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Negative Selection of Immature B Cells by Receptor Editing or Deletion Is Determined by Site of Antigen Encounter

Peter C. Sandel and John G. Monroe*
Department of Pathology and Laboratory Medicine
University of Pennsylvania
Philadelphia, Pennsylvania 19104

Summary

Immature B cells that encounter self-antigen are eliminated from the immune repertoire by negative selection. Negative selection has been proposed to take place by two distinct mechanisms: deletion by apoptosis or alteration of the antigen receptor specificity by receptor editing. While convincing evidence exists for each, the two models are inherently contradictory. In this paper, we propose a resolution to this contradiction by demonstrating that the site of first antigen encounter dictates which mechanism of negative selection is utilized. We demonstrate that the bone marrow microenvironment provides signals that block antigen-induced deletion and promote *RAG* reinduction. In the periphery, the absence of these signals allows the immature B cell to default to apoptosis as a result of BCR engagement.

Introduction

In adult mice, B lymphocytes arise in the bone marrow and proceed through an ordered series of developmental stages during which they upregulate the recombinase genes *RAG-1* and *RAG-2* and rearrange first their heavy chain immunoglobulin and then light chain immunoglobulin gene loci. During their development, B cells are subjected to both positive and negative selection events that shape the peripheral repertoire of antigen-reactive B cells. These selection events are based on the expression and antigen specificity of the B cell antigen receptor (BCR). The complete BCR complex, consisting of both antigen recognition and signaling elements, is first expressed at the immature stage of B lymphocyte development. B cells at this stage of development are highly sensitive to negative selection (King et al., 1998). In vivo studies in normal and immunoglobulin transgenic mice have established that BCR-mediated interactions between immature B cells and endogenous antigen lead to tolerance induction (Nossal and Pike, 1975; Metcalf and Klinman, 1976; Goodnow et al., 1989; Nemazee and Burki, 1989; Erikson et al., 1991; Hartley et al., 1991; Gay et al., 1993; Tiegs et al., 1993).

Immature B cells traffic through two distinct compartments during their transition to mature, immunocompetent B cells. Arising in the bone marrow, immature B cells are phenotypically distinguished from their pre-B cell progenitors by varying levels of surface IgM expression. Functionally, highly purified populations of immature B cells are refractory to BCR-induced positive signals that characterize the response of mature B cells but rather are induced to undergo programmed cell death (Yellen-Shaw and Monroe, 1992; Norvell et al., 1995; Monroe, 1996; Norvell and Monroe, 1996). It is now appreciated that immature B cells emigrating from the bone marrow retain the sensitivity to tolerance induction and apoptotic in vitro response inherent to bone marrow immature B cells (Allman et al., 1992, 1993; Norvell and Monroe, 1996). These peripheral immature B cells are commonly designated as transitional immature B cells (Carsetti et al., 1995). Transitional immature B cells, upon emigrating from the bone marrow, home to peripheral lymphoid organs such as the spleen where they continue the sequence of maturational steps that lead to a mature-stage B cell.

Phenotypically, transitional immature B cells are distinguished from bone marrow immature B cells by increased IgD surface expression. They are distinguished from mature splenic B cells not only by their negative signaling to BCR engagement but also phenotypically by higher surface IgM and heat stable antigen (HSA) expression. The existence of this population of immature B cells in the periphery affords the opportunity for negative selection against the constellation of self-antigens expressed outside of the bone marrow.

Negative selection of autoreactive B cells is known to occur by at least three distinct mechanisms: deletion, anergy, and receptor editing. Deletion of autoreactive B cells has been demonstrated in mice expressing a transgenic immunoglobulin receptor specific for a membrane-bound antigen (Hartley et al., 1991). The deletion of isolated immature B cells can also be modeled in vitro where culture with anti-BCR antibodies results in apoptotic cell death within 16 hr (Yellen-Shaw and Monroe, 1992; Norvell et al., 1995; Norvell and Monroe, 1996). Immature B cells bearing a transgenic immunoglobulin receptor that encounter soluble antigen have been shown to enter a short-lived anergic state in which the cells are refractory to further antigen stimulation (Goodnow et al., 1988, 1989; Fulcher and Basten, 1994; Fulcher et al., 1996). Alternatively, self-reactive immature B cells can be rescued from deletion by upregulating *RAG-1* and *RAG-2* and replacement of the autoreactive BCR by secondary immunoglobulin gene rearrangement (Gay et al., 1993; Tiegs et al., 1993; Prak and Weigert, 1995; Hertz and Nemazee, 1997). In this latter case, continued reactivity to self-antigen would presumably trigger deletion or further immunoglobulin gene rearrangements until self-reactivity was eliminated. However, it should be noted that autoreactive immature B cells that undergo receptor editing rather than deletion are not assured of continued survival, as immunoglobulin gene rearrangement is an error prone process that only results in an in-frame rearrangement one third of the time. Therefore, many immature cells that attempt to undergo receptor editing will fail to produce a functional BCR and will die by neglect.

While significant evidence exists for each mechanism of negative selection, individually the models predict that the ligation of the BCR on an immature B cell leads

*To whom correspondence should be addressed (e-mail: monroe@mail.med.upenn.edu).

to vastly different cell fates: elimination by induction of apoptosis, induction of anergy, or secondary immunoglobulin rearrangement. In order to reconcile the different models of negative selection and accurately predict the fate of autoreactive B cells, we must consider a complex array of factors both intrinsic and extrinsic to the B cell that may constitute the criteria by which the mechanism of negative selection is determined. The type of signal delivered via the BCR, as determined by affinity for the ligand or type of antigen (i.e., membrane-bound versus soluble antigens) can determine whether cells are deleted or rendered anergic (Goodnow et al., 1988, 1989; Hartley et al., 1991). As immature B cells increase their surface IgM density as they mature, a second factor may be their relative position in their transition from pre-B to mature B cell when they encounter antigen. Support for this hypothesis comes from recent studies in which B cells bearing a transgenic immunoglobulin receptor sorted out of IL-7-driven bone marrow cultures on the basis of surface IgM were found to have differential responses to BCR ligation in culture such that IgM^{low} cells underwent secondary immunoglobulin rearrangement and IgM^{high} B cells were induced to die by apoptosis (Melamed et al., 1998). Finally, factors extrinsic to the immature B cells such as environmental influences must be considered.

As described above, immature B cells exist in two distinct compartments, the bone marrow and the peripheral lymphoid organs. In isolation from sources of secondary signals, both bone marrow–derived B cells as well as peripheral transitional immature B cells are induced to die following BCR engagement (Carsetti et al., 1995; Norvell et al., 1995; Norvell and Monroe, 1996). However, in vivo this intrinsic response may be influenced by B cell extrinsic influences provided by the microenvironment in which the immature B cell resides at the time of antigen encounter. While not formally demonstrated, Carsetti et al. (1995) have previously suggested that the bone marrow environment may protect immature B cells and that only transitional immature B cells in the periphery are subject to deletion.

In this paper, we present evidence that the site of first antigen encounter, rather than the developmental stage, can determine the fate of immature B cells. Using culture systems that recapitulate the bone marrow or the splenic environment, we show that immature B cells are protected against BCR-mediated apoptotic signals in the bone marrow but not in the spleen. Further, protection of immature B cells by either pharmacological inhibitors of the apoptotic pathway or bone marrow allows induction of the recombinase machinery in response to BCR cross-linking. These findings allow us to reconcile the deletional and receptor editing models of immature B cell tolerance induction and explain how both mechanisms can be utilized during B cell development in nontransgenic adult mice.

Results

Recapitulation of Bone Marrow Microenvironment

Our previous in vitro studies (Norvell et al., 1995; Norvell and Monroe, 1996) using immature B cells purified from either bone marrow or the periphery argued that the negative response of immature-stage B cells to BCR engagement is a characteristic that is developmental stage-associated and intrinsic to the B cell. Moreover, we concluded that the default response of these cells to antigen receptor engagement in isolation from B cell extrinsic influences from the microenvironment is cell death, resulting in deletion of the antigen-reactive clone. However, we considered that in vivo, immature and transitional immature B cells do not encounter antigen in isolation but rather in the context of complex interactions with other cells and cytokines within the bone marrow and peripheral compartments. As a consequence, there exists the possibility that the influence of these B cell extrinsic factors may modify the fate of the cell after encounter with antigen. In particular, we considered that certain cellular environments might confer protection from BCR-induced death and provide an environment supportive of continued immunoglobulin gene rearrangement and receptor editing.

So as to avoid complications due to different stages of development, we chose to use transitional immature B cells for these studies, as they represent the latest stage of immature B cells. These cells, isolated from the periphery, have left the bone marrow and are therefore free of potential influences of that microenvironment. Furthermore, it is this population that others have postulated are the most likely to undergo deletion upon BCR engagement (Carsetti et al., 1995; Melamed et al., 1998). In order to isolate large numbers of B cells that are highly enriched in transitional immature B cells, we have adapted a previously described system in which sublethally irradiated mice are allowed to reconstitute their peripheral hemopoietic compartments over a period of 14 days. Splenic B cells of these mice are exclusively transitional immature B cells, and following T cell and macrophage depletion, the remaining population is $>$95% $B220^{int}$, HSA^{high}, IgM^{high}, IgD^{int}.

To begin to define the influence of the cellular microenvironment on the fate of B cells targeted for negative selection, we have developed an in vitro system that allows us to manipulate the balance between B cell intrinsic and extrinsic influences on the fate of immature and transitional B cells to BCR engagement. Purified transitional immature B cells are labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and then mixed with unlabeled bone marrow or disassociated spleen. The CFSE label, in conjunction with propidium iodide staining, allowed us to monitor the fate of immature B cells by flow cytometry following BCR cross-linking with anti-IgM $F(ab')_2$ fragments. The CFSE coculture system facilitated our ability to monitor high-avidity antigen encounter in nontransgenic B cells. Finally, the use of non-transgenic B cells for these studies avoids the ambiguity in assigning developmental stages to B cells derived from mice harboring fully rearranged transgenic immunoglobulin loci.

Bone Marrow, But Not Disassociated Spleen, Protects Immature B Cells from BCR-Mediated Deletion

As shown in Figure 1A and consistent with our previous report (Norvell and Monroe, 1996), highly purified populations of transitional immature B cells die via apoptosis

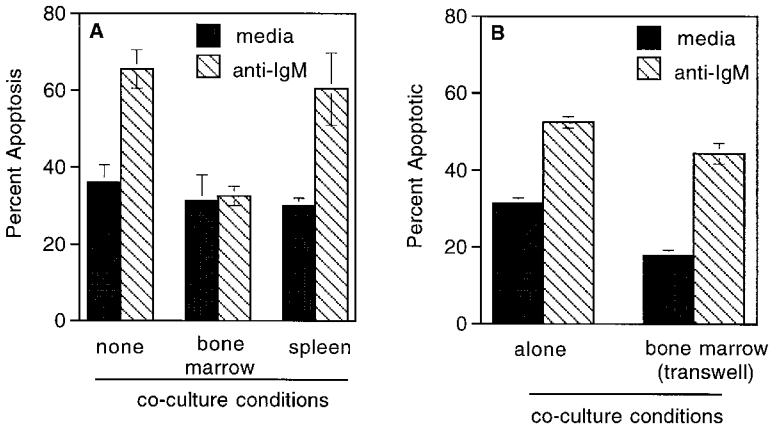

Figure 1. Coculture with Bone Marrow, but Not Spleen, Protects Transitional Immature B Cells from BCR-Mediated Apoptosis in a Contact-Dependent Manner

(A) CFSE-labeled transitional immature B cells were cultured alone with bone marrow or with disassociated spleen at a 1:1 ratio with media (filled bars) or 10 μg/ml anti-IgM $F(ab')_2$ fragments (hatched bars) for 15 hr. Cultures were harvested, fixed, and stained with propidium iodide. Analysis was performed by FACS gating on only $CFSE^{high}$ cells; apoptotic cells represent cells with a sub-2N DNA content. All points represent the mean of triplicate cultures ± SD.

(B) Bone marrow protection requires cell–cell contact. CFSE-labeled transitional immature B cells were cultured alone or with bone marrow from mature mice in transwells, with .4 μM pore size, such that cell–cell contact was prevented. Cultures were treated with 10 μg/ml of anti-IgM (hatched bars) and harvested 16 hr after stimulation.

following BCR cross-linking. In this experiment, transitional immature B cells treated with anti-IgM demonstrated a 2-fold increase in the frequency of apoptotic cells after 15 hr in culture when compared with cells cultured in media without BCR cross-linking. However, when transitional immature B cells were cocultured with whole syngeneic bone marrow at a 1:1 ratio, they did not show a detectable BCR-induced increase in the frequency of apoptotic cells over the same time period. While whole bone marrow provided a protective environment for the immature B cells, coculture with erythrocyte-depleted whole spleen at a 1:1 ratio did not block B cell death induced by BCR engagement. The inability of whole spleen to protect immature B cells from BCR-mediated apoptosis did not appear to be due to a lower frequency of the protective cell in the spleen, as ratios as high as 50:1 (disassociated spleen: immature B cell) also failed to protect immature B cells against BCR-induced apoptosis (data not shown).

We considered the possibility that the inability to detect an increase in the number of apoptotic B cells in bone marrow cocultures, following anti-IgM $F(ab')_2$ treatment, was due to phagocytosis of apoptotic cells, resulting in our inability to monitor and include them in our analysis. Previous in vivo studies have shown that apoptotic cells are quickly phagocytosed by macrophages (Osmond et al., 1994). However, a comparison of the number of cells recovered from stimulated and unstimulated B cell cultures showed no statistically significant variation in the number of cells recovered from cultures of either immature B cells alone or immature B cells cocultured with bone marrow following stimulation with anti-IgM (data not shown). We also examined the ratio of CFSE-labeled cells recovered from stimulated over unstimulated cultures with or without bone marrow; a ratio of 1 would imply no loss of cells due to anti-IgM addition. For these experiments, the ratio of the number of CFSE-labeled cells recovered from stimulated over unstimulated cultures of immature B cells or immature B cells with bone marrow was 1.02 ± 0.17 and 1.02 ± 0.19, respectively. Moreover, we saw no evidence of increased phagocytosis in anti-IgM stimulated cultures by fluorescence microscopy (data not shown). Therefore, we conclude that a cellular component found in the bone marrow environment protects immature B cells from apoptotic signals delivered via the BCR. Furthermore, the ability to mediate this protection is specific to the bone marrow insofar as the other cellular compartment in which immature B cells are found (i.e., the spleen) is not able to do so under these conditions.

Bone Marrow Protection of Transitional Immature B Cells against BCR-Induced Deletion Requires Cell–Cell Contact

In order to begin to characterize the nature of bone marrow–mediated protection of immature B cells against BCR-induced apoptosis, we examined the requirement for cell–cell contact using a transwell culture system and bone marrow conditioned media. As shown in Figure 1A, transitional immature B cells that were cultured in direct contact with bone marrow are protected from BCR-mediated apoptosis; however, transitional immature cells cultured with bone marrow but prevented from physical contact by a porous membrane were found to undergo apoptosis in response to anti-BCR (Figure 1B). Media that were conditioned by culture with bone marrow for 24 hr also failed to protect transitional immature B cells from BCR-induced cell death (data not shown). Therefore, while we cannot exclude a role for soluble factors in bone marrow–mediated protection, these experiments argue that direct cell-cell contact is required for rescue of immature B cells from BCR-induced deletion.

Immature B Cells, Protected by Coculture with Bone Marrow, Express *RAG-2* in Response to BCR Cross-Linking

RAG reinduction and secondary light chain rearrangement has been proposed as a mechanism to eliminate self-reactivity at the immature B cell stage (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993; Ghia et al., 1995; Hertz and Nemazee, 1997; Melamed et al., 1998).

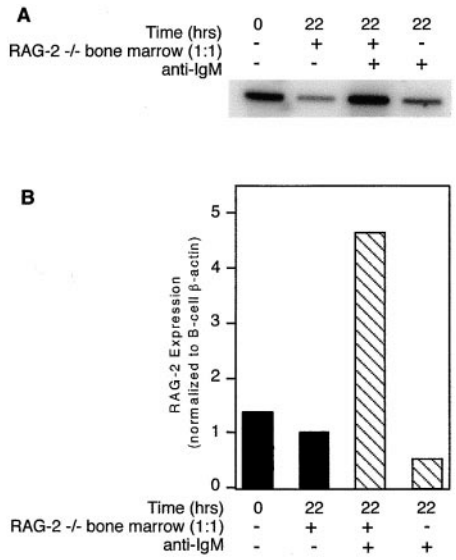

Figure 2. Protected Transitional Immature B Cells Express *RAG-2* in Response to BCR Cross-Linking

Isolated transitional immature B cells were cultured with bone marrow purified from *RAG-2*-deficient mice at a 1:1 ratio (B cells: bone marrow) with media (solid bars) or 10 μg/ ml of anti-IgM (hatched bars). (A) Cultures were harvested after 22 hr and *RAG-2* and β-actin mRNA levels determined by RT-PCR followed by Southern blotting. (B) *RAG-2* expression was normalized to β-actin expression and adjusted for bone marrow addition.

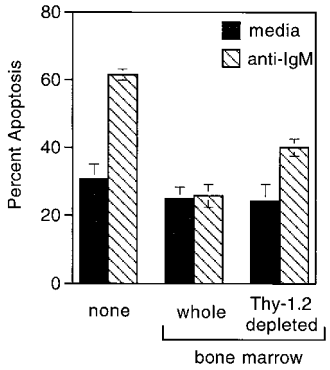

Figure 3. Thy-1.2-Depleted Bone Marrow Does Not Protect Transitional Immature B Cells against BCR-Mediated Apoptosis

Thy-1.2$^+$ cells were depleted from bone marrow using anti-Thy-1.2 and rabbit C′. CFSE-labeled cells were cultured with bone marrow at a 1:1 ratio for 15 hr with media (filled bars) or 10 μg/ml anti-IgM (hatched bars). Apoptotic cells were detected by propidium iodide staining followed by FACS analysis gating on the CFSE^{high} population. Data are presented as the mean of triplicate cultures ± SD.

This mechanism of negative selection seems at odds with our observations that immature B cells (conventional and transitional) are programmed to die following BCR engagement, even after only 30 min of engagement and at low levels of BCR engagement (Yellen-Shaw and Monroe, 1992; Norvell et al., 1995; Norvell and Monroe, 1996; Sater et al., 1998). Given the ability of bone marrow to block the cell death signal induced by BCR cross-linking, we wished to determine whether receptor editing might represent an alternative fate under these conditions. To do so, we measured the degree of *RAG-2* levels in the transitional immature B cells in the presence or absence of the BCR and bone marrow signals.

Whole bone marrow contains both pro-B and pre-B cells that are undergoing heavy and light immunoglobulin gene rearrangement and thus has high *RAG* gene expression (Li et al., 1993). In order to prevent endogenous *RAG* gene expression from masking induced *RAG* expression in immature B cells, we cultured the immature cells with bone marrow from *RAG-2*-deficient BALB/c mice. In these studies, transitional immature B cells were cultured with whole bone marrow from *RAG-2*-deficient mice with or without anti-IgM F(ab′)$_2$ fragments for 22 hr. Following culture, the cells were harvested and *RAG-2* expression determined by RT-PCR and Southern blotting. Figure 2 shows that *RAG-2* expression was induced in transitional immature B cell cultures containing both bone marrow and anti-BCR antibody. Importantly, *RAG-2* induction required both the bone marrow and the BCR inductive signal, as little *RAG-2* was detectable in those cultures containing either alone. Quantitation of PCR product followed by normalization for β-actin and *RAG-2*-deficient bone marrow revealed a 4-fold increase in *RAG-2* mRNA as a result of BCR cross-linking.

Identification of a Thy-1.2^{dull} Bone Marrow Cell that Protects Immature B Cells from BCR-Mediated Death

We (Norvell et al., 1995; Sater et al., 1998) and others (Chang et al., 1991; Brines and Klaus, 1993; Fulcher et al., 1996) have previously reported that factors associated with T cell help such as IL-4 or CD40L will protect immature B cells from BCR-mediated death. We considered it unlikely that the bone marrow protective effect was mediated by recirculating T cells for two reasons. First, erythrocyte-depleted splenocytes, which contain a high number of T cells, did not confer protection, even though taken from the same animal from which the bone marrow was isolated (Figure 1). Second, protection from apoptotic death and *RAG-2* induction occurs using bone marrow from *RAG-2*-deficient mice that do not have mature T cells (Figure 2; data not shown). Nevertheless, in the course of characterizing the phenotype of the bone marrow–derived cell involved in the protective effect, we depleted bone marrow of T cells using anti-Thy-1.2 and rabbit C′ and assayed the remaining population for the ability to protect immature B cells from BCR-mediated apoptosis. Surprisingly, the depletion of Thy-1$^+$ cells eliminated the protective capacity of whole bone marrow (Figure 3). FACS analysis of whole bone marrow revealed that approximately 5% of the total cells are Thy-1.2$^+$. Two populations of Thy-1.2$^+$ cells could be distinguished within the bone marrow, a Thy-1^{dull} population and a Thy-1^{high} population, which make up approximately 4% and 1% of the total bone marrow, respectively (Figure 4A). In order to determine the population involved in the bone marrow protective effect, the two Thy-1$^+$ populations were isolated to high purity by FACS sorting and cultured with CFSE-labeled transitional immature B cells. Coculture with Thy-1.2^{dull} bone marrow cells was found to protect immature B cells

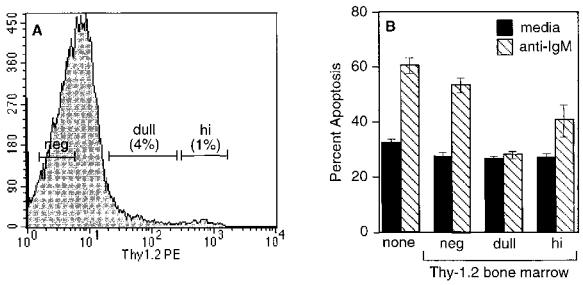

Figure 4. Expression of Thy-1.2 on Bone Marrow Cells and Protection of Transitional Immature B Cells by Sorted Thy-1.2 Populations

(A) Flow cytometric analysis of Thy-1.2 populations within bone marrow from mature BALB/c mice. Analysis was performed by staining with anti-Thy-1.2-PE, and the percentage of Thy-1.2^{dull} and Thy-1.2^{high} cells is indicated.

(B) Thy-1.2^{-}, Thy-1.2^{dull}, and Thy-1.2^{high} bone marrow cells were isolated by FAC sorting. Post-sort analysis determined that the cells were >95% pure. CFSE-labeled transitional immature B cells were cultured alone or with the sorted bone marrow populations at a 10:1 ratio (B cells: bone marrow) with media (filled bars) or 10 μg/ml of anti-IgM f(ab′)$_2$ fragments (hatched bars) for 15 hr. Analysis of apoptotic cells was performed by FACS after propidium iodide staining. Data are presented as means of duplicate cultures ± SD.

against BCR-mediated death, whereas neither the Thy-1.2^{-} nor Thy-1.2^{high} bone marrow populations conferred complete protection against BCR-induced cell death (Figure 4B). Consistent with the inability of splenocytes and the ability of *RAG*-deficient bone marrow to mediate the protective effect, the Thy-1.2^{high} cells represent the recirculating CD3^{+}, CD5^{+} T cell compartment in the bone marrow (data not shown).

Blocking BCR-Induced Caspase-Induced Death in Transitional Immature B Cells Mimics the Influence of Bone Marrow–Derived Protection for *RAG* Gene Induction and Allows for Receptor Editing

The ability of the bone marrow microenvironment to facilitate *RAG* gene induction in conjunction with BCR engagement affords the opportunity to undergo secondary immunoglobulin gene rearrangement as an alternative to deletion of self-reactive developing B cells. One can envision two roles for the bone marrow–associated cell in this process: (1) generation of signals either through cell/cell contact and/or soluble mediators that cooperate with BCR signals for *RAG* induction; or (2) providing a survival signal to the self-reactive B cells so that BCR signals can be redirected toward *RAG* induction. To address these non–mutually exclusive mechanisms, we set up a system whereby BCR-induced apoptosis could be blocked specifically and pharmacologically and then determined whether this manipulation was sufficient to allow BCR-induced *RAG* expression by the transitional immature B cell.

The cysteine protease caspase-3 has been implicated as a central mediator of apoptotic death. The elimination of thymocytes upon signaling via their T cell receptor has recently been shown to require caspase activity (Clayton et al., 1997), and caspase-3 activity is elevated in thymocytes that are undergoing steroid-induced apoptosis (Alam et al., 1997). As shown in Figure 5, transitional immature B cells, like thymocytes, activate caspase-3 in response to antigen receptor cross-linking. Using two different exogenous substrates that contain the preferred caspase-3 recognition site and are cleaved by caspase-3 (Nicholson et al., 1995), DEVD-AMC (Figure 5A) and DEVD-rhodamine (PhiPhiLux, Figure 5B), we were able to detect caspase activity in transitional immature B cells following BCR cross-linking. Lysates prepared from transitional immature B cells following stimulation with anti-BCR antibodies had increasing amounts of detectable caspase-3 activity beginning 10 hr after stimulation and peaking at 14 hr (Figure 5A). Caspase-3 proteolytic activity could also be detected in intact cells using a DEVD-rhodamine substrate that is cell permeable and allows cleavage activity to be assayed by flow cytometry. Confirming the time course of caspase-3 activation as determined in cell lysates, the frequency of cells containing active caspase-3 was significantly higher in the anti-BCR-treated cells 10 hr after stimulation and continued to rise up to 14 hr following BCR engagement (Figure 5B). Mature B cells did not have any detectable BCR-induced increase in caspase-3 cleavage activity over the same time period. Activation of caspase-3 was confirmed by Western blotting lysates from apoptotic immature B cells with a polyclonal anti-caspase-3 antibody. Figure 5C shows the cleavage of the inactive 32 kDa pro-enzyme and the concombinant appearance of the active 17 kDa caspase-3 subunit in transitional immature, but not mature, B cells between 2 and 14 hr after treatment with anti-IgM. The intermediate-sized band (LC) represents the immunoglobulin light chain of the anti-IgM F(ab′)$_2$ antibody fragments used for BCR cross-linking and then detected by the secondary antibody used to develop the Western blot.

The cell permeable tetrapeptide z-DEVD-FMK irreversibly inhibits caspase-3 activity by specific and covalent binding to the enzyme's active site (Nicholson et al., 1995; Margolin et al., 1997; Mittl et al., 1997). Treatment with z-DEVD-FMK prevented BCR-mediated apoptosis of transitional immature B cells in a dose-dependent manner (Figure 5D). The addition of 25 μM of z-DEVD-FMK at the time of anti-BCR stimulation completely abrogated the BCR-induced apoptotic response. Higher doses of the caspase inhibitor reduced the observed apoptosis to below background levels. It should be noted that high doses of DEVD inhibitors have been shown to result in nonspecific inhibition of other caspases (Thornberry et al., 1997; Hirata et al., 1998). Together, the results shown in Figure 5 demonstrates that activation of caspase-3 is required to link BCR signaling to induced cell death.

Last, to directly assess whether pharmacologic inhibition of BCR-induced apoptosis was sufficient to alter the fate of transitional immature B cells so that they initiated the process of receptor editing rather than deletion in response to BCR engagement, we measured *RAG* levels following BCR engagement in the presence of z-DEVD-FMK. Remarkably, protected transitional immature B cells demonstrated a 3- to 4-fold increase in both *RAG-1* and *RAG-2* expression in response to BCR

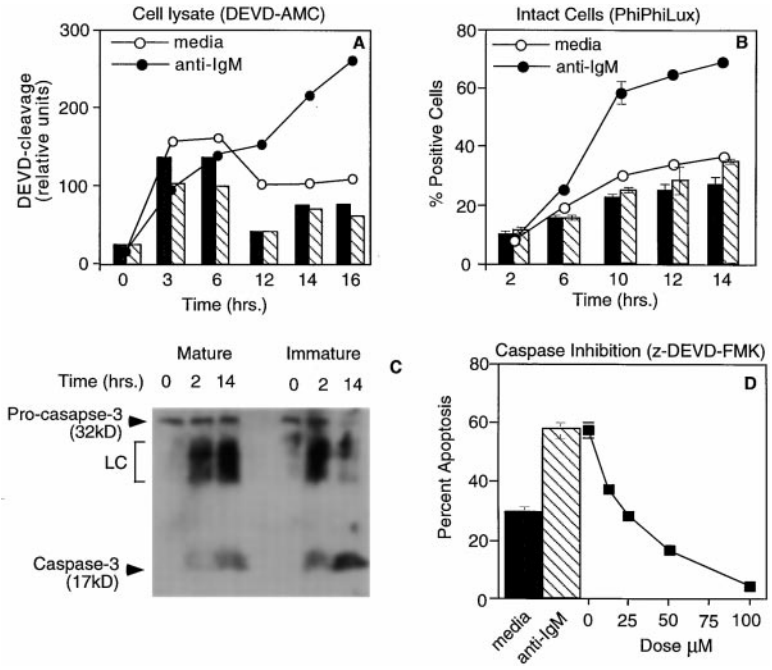

Figure 5. Caspase-3 Is Activated in Response to BCR Cross-Linking and Is Necessary for Transitional Immature B Cell Death

Caspase-3 expression and activation in mature and transitional immature B cells following BCR cross-linking. (A) Cell lysates were prepared from transitional immature B cells and mature B cells stimulated in culture with anti-IgM for 0, 3, 6, 12, 14, or 16 hr. Caspase-3 activity was determined by monitoring cleavage of an exogenous substrate DEVD-AMC over 30 min using a spectrophotometer. Data are presented as relative activity of stimulated immature cell lysates (closed circles) or unstimulated immature cell lysates (open circles) after 30 min at 37°C. Caspase-3 activity in unstimulated and stimulated mature cells is represented by solid and hatched bars, respectively. Data represent one of two experiments. (B) Caspase-3 activity in intact transitional immature or mature B cells was determined after 2, 6, 10, or 14 hr in culture with media or 10 μg/ml anti-IgM by staining with PhiPhiLux (rhodamine-DEVD-rhodamine) followed by FACS analysis. Data are presented as percentage of cells staining positively for PhiPhiLux cleavage and represent the mean of duplicate cultures ± SD. Stimulated immature cells (filled circles), unstimulated immature cells (open circles), unstimulated mature cells (solid bars), stimulated mature B cells (hatched bars). (C) Purified transitional immature and mature B cells were stimulated in culture for either 2 or 14 hr with 10 μg/ml of anti-IgM and expression of caspase-3 detected by Western blotting using rabbit anti-caspase-3 polyclonal antibody. Lanes 1–3, mature B cells; lanes 4–6, transitional immature B cells; lanes 1 and 4, ex vivo cell lysates; lanes 2 and 5, 2 hr after stimulation; and lanes 4 and 6, 14 hr after stimulation. Immunoglobulin light chain from the anti-IgM $F(ab')_2$ fragments is visible in lanes 2, 3, 5, and 6. (D) Inhibition of caspase-3 prevents BCR-mediated death of transitional immature B cells. Isolated transitional immature B cells cultured with media (solid bars) or 10 μg/ml anti-IgM (hatched bars) for 16 hr with either 0, 12.5, 25, 50, or 100 μM z-DEVD-FMK (filled squares). Percentage of apoptosis was determined by FACS after staining with propidium iodide. Data are presented as means of triplicate cultures ± SD.

engagement (Figures 6A and 6B). In this experiment, low *RAG* expression was observed in the transitional immature B cells treated with only the caspase-3 inhibitor. Expression of *RAG* genes in this population is variable but always much lower than in transitional immature B cells treated with the inhibitor and anti-IgM. The addition of z-DEVD-FMK was not responsible for the increased *RAG-2* expression, as mature B cells treated with the caspase-3 inhibitor and anti-IgM showed no increase in detectable *RAG-2* expression (Figure 6B). It

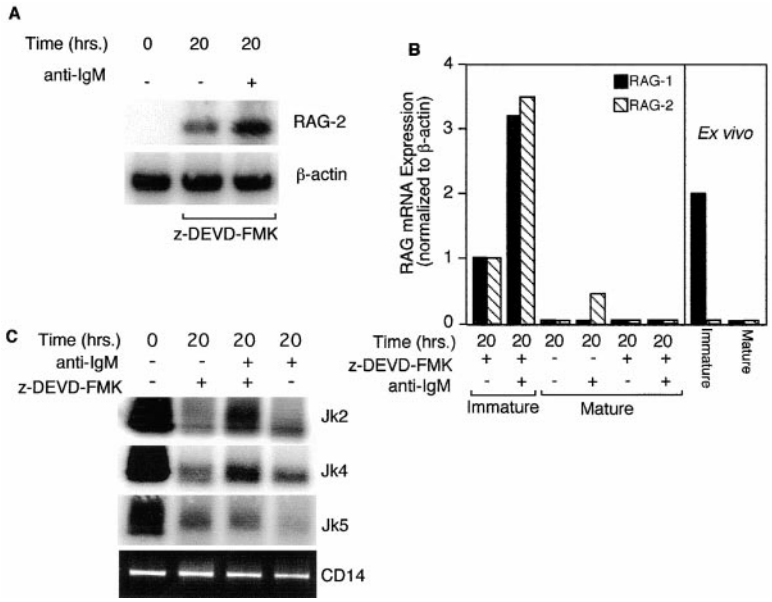

Figure 6. Transitional Immature B Cells Protected by z-DEVD-FMK Upregulate *RAG-1* and *RAG-2* and Initiate Immunoglobulin Light Chain Rearrangement in Response to BCR Cross-Linking

Isolated transitional immature B cells and mature B cells were cultured with 50 μM z-DEVD-FMK and either media or 10 μg/ml of anti-IgM for 20 hr. (A) Expression of *RAG-2* or β-actin mRNA was determined by RT-PCR followed by Southern blotting. (B) *RAG-1* and *RAG-2* mRNA expression from unstimulated (filled bars) or anti-IgM treated (hatched bars) cells was quantitated using a PhosphoImager and normalized to β-actin expression. (C) Expression of SBE from κ light chain rearrangements was detected by LM-PCR from genomic DNA.

should be noted that the level of *RAG* expression in transitional immature B cells ex vivo is variable and likely reflects carryover from the pre-B cell stage (Li et al., 1993) or receptor editing in vivo. In the experiment presented in Figure 6B, *RAG-1* mRNA, but not *RAG-2* mRNA, was detected in transitional immature B cells ex vivo.

RAG Induction in Immature B Cells in which Apoptosis Is Blocked Is Associated with Induced Recombination Events

While previous studies have demonstrated that *RAG* gene reexpression results in secondary immunoglobulin gene rearrangements (Han et al., 1997), we wished to formally demonstrate that immunoglobulin light chain rearrangement was induced in z-DEVD-FMK treated immature B cells in response to BCR cross-linking. During V(D)J recombination, *RAG*-mediated DNA cleavage results in two broken ends, a signal end and a coding end. The signal break ends (SBE) are blunt ended and fairly stable in resting cells. Therefore, in order to determine if V(D)J recombination was being induced in z-DEVD-FMK protected transitional immature B cells following BCR cross-linking, we used a ligation-mediated PCR (LM-PCR) strategy to detect SBE (Schlissel et al., 1993; Constantinescu and Schlissel, 1997). Purified genomic DNA from z-DEVD-FMK treated immature B cells cultured with anti-IgM for 22 hr was ligated to an oligonucleotide linker and then subjected to PCR amplification using primers specific for the linker and specific regions within the immunoglobulin κ locus. Freshly isolated immature B cells had high levels of all SBE, likely as a result of either initial light chain rearrangement or receptor editing in vivo. Treatment with anti-IgM and z-DEVD-FMK resulted in an increase in Jk2 and Jk4 SBE, although we did not see a noticeable increase in Jk5 SBE (Figure 6C). As for *RAG* induction, the induced or continued generation of SBE required the BCR signal as well as a block in caspase-mediated apoptosis.

Discussion

We have sought to reconcile two models of B cell tolerance induction, the deletional model and the receptor editing model. Proponents of negative selection by deletion have shown that immature B cells bearing immunoglobulin transgenes specific for membrane-bound antigens are eliminated in vivo from the B cell repertoire and that highly purified populations of immature B cells die via apoptosis in response to treatment with anti-Ig antibodies in vitro. (Hartley et al., 1991; Carsetti et al., 1995; Norvell et al., 1995). Evidence supporting receptor editing comes from studies demonstrating that immature B cells bearing autoreactive transgenic B cell receptors can be induced to express endogenous immunoglobulin light chains, express novel non–self-reactive antigen receptors on their cell surface, and escape deletion (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993; Hertz and Nemazee, 1997; Pelanda et al., 1997). These two models of negative selection present a paradox in which BCR engagement signals for both receptor editing and cell death by apoptosis. Thus, the two models are incompatible unless either developmental differences exist that function to regulate the intrinsic response of self-reactive immature B cells as they proceed through maturation, or that B cell extrinsic influences modify the fate of the antigen-reactive cell as it traffics through different microenvironments.

Evidence for the developmental model comes from the recent studies of Melamed et al. (1997, 1998). Using BCR^{+} cells developing from IL-7-expanded cultures of bone marrow from the 3-83 immunoglobulin transgenic mice, they observe that in the context of the OP42 stromal cell, BCR engagement by the IgM^{lo} cells leads to *RAG* induction whereas the more mature IgM^{high} cells are induced to undergo apoptosis. They proposed that receptor editing is a property of early self-reactive immature B cells while deletion characterizes the response of the more mature, transitional immature B cells.

While our studies do not specifically address the validity of the conclusions from the above studies, we specifically designed our studies so as to evaluate whether maturation into the transitional compartment determines the fate of the self-reactive immature B cell or if extrinsic factors play a defining role in the mechanism of negative selection. Our studies demonstrate that altering the microenvironment by mimicking distinct anatomic compartments plays a determining role in the fate of the immature B cell to antigen. We have shown that the bone marrow provides a protective environment for immature B cells that allows for reactivation of the recombinase machinery, whereas autoreactive immature B cells in the periphery are not protected and are eliminated from the immune repertoire upon encountering self-antigen. We propose that the mechanism of tolerance induction of immature B cells is determined, not by developmental stage, but by the environment in which immature B cells first encounter antigen, receptor editing in the bone marrow and deletion in the periphery.

Although the peripheral transitional B cells used throughout this study are not normally in contact with the marrow environment, the existence of immature B cells in two distinct compartments has allowed us to dissect the influences of in vivo environments on immature B cell tolerance induction. Using isolated transitional immature B cells, we have demonstrated that even these IgM^{high}, and developmentally advanced, immature B cells will upregulate their *RAG* genes and thus are capable of undergoing receptor editing provided that they are protected from apoptosis. We propose the model illustrated in Figure 7 based upon our findings. In the bone marrow, pre-B cells progress to the immature B cells stage upon successful rearrangement of their light immunoglobulin gene loci and expression of the mature BCR on the cell surface. Within the bone marrow, a population of Thy-1^{dull} cells protects immature B cells against BCR-mediated apoptotic signals. Upon BCR engagement, immature B cells in the bone marrow upregulate their *RAG-1* and *RAG-2* genes and attempt to escape self-reactivity by undergoing further immunoglobulin gene rearrangements. Immature B cells that successfully produce a non–self-reactive BCR, either initially or as a result of secondary immunoglobulin gene rearrangement, exit the bone marrow and transit to the

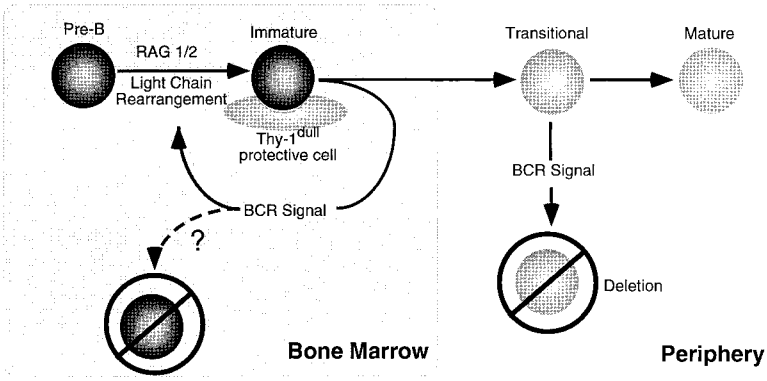

Figure 7. Model of Site-Dependent Autoreactive B Cell Negative Selection

peripheral lymphoid organs, whereas immature B cells that fail to produce a functional immunoglobulin receptor or fail to escape autoreactivity die in the bone marrow. Upon arriving in the peripheral lymphoid organs, transitional immature B cells are subject to further scrutiny. Transitional immature B cells are not protected in the peripheral lymphoid organs; thus, encounters with self-antigens in the spleen or lymph nodes result in deletion and the elimination of these autoreactive cells from the immune repertoire. Transitional immature B cells that do not engage self-antigen in the periphery develop into fully immunocompetent IgM^{lo}, IgD^{high}, HSA^{lo} mature B cells.

While we have determined that transitional immature B cells upregulate *RAG* genes in response to BCR cross-linking and thus are capable of undergoing secondary immunoglobulin gene rearrangement, we have not attempted to determine the frequency of immature cells that undergo receptor editing or deletion in vitro or in vivo. Operationally, it is difficult to distinguish immature B cells that have downregulated their surface BCR and are undergoing receptor editing from pre-B cells that also have no surface BCR and express *RAG-1* and *RAG-2*. In order to accurately determine the frequency of receptor editing, it will be necessary to distinguish between these two cell types in an in vivo model. While mice expressing immunoglobulin transgenes have provided great insight into the nature of B cell development and tolerance induction, these model systems suffer from development artifacts that make them unsuitable for these studies. The presence of a previously rearranged immunoglobulin transgene accelerates B cells through development such that the stages of development become difficult to distinguish. The premature expression of a functional surface BCR implies that immature B cells with low to moderate surface IgM expression may retain many of the functional characteristics of pre-B cells such as the ability to undergo further immunoglobulin gene rearrangements. For example, mice expressing the 3-83 immunoglobulin transgene have a reduced pre-B cell population; however, when crossed onto an autoreactive background ($H\text{-}2^b$), the pre-B cell pool is recapitulated and appears to be the site of secondary immunoglobulin gene rearrangement (Pelanda et al., 1997). While a direct measurement of the frequency of immature B cells undergoing receptor editing in vivo is difficult, a recent study determined that the frequency of λ light chain bearing mature B cells with rearranged κ light chains, indicating secondary immunoglobulin rearrangement, was up to 47% (Retter and Nemazee, 1998), implying that receptor editing is a major mechanism of tolerance induction.

The identity of the protective bone marrow cell and the molecular interactions necessary for bone marrow–mediated protection of immature B cells are areas of current study. As shown above, we have identified one marker, Thy-1.2, which is expressed on these cells at low levels. Previous studies have described bone marrow stromal cells such as BMS.1 and BMS.2 (Pietrangeli et al., 1988) that express Thy-1.2, and we are currently examining these lines for their ability to protect immature B cells against BCR-mediated death. We have determined that the protective effect of Thy-1.2^{dull} bone marrow cells requires cell–cell contact but have not identified the signaling elements necessary for rescue of immature B cells. There are two possible mechanisms by which this surface receptor may allow for *RAG* expressing and receptor editing in response to BCR cross-linking: (1) signaling via the bone marrow survival receptor may alter BCR signaling events to favor receptor editing rather than apoptosis, or (2) the signals delivered by the bone marrow may allow the immature B cell to survive long enough to produce a nonautoreactive BCR and exit the bone marrow. While these possibilities are not mutually exclusive, we favor the second mechanism based upon our finding that inhibition of caspase-3 activation is sufficient to allow *RAG-2* expression in response to BCR engagement and previously published findings that immature B cells expressing a bcl-2 transgene exhibit increased receptor editing (Lang et al., 1997; Mandik et al., 1997).

Experimental Procedures

Reagents

$F(ab')_2$ fragments of polyclonal rabbit anti-mouse IgM were generated in our laboratory and have been previously described (Monroe and Kass, 1985). Rabbit anti-human caspase-3 was kindly provided by Dr. Rafick-Pierre Sékaly (University of Montreal, Canada). z-DEVD-FMK, DEVD-CHO, and DEVD-AMC were from Calbiochem. PhiPhiLux was purchased from OncoImmunin. BALB/c *RAG-2*$^{-/-}$ mice were the kind gift of Dr. Jan Erikson (Wistar Institute).

B Lymphocyte Purification

Wild-type BALB/c mice were obtained from The Jackson Laboratory or bred in our colony. Transitional immature B cells were isolated

from the spleens of mice that had been subjected to 500 rad of whole body irradiation and allowed to reconstitute the bone marrow and peripheral compartments for 13–14 days (Allman et al., 1992, 1993; Norvell and Monroe, 1996). Transitional immature B cells isolated from the spleen were IgM^{high}, IgD^{int}, HSA^{high} and consistently found to be >95% $B220^{+}$. Mature splenic B cells were isolated from unmanipulated adult (8- to 10-week-old) BALB/c mice as previously described (Yellen et al., 1991).

Bone Marrow/Disassociated Spleen Coculture

Whole bone marrow was prepared by grinding femurs from mature BALB/c mice in a mortar and pestle in 10 ml of HBSS + 2% FCS followed by RBC lysis in Gey's solution. Disassociated spleen was prepared from mature BALB/c mice by grinding spleens between frosted glass slides followed by RBC lysis. Isolated immature B cells were washed in serum-free HBSS and then resuspended in serum-free RMPI at a density of 10×10^6 cells/ml in preparation for carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling. Immature B cells were labeled with 2 μM CFSE at 37°C for 12 min, and excess CFSE was removed by washing cells in ice-cold RMPI + 10% FCS. 2×10^5 CFSE-labeled transitional immature B cells were cultured with bone marrow or disassociated spleen, at a 1:1 ratio in RPMI + 10% FCS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 U/ml streptomycin, at a final density of 1×10^6 cells/ml in round-bottom wells.

***RAG-2* RT-PCR**

Total RNA was prepared from B cells cultured alone or with *RAG-2*$^{-/-}$ bone marrow after 22 hr in culture using RNA STAT-60 (Tel-Test Inc.). cDNA was prepared using 6 μg of total RNA, 500 ng oligo 15-dT primer (Promega), and 400 U Superscript II reverse transcriptase (GIBCO-BRL). *RAG-2* and β-actin mRNA expression was determined by PCR using previously published primers and amplification conditions (Li et al., 1993). Amplified products were resolved on 1.5% agarose gels, transferred to Genescreen Plus (NEN, Boston, MA), and detected by Southern analysis using a ^{32}P-labeled oligonucleotide specific to bases 260–284 of the *RAG-2* cDNA or 414–434 of β-actin. Quantitation was performed on a Molecular Dynamics Storm 860 PhosphoImager.

LM-PCR

Genomic DNA was purified by SDS/proteinase K digestion followed by phenol/chloroform extraction as described (Sambrook et al., 1989). The BW linker was prepared as previously described (Schlissel et al., 1993), and 2 μg of template DNA was ligated overnight with 40 U of T4 DNA ligase (New England Biolabs). 100 ng of ligated DNA was subjected to 40 cycles of amplification using primers BW-1H, 5′-CCGGGAGATCTGAATTCCAC-3′ and either Jk910F, 5-CCGGATCCTCTTGTGGGACAGTTTTCCCTCC-3′, Vk1474F, 5′-GGTCCCATTGTGTCCTTTGTATGAGTTTGTGG-3′, or Jk1847F, 5′-GCCATTCCTGGCAACCTGTGCATCA-3′. Amplified DNA was run on 3% agarose gels (NuSieve, FMC Bioproducts), transferred to Genescreen Plus (NEN), and hybridized using ^{32}P-labeled oligonucleotides specific for Jk1-2, Jk4, or Jk5 and analyzed with a Storm 860 PhosphoImager. CD14 was amplified from BW ligated DNA in a control reaction using previously published primers (Schlissel et al., 1993).

B Cell Apoptosis Assay

B lymphocytes were cultured alone or with bone marrow and harvested 14–16 hr after addition of 10 μg/ml rabbit anti-mouse IgM $F(ab')_2$ fragments. Cells were washed in FACS buffer (1× PBS, 2% FCS, and 0.02% NaN_3) and fixed in ice-cold 70% EtOH at −20°C overnight. Cells were washed as above and stained with 10 μg/ml propidium iodide and 50 μg/ml RNAse for 8 hr at room temperature. Cells were analyzed by flow cytometry performed on Becton Dickinson FACScan at the University of Pennsylvania Flow Cytometry Facility, and the number of sub-diploid cells was determined by cell cycle analysis using CellQuest software.

Isolation of Thy-1 Bone Marrow Subsets

Whole bone marrow was prepared as above and stained with anti-Thy-1.2-PE (Pharmingen). Stained cells were live gated and sorted on the basis of Thy-1.2 expression into Thy-1.2^{-}, Thy-1.2^{dull}, and Thy-1.2^{high} populations using a Becton Dickinson FACStar Plus or FACS Vantage TSO at the University of Pennsylvania Cytometry. Post sort purity was consistently determined to be >95%. Isolated bone marrow populations were rested in culture overnight and then cocultured with CFSE-labeled transitional immature B cells as described above except that the ratio of immature B cells to bone marrow was increased to 10:1 (immature B cells: sorted bone marrow cells).

Quantitation of Caspase-3 Activity

Lysate Assay

Following stimulation in culture, mature or transitional immature B cells were washed in 1× PBS and lysed by repeated rapid freeze/thaw cycles in a dry ice/EtOH bath. The nuclei were removed by centrifugation at 8,000 × g and the protein concentration of the lysates determined by Bradford assay (Biorad). Lysate containing 50 μg of protein was added to 1 ml of 20 μM DEVD-AMC in PBS and the cleavage of DEVD-AMC monitored over 30 min at an excitation of 380 nm and an emission of 460 nm using a Perkin Elmer Luminance Spectrometer (Nicholson et al., 1995). Results were reported as relative units of fluorescence at 460 nm after 30 min at 37°C.

PhiPhiLux

Following stimulation in culture by anti-IgM $F(ab')_2$ fragments, 2×10^5 B cells were pelleted and resuspended in 10 μl of PhiPhiLux in RPMI. After a 1 hr incubation at 37°C, the reaction was stopped by the addition of 300 μl of ice-cold FACS buffer and the cells analyzed immediately by flow cytometry. The frequency of cells containing active caspase-3 was determined by monitoring increased free rhodamine on FL2.

Western Blotting

Transitional immature B cells and mature B cells were harvested after culture and washed in cold 1× PBS and lysed in 1% NP40 lysis buffer as previously described (Wechsler and Monroe, 1995) with the addition of 20 μM DEVD-CHO to the lysis buffer. Cytosolic extracts were resolved on 15% SDS-PAGE and transferred to Hybond-ECL (Amersham). Blots were probed with rabbit anti-human caspase-3 as previously described (Alam et al., 1997) with the exception that all washes were performed in Tris buffered saline + .05% Tween-20. Pro-caspase-3 (32 kDa) and the 17 kDa active subunit of caspase-3 were visualized using donkey-anti-rabbit-HRP (Jackson ImmunoResearch) followed by enhanced chemiluminescence (ECL) detection reagents (Amersham).

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