA levels in IgM^{lo}/IgD$^-$ and IgM^{hi}/IgD$^-$ cell populations could also reflect the levels of sort contamination by preB cells or the specific down-regulation of sIgM in autoreactive B cells undergoing receptor editing. To simplify the analysis, subsequent experiments used BM from BCR Tg mice. The advantage of this approach is that all cells have preformed genes encoding a receptor of known specificity that suppresses endogenous rearrangements in the absence of antigens.

3–83 Tg IgM^{lo}/IgD$^-$ B Cells Develop In Vitro in the Absence of Antigen and Acquire Maturation Markers

To determine if IgM levels distinguish B cell populations that could respond to self-antigen by receptor editing or apoptosis, we chose to analyze cultures derived from BM of 3–83 immunoglobulin Tg mice, which have a BCR of known specificity that reacts with certain alleles of mouse class I major histocompatibility complex (MHC) antigens (Russell et al., 1991). Because of the effect of the immunoglobulin transgenes (Spanopoulou et al., 1994; Melamed et al., 1997), few sIgM$^-$ cells were present in these cultures but, like cultures derived from non-Tg BM, they contained IgM$^+$/D$^+$ cells and were enriched in IgM$^+$/IgD$^-$ cells (Figure 2A, left panel). Cells bearing the IgM^{lo}/IgD$^-$ and IgM^{hi}/IgD$^-$ phenotypes were sorted as shown and recultured on S17 stromal cells in the absence of IL-7 to promote differentiation. Cells were then stained with fluorescent-labeled monoclonal antibodies (MAb) and analyzed by flow cytometry for the acquisition of maturation markers. These experiments demonstrated the predicted precursor/product relationship among these populations (Figure 2A, right four panels): IgM^{hi}/IgD$^-$ cells rapidly expressed IgD (Figure 2A, top right panels), while IgM^{lo}/IgD$^-$ cells first increased their IgM levels and then acquired IgD (Figure 2A, lower right panels). For many cells, at least two days were required for full differentiation to the IgM$^+$/IgD$^+$ phenotype. Consistent with their earlier stage of maturity, IgM^{lo}/IgD$^-$ expressed lower levels of B220, HSA, and CD22 relative to the IgM^{hi}/IgD$^-$ cells (Figure 2B). During culture, acquisition of IgD was accompanied by increasing levels of these markers as well as by the appearance of the additional maturation markers CD21 and CD23 (data not shown). However, in the presence of antigen (op42 cells), maturation, as defined by the appearance of these markers, was inhibited, consistent with previous reports (Hartley et al., 1991; Melamed and Nemazee, 1997). Subsequent experiments probed the functional differences in immune tolerance responses between IgM^{hi}/IgD$^-$ and IgM^{lo}/IgD$^-$ subpopulations.

Self-Antigen Induces Receptor Editing in 3–83 Tg IgM^{lo}/IgD$^-$ and Rapid Apoptosis in IgM^{hi}/IgD$^-$ B Cells

We next assessed the ability of antigen binding to the 3–83 Tg BCR to stimulate markers of receptor editing in the IL-7 BM culture-derived IgM^{hi}/IgD$^-$ and IgM^{lo}/IgD$^-$ cells. Upon exposure to the stromal cell line op42, which expresses on its surface MHC antigens to which the 3–83 BCR reacts, sorted IgM^{lo}/IgD$^-$ cells responded by rapid and substantial increases in *RAG2* mRNA levels (Figure 3A, left). *RAG2* levels increased 7-fold after 24 hr and peaked at 14-fold after 48 hr. Antigen stimulation of the IgM^{lo}/IgD$^-$ cells also induced new Vκ-to-Jκ1 L chain gene rearrangements, which became evident by 48 hr (Figure 3A, right), demonstrating that the *RAG* gene up-regulation was functionally relevant. In contrast, IgM^{hi}/IgD$^-$ cells failed to significantly up-regulate these markers of receptor editing in the first 24 hr (Figure 3A, left and right). As less than 10% of the IgM^{hi}/IgD$^-$ cells cultured on op42 were recovered after 48 hr (see below), receptor editing markers were not tested in these cells at this time point. The finding that sorted IgM^{lo}/IgD$^-$ cells incubated on S17 had low recombinase activity indicated that the antibodies used to stain cells for sorting were insufficient to stimulate significant receptor editing. Overall, these data suggested that receptor editing was antigen inducible in IgM^{lo}/IgD$^-$ cells, but not in IgM^{hi}/IgD$^-$ cells.

We next assessed the effect of antigen on the survival and apoptosis of the IgM^{hi}/IgD$^-$ and IgM^{lo}/IgD$^-$ cells. These sorted IgM$^+$/IgD$^-$ populations survived well when recultured for 24 hr on S17 stromal cells, which lack cognate antigens reactive with the 3–83 BCR (Figure 3B, left panels). In contrast, 42.5% of the IgM^{hi}/IgD$^-$ cells

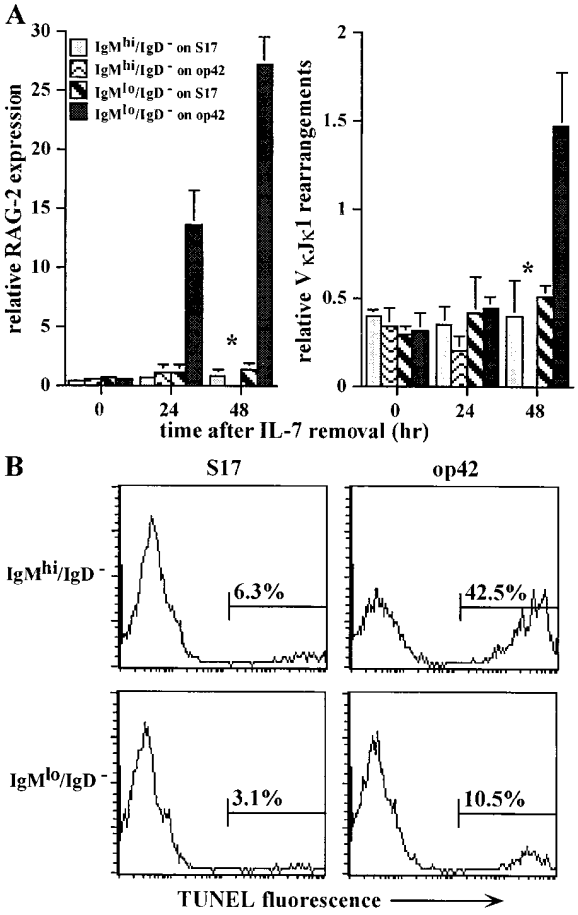

Figure 3. Self-Antigen Stimulation of 3–83 Tg B Cell Cultures Induces Secondary Rearrangements in IgM^{lo}/IgD^{-} Cells and Apoptosis in IgM^{hi}/IgD^{-} Cells

IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} cells were sorted from 3–83 Tg BM cultures as in Figure 2 and recultured with S17 or op42 stroma in the absence of IL-7.

(A) Left panel, RT–PCR analysis of mRNA expression of *RAG2* normalized to that of CD19. Results are shown as mean ± SEM of three separate experiments. Right panel, DNA PCR analysis of Vκ-to-Jκ1 rearrangements in cell lysates normalized to that of the 3–83 Tg H chain gene, Vgam 3.8-JH2. Results are shown as mean ± SEM of three separate experiments. Asterisk, values were not determined because of excessive cell death in these samples.

(B) 3–83 Tg IgM^{hi}/IgD^{-} (upper panels) and IgM^{lo}/IgD^{-} (lower panels) B cells were sorted as in Figure 2 and recultured for 24 hr in the absence of IL-7 on irradiated S17 (left panels) or op42 (right panels) stroma. The presence of apoptotic cells was quantified by a TUNEL assay. In control cultures in which apoptosis was induced with 5 μM Beauvericin, 80%–90% of the cells were apoptotic.

exposed to antigen-bearing op42 cells were apoptotic at 24 hr as determined by TUNEL assay (Figure 3B, top right). At 48 hr, fewer than 10% of the IgM^{hi}/IgD^{-} cells cultured on op42 were recovered as viable cells compared to 60%–70% recovery in the other cultures, indicating that most of the antigen-treated IgM^{hi}/IgD^{-} cells underwent antigen-induced programmed cell death by 48 hr (data not shown). The observed lack of apoptosis of the IgM^{hi}/IgD^{-} cells in the S17 cultures, combined with their death in op42-containing cultures, again suggested that the sorting antibodies had little functional effect on apoptosis in the subsequent cultures; cells sorted using

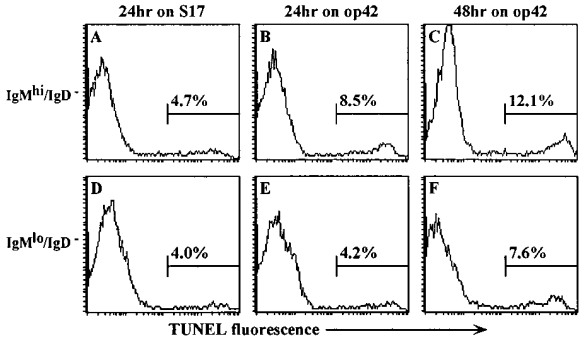

Figure 4. Enforced Expression of Bcl-2 Protects IgM^{hi}/IgD^{-} B Cells from Antigen-Induced Apoptosis

3–83/Bcl-2 double Tg IgM^{hi}/IgD^{-} (A–C) and IgM^{lo}/IgD^{-} (D–F) B cells were isolated as in Figure 2 and cultured for 24–48 hr on irradiated layers of S17 stroma (A and D) or op42 stroma (B, C, E, and F). The presence of apoptotic cells was determined by a TUNEL assay, and data shown is representative of three different experiments. In control cultures treated with 5 μM Beauvericin (Sigma), 80%–90% of the cells were apoptotic.

Fab or IgG anti-IgM behaved similarly in this assay (not shown). In contrast to the IgM^{hi}/IgD^{-} cells, IgM^{lo}/IgD^{-} cells were resistant to antigen-induced apoptosis (Figure 3B, lower right panel). These data demonstrated that sIgM density was inversely correlated with the ability to undergo antigen-induced receptor editing and directly correlated with the ability to undergo apoptosis: IgM^{hi}/IgD^{-} cells were receptor editing-incompetent and apoptosis-sensitive, whereas IgM^{lo}/IgD^{-} cells were receptor editing competent and apoptosis resistant. As sIgM expression is also correlated to developmental progression, these results suggested that as the B cells advanced in development their competence to undergo tolerance-induced receptor editing decreased, whereas sensitivity to antigen-mediated death signals increased.

IgM^{hi}/IgD^{-} B Cells Protected from Antigen-Induced Apoptosis by Enforced Bcl-2 Expression Do Not Undergo Receptor Editing

To determine if the inability of IL-7 BM culture-derived IgM^{hi}/IgD^{-} cells to undergo receptor editing upon antigen stimulation was simply the result of their premature death, apoptosis in these cells was suppressed through the introduction of a constitutively expressed Bcl-2 transgene. That this experimental maneuver was successful is illustrated in Figure 4, which shows that IgM^{hi}/IgD^{-} cells from 3–83/Bcl-2 double Tg BM cultures were apoptosis resistant upon prolonged exposure to antigen (compare Figures 4B and 4C to Figure 3B, top right panel). Control cultures of IgM^{hi}/IgD^{-} cells cultured without antigen on S17 cells (Figure 4A) had low levels of apoptosis, as expected, and inclusion of the apoptosis-inducing drug Beauvericin resulted in >80% apoptosis (data not shown), indicating the efficiency of the assay. Using cells from these 3–83/Bcl-2 double Tg cultures, it was also found that op42-exposed IgM^{lo}/IgD^{-} cells (Figures 4E and 4F) were apoptosis resistant, as was expected from the results with cultures from 3–83 cells lacking the Bcl-2 transgene.

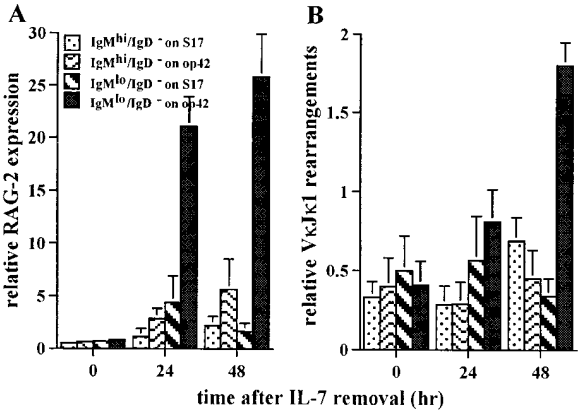

Figure 5. IgM^{hi}/IgD^{-} B Cells from 3–83/Bcl-2 Double Tg BM Cultures Fail to Undergo Receptor Editing in the Presence of Self-Antigen

IgM^{hi}/IgD^{-} and IgM^{lo}/IgD^{-} 3–83/Bcl-2 double Tg B cells were sorted from IL-7 BM cultures and recultured without IL-7 on S17 or op42 stroma.

(A) RT–PCR analysis of mRNA expression of *RAG2* normalized to that of CD19. Results are shown as mean ± SEM of three separate experiments.

(B) DNA PCR analysis of Vκ-Jκ1 rearrangements in cell lysates normalized to that of the 3–83 H chain transgene Vgam3.8-JH2. Results are shown as mean ± SEM of three separate experiments.

The ability of the Bcl-2 transgene to rescue IgM^{hi}/IgD^{-} cells from op42-induced apoptosis allowed us to determine if this antigenic stimulus resulted in a receptor editing response. Figure 5 shows that, despite their resistance to apoptosis, 3–83/Bcl-2 double Tg IgM^{hi}/IgD^{-} cells failed to significantly up-regulate either *RAG2* expression (Figure 5A) or L chain gene recombination in response to antigenic stimulation (Figure 5B). In contrast, 3–83/Bcl-2 double Tg IgM^{lo}/IgD^{-} cells expressed a significant 12- to 15-fold increase in *RAG2* mRNA and up to a 3-fold increase in Vκ-Jκ1 DNA rearrangements when cultured on op42 for 24–48 hr (Figures 5A and 5B, respectively). These results suggested that the apoptosis sensitivity of IgM^{hi}/IgD^{-} cells was independent of their loss of competence to undergo receptor editing and that both of these functional responses were correlated with developmental progression.

The Differential Functional Responses of IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} 3–83 Tg B Cells to BCR Cross-linking Do Not Result from Differences in BCR-Mediated Ca^{2+} Mobilization

We next studied the possibility that the differential responses of IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} cells to antigen stimuli resulted from distinct Ca^{2+} mobilization responses as a consequence of quantitative differences in BCR expression rather than differentiation status. In order to identify these cell populations without perturbing sIgM, we took advantage of the fact that in these cells CD45R (B220) expression level is correlated to sIgM density (Figure 6A). We therefore used B220 levels to gate IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} cells responding to different stimuli through the BCR. IL-7 BM culture-derived 3–83 Tg B cells were stained for IgD and B220, loaded with Indo-1AM, and stimulated with anti-IgM MAb, 3–83 cognate

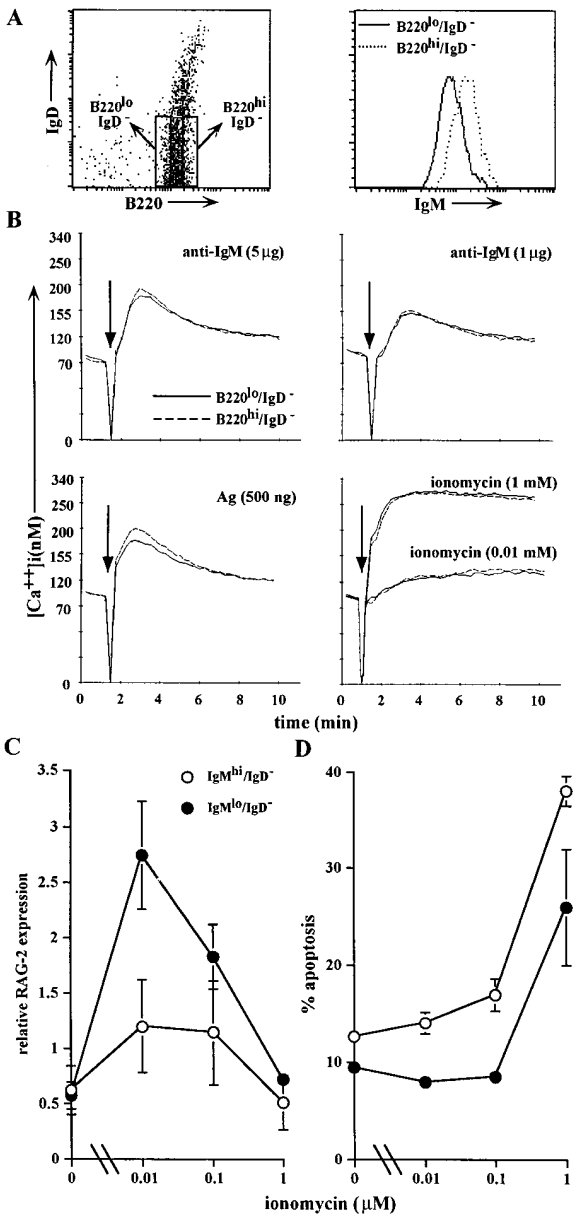

Figure 6. Induction and Functional Consequences of Ca^{2+} Mobilization in IgM^{hi}/IgD^{-} and IgM^{lo}/IgD^{-} B Cells

(A) Sorting of B220^{lo}/IgD^{-} and B220^{hi}/IgD^{-} subsets. Left panel, sorting gates; right panel, sIgM expression levels in the sorted populations.

(B) IgM^{hi}/IgD^{-} and IgM^{lo}/IgD^{-} cells have similar Ca^{2+} responses to BCR stimuli. 3–83 Tg B cells generated in IL-7 cultures were stimulated with either 500 ng/ml of antigen coupled to dextran, anti-IgM MAb (b-7–6) at 1 or 5 μg/ml, or with ionomycin, and mean intracellular free calcium concentration was evaluated in gated B220^{lo}/IgD^{-} and B220^{hi}/IgD^{-} cells.

(C and D) Receptor editing and apoptosis in ionomycin-stimulated IgM^{hi}/IgD^{-} and IgM^{lo}/IgD^{-} cells. IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} cells were sorted from 3–83 Tg BM cultures as in Figure 2 and recultured on S17 stroma with 0.01–1 μM of ionomycin. Cells were collected after 16 hr and *RAG2* expression (C) and apoptosis (D) were determined.

antigen coupled to dextran, or ionomycin. Figure 6B shows that B220^{lo}/IgD^{-} and B220^{hi}/IgD^{-} cells underwent similar Ca^{2+} mobilization responses to the different stimuli tested in this experiment. Therefore, with respect to

their Ca^{2+} response, which represents an early event in BCR signaling, the cell populations appeared to be identical despite expression of different amounts of receptor.

Ca^{2+} Mobilization Induces, Respectively, *RAG2* Expression and Apoptosis in IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} B Cell Populations

To determine if the differential responses of IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} B cells were caused by differences in maturational status or BCR level, we tested whether or not these cell populations responded differently to an intracellular signaling stimulus that should bypass the BCR and trigger an equivalent Ca^{2+} response. We cultured sorted IgM^{lo}/IgD^{-} and IgM^{hi}/IgD^{-} 3–83 Tg B cells for 16 hr on S17 stroma in the presence of 0.01–1 μM ionomycin and then assayed for *RAG2* mRNA up-regulation as a marker for receptor editing (Figure 6C) and apoptosis (Figure 6D). Low doses of ionomycin stimulation (0.01–0.1 μM), which stimulated a low-level Ca^{2+} response (Figure 6B), efficiently induced 3- to 4-fold increases in *RAG2* gene expression in IgM^{lo}/IgD^{-} cells in the absence of cell death (Figures 6C and 6D). Similarly treated IgM^{hi}/IgD^{-} cells did not express significant levels of *RAG2* mRNA (Figure 6C), despite their similar Ca^{2+} influx response, and were more sensitive to apoptosis at the same ionomycin concentrations (Figure 6D). At high doses of ionomycin, *RAG2* expression was diminished in IgM^{lo}/IgD^{-} cells and increased apoptosis was monitored in both cell populations, consistent with the possibility that high concentrations of ionomycin are toxic. It is also possible that high doses of ionomycin might stimulate cell cycle events, which are known to down-regulate *RAG* expression (Li et al., 1996). Thus, upon BCR-independent Ca^{2+} mobilization, IgM^{lo}/IgD^{-} B cells were more competent to undergo receptor editing than IgM^{hi}/IgD^{-} cells, suggesting that the differential responses of the IgM^{lo} and IgM^{hi} populations were the result of developmental differences rather than distinct signaling responses of cells at a single stage of differentiation. Finally, these results demonstrated that Ca^{2+} mobilization following BCR cross-linking is likely responsible for both receptor editing and apoptotic responses.

Discussion

Receptor Editing and Apoptosis Sensitivity Are Developmentally Regulated

This paper attempts to integrate two opposing lines of evidence about the mechanisms of central B cell tolerance: one indicating that receptor editing and developmental arrest are the dominant responses (Gay et al., 1993; Tiegs et al., 1993; Radic and Zouali, 1996; Hertz and Nemazee, 1997; Melamed and Nemazee, 1997), the other indicating that apoptosis can be a major mechanism (Nossal, 1994; Goodnow, 1996; Klinman, 1996; Monroe, 1996). The results presented reconcile the disparate findings of the prior studies and demonstrate the existence of two sequential differentiation stages among IgM^{+}/IgD^{-} cells. We show here that upon antigen binding the newly generated IgM^{lo}/IgD^{-} cells undergo receptor editing, whereas the more developed IgM^{hi}/IgD^{-} cells die rapidly. We therefore suggest that receptor editing in B cells is developmentally regulated and that B cells advancing in their development lose the ability to undergo receptor editing and concomitantly acquire sensitivity to antigen-mediated apoptosis.

Our interpretation of the data is illustrated in the model presented in Figure 7A. A newly emerging IgM^{lo}/IgD^{-} cell that is generated from a preB cell retains the signaling properties of a preB cell, including competence to express recombinase and to rearrange L chain genes. When such a cell encounters autoantigen, survival is not immediately impaired (this study; Hartley et al., 1993; Hertz and Nemazee, 1997; Melamed and Nemazee, 1997), but V_LJ_L recombination persists as a result of developmental arrest, resulting in receptor editing. If the IgM receptors expressed by a receptor editing–competent B cell fail to bind antigens, the cell eventually undergoes a critical decision committing it to its receptor. This commitment is comprised of further differentiation to receptor editing incompetence and apoptosis hypersensitivity. This now-committed B cell should generally have higher sIgM density than an immature B cell and, if it subsequently encounters autoantigen, will also lack the ability to undergo receptor editing, but instead should rapidly undergo apoptosis (Figure 3B). These cells probably correspond to the "transitional" B cells described by several investigators, which die rapidly when acutely treated with antigens or anti-BCR antibodies (Brines and Klaus, 1992; Carsetti et al., 1995; Norvell et al., 1995; Monroe, 1996). Assuming that this scenario is correct, it implies that most B cell tolerance naturally occurring in the BM (central tolerance) should be through the mechanism of receptor editing, because cells at this site are unlikely to mature to the "committed B cell" stage in the presence of antigens (Hartley et al., 1993; Tiegs et al., 1993; Melamed and Nemazee, 1997).

Consistent with this prediction, receptor editing is extensive in mice with germline-targeted anti-DNA or anti-MHC class I autoantigen genes in the natural genomic context, and, as a consequence, peripheral B cell numbers are not drastically reduced (Chen et al., 1997; Pelanda et al., 1997). However, because of the error-prone nature of V(D)J recombination, cells undergoing receptor editing are not always able to correct their autoreactive antigen receptors to relieve their arrested development and eventually die by default, probably at a rate characteristic of preB cells that fail to generate functional L chains (Lu and Osmond, 1997). Cells at the IgM^{+}/IgD^{-} stage have little or no expression of the important death inhibitory proteins Bcl-2 and Bcl-x (Li et al., 1993; Choi et al., 1996), which are highly expressed in other B cell developmental stages and are required for normal lymphoid development and maintenance (Nakayama et al., 1993; Veis et al., 1993; Rajewsky, 1996). On the other hand, committed B cells should be permitted to exit to the periphery where they would undergo an apoptotic or anergy response when they encounter autoantigens not present in the BM (Russell et al., 1991; Goodnow, 1996; Lang et al., 1997).

An alternative interpretation of the differential responses of immature and committed B cells to self-antigen is that they represent distinct signaling responses of a single cell type that are regulated by the quantitative interactions between antigens and the BCR

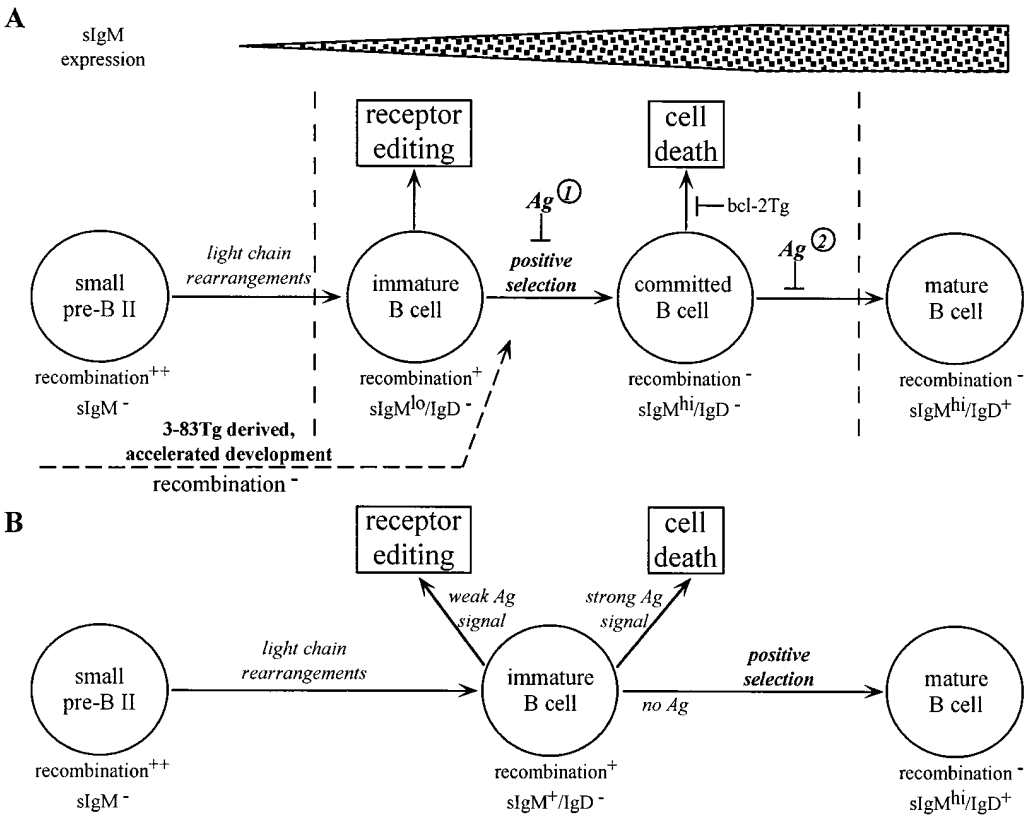

Figure 7. Alternative Models for the Regulation of Central Tolerance

(A) Developmental model postulates that IgM^+IgD^- cells comprise two developmentally distinct compartments that differ in their response to autoantigen by undergoing either receptor editing (pathway "1") or cell death (pathway "2"). The latter response is blocked by enforced Bcl-2 expression. It is further postulated that cells undergoing receptor editing fail to progress in development unless they alter their receptors such that they lose autoreactivity and that cells failing to do so eventually die by default. In this model, the type of tolerance response is regulated by the developmental stage at which the cell encounters antigen.

(B) Signaling model postulates that the specific interaction between a particular antigen and the levels of signaling molecules, including IgM and other surface molecules, regulates cell death and receptor editing among IgM^+IgD^- cells. This model predicts that a single cell type is capable of either antigen-induced receptor editing or apoptosis depending on the nature of the antigen stimulus. In this model, the type of tolerance response is regulated by the nature of the signal elicited by antigen.

(Figure 7B). According to this idea, the nature of the antigen, sIgM levels, and perhaps also the levels of associated signaling molecules such as CD45R could regulate the BCR signal, which in turn would affect whether the cell undergoes receptor editing or apoptosis in response to antigens. The data presented here do not favor this possibility by showing that both cell types have similar Ca^{2+} responses to BCR ligation despite their differing biological responses. Furthermore, a BCR-independent, Ca^{2+} mobilization signal (mimicked by ionomycin treatment) stimulated significant levels of *RAG2* mRNA without cell death in IgM^{lo} cells, but not in IgM^{hi} cells (Figures 6C and 6D). Also arguing against a simple role for signaling intensity on the commitment to receptor editing or apoptosis is our finding that a wide range of antigenic stimuli can stimulate receptor editing in immature B cells. For example, different naturally occurring MHC class I antigens that bind to the 3–83 receptor with a wide range of affinities can all stimulate receptor editing in vivo (Lang et al., 1996). In addition, titration experiments using a wide concentration range of anti-BCR antibodies have failed to define a threshold above which receptor editing fails to occur or at which apoptosis becomes significant (Hertz and Nemazee, 1997). Taken together, we conclude that the distinction between the receptor editing or apoptosis responses in IgM^+/IgD^- B cells is not based on distinct signaling responses of a single cell type, but rather represent a developmentally regulated change in biologic response.

Our conclusions were reached by taking advantage of an immunoglobulin Tg mouse strain that allows control of BCR specificity and an IL-7 BM culture system that facilitates the isolation and synchronization of B cell differentiation. As these experimental maneuvers perturb B cell development in several ways and only allow us to follow B cell fate for a limited period of time, it will be important to confirm these results in a more physiological system. The use of independent, antigen-specific BCR Tg mice may help in testing whether these results are generalizable. While the use of non-Tg immature B cells to study these issues would be ideal, such an approach is confounded by two problems. The heterogeneity of their antigen receptors generates an unknown background frequency and extent of BCR stimulation, and specific stimulation of their antigen receptors requires the use of anti-BCR antibodies that may poorly

mimic natural antigens. Our studies also made use of BCR-reactive antigen that was expressed at a significant epitope density on the surface of a stromal cell line. It remains to be seen if soluble antigen or a lower density of membrane antigen could drive similar functional responses, although studies using BCR antibodies as surrogate antigens suggest that bivalent ligands of high affinity are sufficient for both receptor editing and apoptotic responses.

The BCR Signals Both Positive and Negative Selection

As BCR expression levels increase during B cell development (Hardy et al., 1991), we used this marker in the present study to distinguish immature from committed B cells. While we have identified a correlation between sIgM expression and B cell commitment to its receptor, it is unclear if sIgM density per se constitutes the signal for B cell commitment, or if it is merely a convenient marker. Clearly, some level of sIgM and its consequent signaling is required for completion of the later stages of B cell development, as is indicated by a variety of mutational studies (reviewed in Rajewsky, 1996), suggesting that in the absence of receptor cross-linking the basal signaling of a high level of assembled IgM receptor complex could be sufficient to drive developmental progression (see also Shaffer and Schlissel, 1997). But, in a diverse repertoire, it is also potentially possible that for some receptors weak BCR:ligand interactions can drive development at low surface IgM densities. In the case of the sorted 3–83 Tg B cells studied here, all cells had identical antigen specificity and only sIgM levels differed, albeit by only ~10-fold. The quantitative differences in IgM levels and biological responses among these genetically identical cells is probably attributable to their asynchronous proliferative arrest and subsequent developmental progression following IL-7 withdrawal. The kinetics of development of these two populations suggested that the IgM^{lo}/IgD^{-} cells required more time to acquire sIgD than did the IgM^{hi}/IgD^{-} cells. Thus, it is also possible that the duration of a basal, unligated sIgM signal could direct developmental progression.

In this study, we have been able to show that cycling, IL-7-responsive BCR Tg cells expressing low levels of recombinase mRNA could be induced to increase dramatically these levels upon the concerted stimulation by specific antigen and IL-7 withdrawal. In normal B cell development, nondividing, recombinationally active preB cells give rise to $sIgM^{+}$ cells (Hardy et al., 1991; Boekel et al., 1995). It is therefore likely that self-antigen-induced receptor editing in normal developing B cells occurs through the maintenance of recombinase rather than its reexpression. This notion implies that the preB and immature IgM^{+} cells constitute a single stage of differentiation with virtually identical signaling machinery and transcriptional control. We are aware of no surface markers other than sIgM itself that distinguish these cells. Generation of an appropriate antigen receptor through successful VJ recombination and expression of L chains that complete a nonautoreactive BCR is an important signal that permits commitment to maturation (Melchers et al., 1995; Rajewsky, 1996). On the other hand, cells with self-reactive BCRs will fail to receive positive signals required for maturation and, like preB cells that fail to generate a productive L chain, will continue to rearrange L chain genes. The present study suggests that this developmental commitment step can be functionally defined by heightened apoptosis sensitivity and by the loss of competence to undergo receptor editing. These differences in the biological response to antigen stimulation suggest that as B cells advance in development the mechanism of immune tolerance progresses from receptor selection to clonal selection.

Experimental Procedures

Mice

Mice used for the experiments were strain B10.D2nSn/J that expressed the 3–83 transgene, which encodes a BCR reactive to the mouse class I MHC antigens K^{k} and K^{b} (Russell et al., 1991), or their non-Tg littermates. In some experiments, mice Tg for both the 3–83 genes and the Eμ2–22 Bcl-2 transgene (Lang et al., 1997; Strasser et al., 1991) were used. Mice were housed and bred at the National Jewish Center facility and used at 4–8 weeks of age.

Cell Culture

B cell precursors from Tg and non-Tg mice were grown in vitro as described (Melamed and Nemazee, 1997; Melamed et al., 1997). Briefly, BM cells were depleted of erythrocytes and were cultured in IMDM medium (GIBCO-BRL) supplemented with 10% fetal calf serum (Gemini Bio-Products, Calabasas, CA), 5×10^{-5} M 2-mercaptoethanol, and 50–100 U/ml of rIL-7 (Winkler et al., 1995) at a concentration of 2×10^{6} cells/ml for 5–6 days. These culture conditions allow preferential growth of IL-7-responsive B cell precursors, and after 5–6 days the culture contains >98% of $B220^{+}$ cells (Melamed et al., 1997). Cultured cells were stained and sorted by FACS into two populations (see Results). Sorted populations were then cultured for 16–48 hr in the absence of IL-7 (conditions that promote differentiation [Hardy et al., 1991; Rolink et al., 1991, 1993; Melamed et al., 1997]) on plates with adherent 2000 rad treated antigen-negative stromal cell line S17 (Cumano et al., 1990) or antigen-bearing stromal cell line op42 (Smithson et al., 1995). Stromal cell lines were tested for the expression of the (3–83 BCR-reactive) mouse class I MHC antigens K^{k} and K^{b} by staining with the high-affinity antibody Y3 (Lang et al., 1996). The relevant antigen on these cells is the mouse MHC class I alloform H-$2K^{k}$. In some experiments, sorted cells were cultured on S17 stroma and stimulated for 16 hr with 0.01–1 μM ionomycin.

Flow Cytometry and Sorting

Antibodies used for cell staining were: goat anti-mouse IgM-FITC (Zymed, San Francisco, CA), anti-mouse IgD^{a} AMS 9.1-FITC or -biotin (Pharmingen), anti-mouse IgD JA12.5-biotin, anti-mouse CD21 7E9-biotin (Kinoshita et al., 1988), anti-mouse CD22 CY34.1.2-biotin (American Type Tissue Culture Collection, Rockville, MD), anti-mouse B220 RA3–6B2-phycoerythrin (PE) (Pharmingen), goat anti-mouse IgM-PE (Caltag), and anti-mouse HSA (CD24) M1/69 PE (Pharmingen). Biotinylated antibodies were visualized with Streptavidin PE (Becton Dickinson) for two-color analysis or with Streptavidin-Tri Color (Caltag) for three-color analysis. FACS analysis was performed on FACScan (Becton Dickinson) with forward and side-scatter gates adjusted to include only nucleated viable cells. For cell sorting, Tg and non-Tg B cell precursors were collected after 5–6 days of culture and stained for expression of sIgD and sIgM (or, for the data presented in Figure 6, B220). Based on these two markers, IgM^{hi}/IgD^{-} and IgM^{lo}/IgD^{-} cell populations were sorted using an Elite flow cytometer (Coulter). Sorted cells were washed and then cultured as described in Results.

Apoptosis Assay

Antigen-mediated death of B cell precursors in the cultures was determined by the terminal deoxynucleotidyltransferase-mediated

uridine triophosphate end labeling (TUNEL) assay (Boehringer Mannheim) as described (Melamed and Nemazee, 1997). As a positive control for the activity of the assay, in each experiment some samples were treated with the apoptosis-inducing drug Beauvericin (cyclo-[D-α-Hydroxyisovaleryl-L-methylphenylalanyl]$_3$) (Ojcius et al., 1991) (Sigma) at a final concentration of 5 μM.

DNA and RNA Analysis

DNA templates for PCR were prepared in cell lysates as described (Melamed and Nemazee, 1997). cDNA templates were generated from total RNA purified using the RNAzol method (Tel-Test, Friendswood, TX) as described (Melamed and Nemazee, 1997).

Quantitation of endogenous Vκ-Jκ1 rearrangements and of the 3–83 H chain transgene (Vgam3.8-JH2) in DNA samples was determined by a PCR assay. Levels of *RAG2* and CD19 mRNA were determined by a reverse transcriptase (RT)–PCR assay. The PCR conditions and primer sequences were as described (Melamed and Nemazee, 1997). PCR products were fractionated on 1% agarose gels, transferred to nylon membranes, and hybridized with specific *RAG2*, CD19, Jκ, or JH specific probes. Blots were scanned in a Phosphor-Imager (Molecular Dynamics) to quantitate signal intensity. The results are expressed as a semiquantitative estimate where signal intensity of *RAG2* product was normalized to that of CD19 product signal and that of the Vκ-Jκ1 PCR was normalized to the signal intensity of the Vgam3.8-JH2 (the Tg H chain) product.

Analysis of Calcium Mobilization

IL-7 BM culture-derived 3–83 Tg B cells were surface stained for B220 (CD45R) and IgDa and subsequently loaded with Indo-1AM (Molecular Probes) for 45 min at 37°C. Cells were washed twice and resuspended at a concentration of 10^6 cells/ml in IMDM and 5% FCS and stimulated with either 500 ng/ml 3–83 cognate antigen (an antigen mimetic peptide [SGFGGFQHLCCGAAGA] multimerized on ν-ethylmaliamide-activated dextran in a 64:1 peptide:dextran molar ratio [Vilan et al., 1997]), 1 μM ionomycin, or anti-BCR (b-7–6) antigen (1–5 μg/ml). Mean intracellular free calcium concentration was evaluated in CD45R^{lo}/IgD$^-$ and CD45R^{hi}/IgD$^-$ cells using a flow cytometer (ELITE, Coulter) with appended data acquisition system and MultiTime software (Phoenix Flow Systems).

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