# Loss of the proapoptotic protein, Bim, breaks B cell anergy

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**Although B cells that respond with high avidity to self–antigen are eliminated early in their development, many autoreactive B cells escape elimination and are tolerized later in their lives via anergy. Anergic B cells are unresponsive to antigen and die prematurely. It has been suggested that the proapoptotic protein, Bim, controls the fate of anergic B cells. To test this idea, mice lacking Bim were crossed with mice that express soluble hen egg lysozyme and whose B cells bear receptors specific for the protein. In Bim$^{+/+}$ mice these B cells are anergic and die rapidly. If the mice lack Bim, however, the B cells live longer, are more mature, respond to antigen, and secrete anti–hen egg lysozyme antibodies. This break of tolerance is not due to expression of endogenous B cell receptors, nor is it dependent on T cells. Rather, it appears to be due to a reduced requirement for the cytokine BAFF. Normal B cells require BAFF both for differentiation and survival. Bim$^{-/-}$ B cells, on the other hand, require BAFF only for differentiation. Therefore, autoreactive B cells are allowed to survive if they lack Bim and thus accumulate sufficient signals from differentiating factors to drive their maturation and production of autoantibodies.**

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Abbreviations used: BCR, B cell receptor; Dbl Tg, double transgenic; FO, follicular; HRP, horseradish peroxidase; MZ, marginal zone; sHEL, soluble hen egg lysozyme; T2, transitional 2.

The immune system must eliminate B cells that respond to self while providing protection against a broad range of foreign pathogens. In B cells, self-tolerance is achieved in several ways. Immature B cells that bind with high avidity to self-antigens they encounter are stimulated to rearrange their Ig genes again, with the goal of substituting genes coding for harmless Ig receptors for those coding for autoreactive Ig (1–8). Those B cells that fail to edit their receptors away from autoreactivity are eliminated by clonal deletion (1, 8, 9). B cells that react with self-antigens with low avidity, or with soluble self-antigens, become anergic.

Several mouse models have been produced that aid the study of B cell anergy (10–14). Although the B cells generated in these various models differ from each other in some aspects, they share some important properties. In most models, anergic B cells bear low levels of surface IgM (mIgM) and appear to be blocked at the transitional 2 (T2) stage of B cell development (12, 15–17). Thus, the cells are CD21$^{low}$, CD23$^+$, IgD$^+$, CD93$^+$, and IgM$^{low}$. Additionally, the cells exhibit features of activated B cells, such as elevated CD86 and CD95, but other markers of activation are not apparent

(18). Hence, anergic B cells have physical characteristics marking them as recent bone marrow immigrants that are chronically stimulated by antigen.

Anergic B cells also have functional characteristics that distinguish them from normal cells and are probably caused by chronic exposure to antigen. Amongst these are a reduction in their ability to produce antibody in response to antigen and a shortened life expectancy (10, 12, 13). Perhaps contributing to these two characteristics is an increased level of intracellular calcium in these cells at rest, coupled with an inability to raise their calcium levels in response to mIgM cross-linking (12, 19).

The best-characterized model of B cell anergy is the so-called double transgenic (Dbl Tg) model in which mice express transgenes coding for soluble hen egg lysozyme (sHEL) and for an Ig receptor that can bind to sHEL (HEL-Ig; reference 10). This model allows comparison of anergic B cells in the Dbl Tg mice to their naive counterparts in mice expressing only the HEL-Ig Tg and provides a system for stimulating cells in vitro with a well-defined antigen.

Early work in this model suggested that anergic B cells might have shortened life spans because they were killed by CD4 cells expressing Fas and interacting with Fas ligand on the surface of the anergic cells (20). Later work, however, suggests that the life span of anergic B cells and autoreactive B cells destined for death may be mainly under the control of members of the Bcl-2 family (21). Overexpression of Bcl-2 or Bcl-xl protects autoreactive B cells from death, although they remain anergic (15, 22, 23). Anergic B cells contain higher levels of the proapoptotic Bcl-2–related protein, Bim, than wild-type cells do (24), and absence of Bim inhibits the death of autoreactive B cells and prolongs the survival of HEL-reactive B cells in vivo (25). In theory, once B cells are rendered anergic, they should remain so, providing antigen levels are maintained. However, the longer their life expectancy, the greater the risk that an anergic B cell will "break through" and respond to antigen, so presumably this shortened life expectancy of anergic B cells, controlled by Bcl-2–related proteins, is important to the maintenance of B cell tolerance.

The survival and differentiation of B cells is affected by the cytokine BAFF and this cytokine also affects the tolerance status of B cells. In animals that contain a mixed population of anergic and naive B cells, anergic B cells do not compete very well for BAFF and remain unresponsive (17, 24). However, in the absence of competitor naive B cells, overexpression of BAFF converts anergic B cells into cells that are no longer unresponsive and that secrete autoantibody. Based on these in vivo observations, it has been proposed that, to stay alive and maintain function, anergic cells may require more BAFF than wild-type cells do.

Collectively, these results suggest some intersection between the effects of Bcl-2–related proteins and BAFF on the survival and function of anergic B cells. To address this, we created Bim$^{-/-}$ Dbl Tg mice. Loss of Bim increased B cell numbers in such animals and the B cells that were present were more mature, based on expression of IgM and CD21. These mature B cells responded to HEL in vitro and secreted antibody in vivo, indicating a breach of tolerance. This break of tolerance did not depend on T cells or coexpression of endogenous B cell receptors (BCRs). Rather, our data suggested that loss of Bim allowed the B cells to survive independently of their normal survival factor, BAFF. This longer life expectancy allowed the B cells to accumulate differentiation signals from factors such as BAFF. Once differentiated, the B cells were able to respond normally to antigen and secrete antigen-specific Ig. Thus, in normal mice, Bim prevents maturation and activity of autoreactive anergic B cells by decreasing their life expectancy and exposure to differentiation-inducing signals.

## RESULTS
### Lack of Bim increases the number of B cells in Dbl Tg mice
B cell numbers are governed by the frequency with which they enter the periphery, their proliferation, and the length of time they survive. Dbl Tg mice have 20–35% fewer splenic B

cells than either their naive (HEL-Ig) or wild-type (C57BL6) counterparts. It has been shown that this is the result of a shortened B cell life span (26–28). BCR signaling induces B cell death both directly, by activating Bim (25), and also indirectly, by increasing the cell's requirement for cytokines, such as BAFF, that promote survival of the cells (24).

Bim is involved in the deaths of B cells deprived of cytokine-mediated survival signals and of B cells exposed to antigen (25, 29). Thus, we reasoned that Bim could play an important role in the death of anergic B cells. To find out whether this is so, we crossed Bim$^{-/-}$ mice to Dbl Tg animals and analyzed the resulting offspring. Spleens and lymph nodes from Bim$^{-/-}$ Dbl Tg mice were visibly larger than those in their Bim$^{+/+}$ counterparts. Microscopic evaluation revealed an increased size of the white pulp regions (Fig. 1 A), and these regions of the spleen contained expanded populations of both B and T cells (Fig. 1 B). This latter result was confirmed by flow cytometry, which showed that the spleens of Bim$^{-/-}$ Dbl Tg mice contained increased numbers of B cells, exceeding even those of wild-type controls (Fig. 1 C). This phenomenon became more pronounced in older mice. Although we observed significant variability, on average Bim$^{-/-}$ Dbl Tg spleens contained ∼2.5 times more B cells than did the spleens of Bim$^{+/+}$ Dbl Tg control animals (P < 0.003).

### Bim$^{-/-}$ Dbl Tg B cells are not tolerant
Bim$^{-/-}$ mice have high levels of circulating autoantibodies and die prematurely of antibody complex–mediated disease (29). It has been shown that autoreactive B cells in Bim$^{-/-}$ mice are less likely to die during negative selection in the bone marrow (25). However, this observation cannot account for the high serum autoantibody levels in Bim$^{-/-}$ mice because the autoreactive cells should become anergic and hence unable to secrete autoantibody. Thus, the presence of autoantibody in Bim$^{-/-}$ mice suggests that the mechanisms maintaining anergy are defective in such animals. To find out if this was the case, we measured serum anti-HEL antibody titers in various types of mice expressing or lacking Bim.

Mice that did not express HEL-Ig had virtually no soluble anti-HEL antibodies, whether or not the mice also expressed Bim. On the other hand, single Tg mice, expressing only HEL-Ig, contained high levels of anti-HEL antibody in their sera. Again, this result was independent of Bim expression. As has been reported previously, Dbl Tg Bim$^{+/+}$ mice contained very little serum anti-HEL, reflecting the anergic status of their B cells. Dbl Tg mice lacking Bim, on the other hand, contained almost as high levels of serum anti-HEL as mice expressing just HEL-Ig (Fig. 2 A; P < 0.001). Thus, the absence of Bim inhibited anergy in Dbl Tg mice.

The antibody in the Bim$^{-/-}$ mice could have been secreted by plasma cells in the bone marrow. To find out if this was possible, we stained bone marrow cells from these and control mice, and compared plasma cell numbers. As shown in Fig. 2 B, and as reported previously (30), Bim$^{+/+}$ Dbl Tg mice contained very few early differentiating (B220$^{hi}$, CD138$^{+}$) or mature (B220$^{lo}$, CD138$^{+}$) plasma cells by
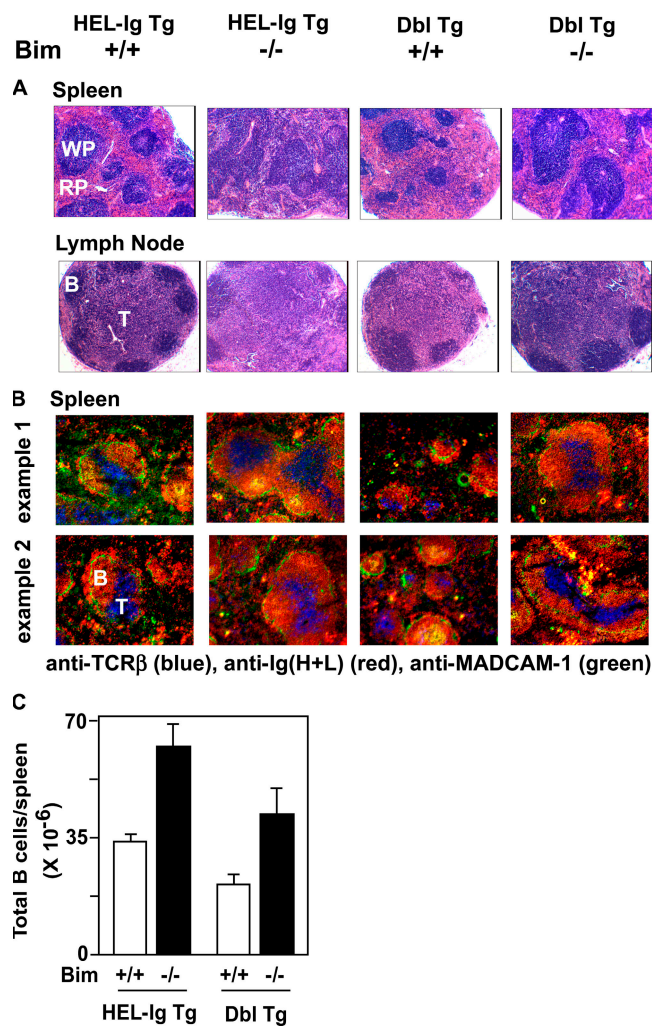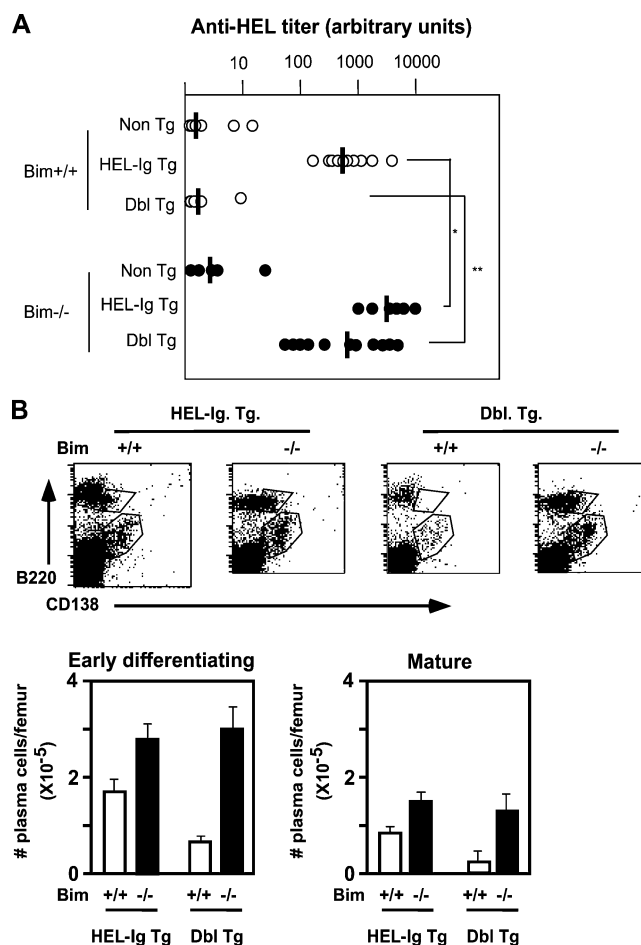
**Figure 1. Loss of Bim increases B cell number in Dbl Tg mice and improves survival.** (A) Spleens and lymph nodes were isolated from Bim$^{+/+}$ and Bim$^{-/-}$ Dbl Tg animals as well as from their naive HEL-Ig Tg counterparts. Tissues were sectioned and stained with hematoxylin and eosin. Tissue images were taken using a 10× objective. (B) Sections of frozen spleen were stained with antibodies to TCR (blue), mIg H+L (red), and MadCam-1 (green) and examined by fluorescence microscopy. Examples from two different experiments are shown. (C) The total number of B cells per spleen was determined by flow cytometry measuring the percentage of total B220$^+$ cells.



**Figure 2. B cells from Bim$^{-/-}$ Dbl Tg mice are not tolerant.** (A) Sera from 8–24-wk-old mice were analyzed by ELISA for reactivity to HEL. The level of reactivity for each sample was normalized to that of a known HEL-Ig Tg Bim$^{+/+}$ control sample. Each dot represents an individual mouse. Lines represent mean values for each genotype. (B) Bone marrow cells were isolated from the various genotypes and plasma cells were analyzed by flow cytometry for expression of B220 and CD138 (syndican). Early differentiating (B220$^{hi}$, CD138$^+$ cells) and mature (B220$^{lo}$, CD138$^+$) plasma cells were gated to determine their frequency. (C) Mature plasma cell numbers/femur were calculated, and the mean and standard deviations of the numbers derived from three to four animals from each genotype are shown. *, P < 0.05; **, P < 0.001.

comparison with their HEL-Ig counterparts. Bim$^{-/-}$ Dbl Tg mice, on the other hand, contained as many plasma cells of both types as Bim$^{+/+}$ or Bim$^{-/-}$ HEL-Ig mice did.

Based on these data, we suggest that loss of Bim allows B cells to escape anergy as witnessed by the increased number of bone marrow plasma cells and by the high levels of circulating autoantibody in Bim$^{-/-}$ Dbl Tg animals.

## B cells in Bim$^{-/-}$ Dbl Tg mice are more mature than their anergic counterparts
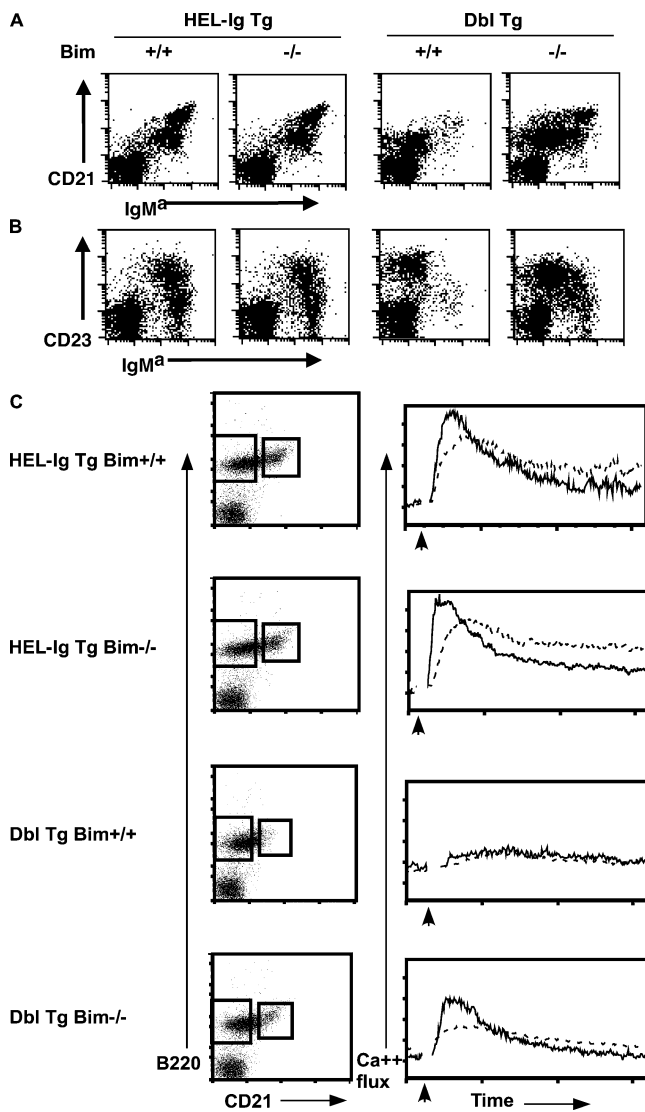
After B cells leave the bone marrow, they continue to mature, passing from the T1 stage through the T2 stage to be-

come naive follicular (FO) B cells (31). Previous examination of B cells from Bim$^{+/+}$ Dbl Tg mice showed that the anergic B cells in these mice do not mature beyond the late T2 stage, as demonstrated by their expression of low levels of CD21 and high levels of CD23 (Fig. 3, A and B; reference 15). These anergic cells also bear low levels of IgM, presumably caused by chronic exposure to antigen. On the whole, Bim$^{+/+}$ Dbl Tg spleens contain fewer FO B cells and have only a small number of marginal zone (MZ) B cells, as defined by FO localization or surface expression of CD21 and CD23 (FO cells are CD21$^{int}$ and CD23$^{high}$, whereas MZ cells are CD21$^{high}$ and CD23$^{low}$).

The presence of plasma cells and serum antibody in Bim$^{-/-}$ Dbl Tg mice suggested that the B cells in these mice were developing beyond the T2 cell stage. Accordingly, we analyzed spleen and lymph node B cells for markers characteristic of maturation. Bim$^{-/-}$ Dbl Tg mice were found to contain a population of cells with increased levels of CD21 and mIgM (Fig. 3, A and B), indicating that these cells had matured beyond the T2 stage. These cells were found to have

varying levels of CD23 expression, suggesting that both FO and MZ populations had increased in number.

The elevated levels of CD21 and mIgM on the Bim$^{-/-}$ Dbl Tg B cells suggested that the cells were not anergic. Therefore, the cells were tested for their ability to flux calcium in response to antigen. We found that the cells that expressed elevated levels of CD21 fluxed calcium in response to HEL significantly better than either the CD21$^{low}$ cells from the same mice or their Bim$^{+/+}$ anergic counterparts (Fig. 3 C).

Collectively, these data show that there is a population of B cells in the Bim$^{-/-}$ Dbl Tg animals that mature, even in the presence of autoantigen. These cells display increased CD21 and respond to HEL almost as well as their naive counterparts; therefore, they are not anergic.

### The loss of tolerance is not due to expression of endogenous receptor

We wished to find out how loss of Bim allowed autoreactive B cells to survive and avoid anergy. One possibility is that lack of Bim reduces the efficiency of allelic exclusion. If this were so, Bim$^{-/-}$ B cells expressing anti-HEL might be allowed to rearrange their endogenous Ig genes more often than Bim$^{+/+}$ anti-HEL B cells do. Because simultaneous expression of two different antigen receptors has been shown to allow autoreactive B cell clones to progress through development (32), such a scenario would account for the fact that Bim$^{-/-}$ B cells are not anergic in Dbl Tg mice.
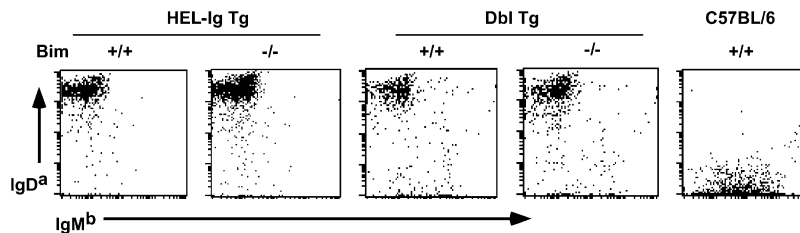
The anti-HEL Ig in Dbl Tg mice is IgM$^a$ and IgD$^a$, whereas the endogenous μ heavy chain alleles in Dbl Tg mice backcrossed to C57BL6 is IgM$^b$. Cells that avoided allelic exclusion in these mice could therefore be identified by their expression of mIgM$^b$. B cells from Dbl Tg mice that did or did not express Bim were stained with anti-IgD$^a$ and anti-IgM$^b$. We chose to analyze IgD$^a$ because, unlike IgM$^a$, its cell surface expression is not reduced in anergic cells.

There was no increase in the percentage of cells expressing an endogenous IgM$^b$ receptor in Bim$^{-/-}$ Dbl Tg mice versus Bim$^{+/+}$ Dbl Tg controls. Likewise, we did not observe considerable numbers of cells expressing both receptors simultaneously (Fig. 4). This analysis was also applied to cells from older mice to see if the emergence of these cells takes time to detect. Again, we found no difference between Bim$^{+/+}$ or Bim$^{-/-}$ mice (not depicted). Thus, we conclude that the break of tolerance observed in these mice is not due to dual usage of both the Tg and endogenous heavy chains.

### T cells are not required for Bim$^{-/-}$ B cells to break tolerance

It is thought that T cell help might break B cell anergy (30, 33, 34). Because Bim affects T as well as B cell tolerance, it is possible that B cells escape anergy in Bim$^{-/-}$ Dbl Tg mice because they receive help from T cells that escaped tolerance and not because loss of Bim affects the B cells directly. To test this idea, Bim$^{-/-}$ Dbl Tg mice were crossed to mice lacking TCRα. The resultant progeny were intercrossed to produce mice lacking both Bim and TCRα and expressing either or both of the HEL-Ig and sHEL transgenes. We compared



**Figure 3. B cells from Bim$^{-/-}$ Dbl Tg mice are more mature than B cells from their Bim$^{+/+}$ anergic counterparts.** (A and B) Cells were isolated from the spleens of HEL-Ig Tg or Dbl Tg mice that were Bim$^{+/+}$ or Bim$^{-/-}$. The cells were analyzed by flow cytometry for expression of (A) CD21 and IgM$^a$, and (B) CD23 and IgM$^a$. (C) Spleen cells from mice of the various genotypes were loaded with Indo-1 and stimulated with 1 μg/ml HEL. Samples were gated on B220 and either CD21$^{low}$ (dashed lines) or CD21$^{hi}$ (solid lines) populations and analyzed for Ca$^{2+}$ flux over time. All Ca$^{2+}$ flux histograms are plotted on the same scale. Arrows indicate when HEL was added to the samples. Data are representative of two independent experiments.

**Figure 4. Expression of endogenous Ig genes does not contribute to the loss of anergy of Bim$^{-/-}$ Dbl Tg B cells.** Cells were isolated from the spleens of mice of the various genotypes. B220$^+$ cells were ana- lyzed by flow cytometry for expression of IgD$^a$ (Tg BCR), and IgM$^b$ (endogenous BCR) was determined. These data shown are representative of four animals from each genotype analyzed in two independent experiments.

these to similar mice expressing Bim. Spleens from these mice were analyzed for the presence of B cells expressing increased levels of IgM$^a$ and CD21 as compared with naive controls, as indicators of loss of anergy. Surprisingly, we found that Bim$^{-/-}$ B cells escaped tolerance induced by anergy even in the absence of T cells (Fig. 5). B cell numbers in Dbl Tg TCR$\alpha^{-/-}$ mice lacking Bim were substantially higher than in cohorts expressing Bim ($5.5 \times 10^7$ vs. $3.8 \times 10^7$ B220$^+$ cells/spleen, respectively). Furthermore, the B cells from Bim$^{-/-}$ Dbl Tg animals lacking T cells expressed higher levels of both IgM and CD21 (Fig. 5 C). Likewise, titers of serum antibodies against HEL were unaffected by the lack of T cells, with Bim$^{-/-}$ Dbl Tg TCR$\alpha^{-/-}$ mice containing the same high levels of anti-HEL as their TCR$\alpha^{+/+}$ counterparts (Fig. 5 D).

These data indicate that T cells do not play a prominent role in the escape of Bim$^{-/-}$ B cells from anergy.

### Serum HEL levels are not lower in Bim$^{-/-}$ Dbl Tg mice

Peripheral tolerance is broken in Bim$^{-/-}$ B cells in the absence of T cell help. Thus, the defect that allows B cells to break anergy appears to be cell intrinsic. One possible explanation is that the increased number of B cells in the Dbl Tg Bim$^{-/-}$ animals reduces the amount of available HEL in the serum below a threshold required to maintain anergy. To test this, we measured the amount of HEL in these mice that was available to bind B cells. A sandwich ELISA was used to do this. The HEL in the mouse sera was captured using the HyHEL9 anti-HEL monoclonal antibody. This antibody recognizes an epitope on HEL that is not seen by the anti-HEL Tg BCR (35–37). We then measured the amount of captured HEL that was available to bind the anti-HEL Tg receptor using biotinylated-HyHEL10, an antibody that recognizes the same epitope as the anti-HEL Tg BCR (35), and detected the complex with streptavidin–horseradish peroxidase (HRP). As shown in Fig. 6, levels of available HEL did not differ significantly between mice expressing or lacking Bim, although the available HEL in Dbl Tg Bim$^{-/-}$ sera tended to be slightly lower than that in their Bim$^{+/+}$ counterparts. The concentration of HEL in serum required to induce anergy has been reported previously to be >0.5 ng/ml and less than the 9.5 ng/ml reported in Dbl Tg mice (27, 38, 39), and the concentration of HEL in the Bim$^{-/-}$ Dbl Tg averaged 8.3 ng/ml.

If a lowering of the available HEL was required to break B cell tolerance, the Bim$^{-/-}$ Dbl Tg mice that express the highest levels of HEL should remain tolerant. This turns out not to be the case. In the three Bim$^{-/-}$ Dbl Tg mice that showed the highest levels of available HEL (Fig. 6 A), 10–20% of the splenic B cells were not anergic and expressed higher levels of CD21 and IgM.

The idea that lower HEL levels affected tolerance was tested in another way by analyzing the amount of HEL bound to B cells in Bim$^{-/-}$ Dbl Tg mice and Bim$^{+/+}$ Dbl Tg controls. To measure the amount of HEL bound to the B cell surface, we used the HyHEL9 monoclonal antibody, the antibody that does not compete for binding to HEL with the HEL-Ig Tg receptor. For the sake of comparison, and as a positive control for HEL binding, we also analyzed HEL-Ig Tg B cells that had been incubated with soluble HEL before the analysis. As shown in Fig. 6 B, HEL-Ig Tg B cells preincubated with HEL bound plenty of HyHEL9. There was a significant amount of HEL bound to the B cells in Dbl Tg Bim$^{-/-}$ mice. These levels were not significantly different from those on B cells from Dbl Tg Bim$^{+/+}$ animals (mean fluorescence intensity of 11.2 vs. 13.4).

Although these data clearly show that there is available HEL in the serum of Dbl Tg Bim$^{-/-}$ mice (Fig. 6 A) and that HEL is bound to B cells isolated from these mice (Fig. 6 B), we have not ruled out the possibility that the amount of available HEL drops below a threshold such that newly emerging B cells do not see enough HEL to be fully anergized. To test this, we isolated HEL-Ig Tg B cells from a Bim$^{+/+}$ mouse, labeled the cells with CFSE to ensure that we could distinguish them from the B cells in the recipients, and transferred them into a Dbl Tg Bim$^{-/-}$ mouse. We performed this transfer during the period that B cells in the recipient mouse were beginning to break tolerance (8–10 wk of age). To evaluate whether or not the transferred cells were binding antigen, we compared the levels of surface IgM on these cells with those on HEL-Ig Tg B cells and Dbl Tg B cells. Levels of IgM on these transferred cells fell to those on Dbl Tg Bim$^{+/+}$ or Dbl Tg Bim$^{-/-}$ cells (Fig. 6 C). Thus, the levels of HEL in Dbl Tg Bim$^{-/-}$ mice are sufficient to reduce surface IgM levels to those of tolerized cells.

Collectively, these data suggest that the break in B cell tolerance in Bim$^{-/-}$ mice is not caused by a reduction in available HEL.
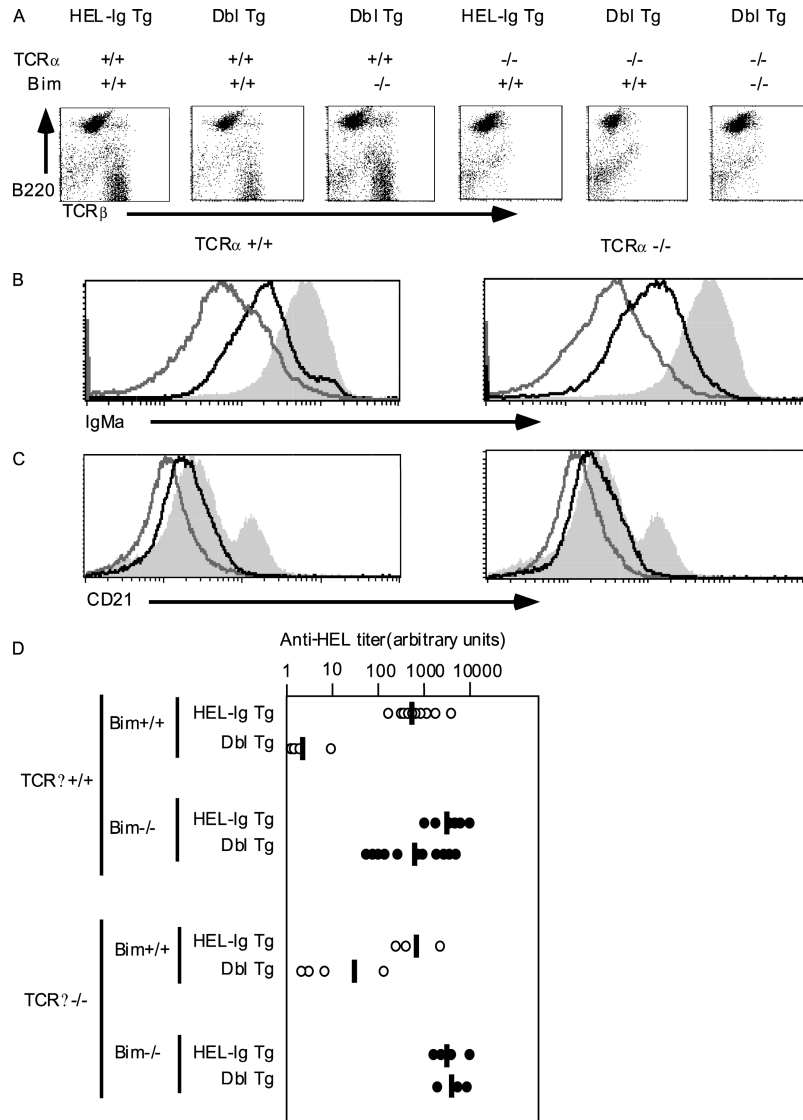
**Figure 5. T cells are not required for loss of B cell tolerance in Bim$^{-/-}$ Dbl Tg mice.** (A–C) Cells were isolated from the spleens of mice of the various genotypes. (A) Cells were stained with antibodies against B220 and TCRβ to confirm the absence of T cells. (B and C) B220$^+$ cells were analyzed for expression levels of (B) IgM$^a$ and (C) CD21. Anti-HEL serum antibody titers were measured using ELISAs. Data from TCRα$^{+/+}$ mice were obtained at the same time as that from TCRα$^{-/-}$ mice using the HEL-Ig Tg sample used previously for normalization. The data from TCRα$^{+/+}$ mice are from Fig. 2 A and are shown again here for the sake of comparison. Each dot represents an individual mouse. Lines represent the mean values for each genotype. Mice were between 8 and 24 wk of age. Each genotype contained animals spanning this age range.

## Bim$^{-/-}$ Dbl Tg B cells survive in the absence of BAFF

Normal B cells depend upon BAFF both for survival and maturation as indicated by the fact that BAFF$^{-/-}$ animals contain normal numbers of immature B cells but are devoid of mature FO and MZ populations (40, 41). Anergic B cells are also highly dependent on BAFF. Overexpression of BAFF in mice allows the survival and maturation of B cells that would otherwise be anergic, and also allows the cells to break tolerance and secrete antibody (17, 24). The observation that anergic B cells do not compete well with normal cells for BAFF suggests that these cells are more dependent than wild-type cells on this cytokine.

These experiments did not demonstrate, however, whether BAFF effected loss of anergy by increasing the life expectancy of anergic B cells or by stimulating their maturation, or both.
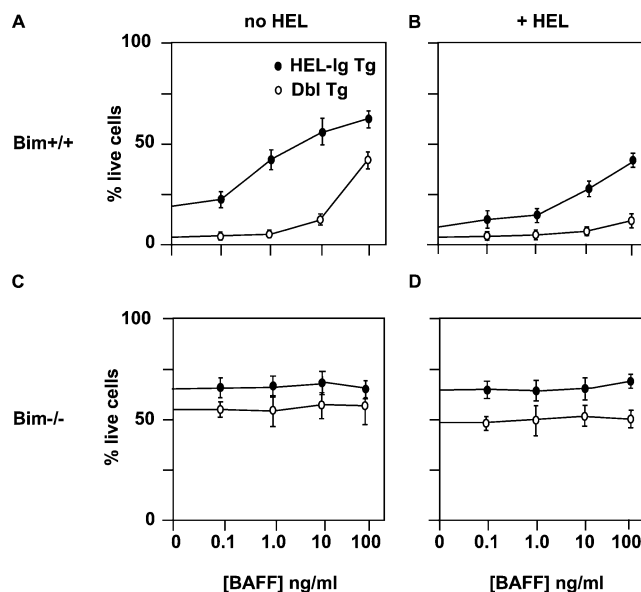
To find out which feature of anergic B cells was affected by BAFF and the relative concentrations of BAFF involved, we isolated B cells from HEL-Tg or Dbl Tg mice that did or did not express Bim and cultured the cells without or with HEL with titrated concentrations of BAFF. 4 d later we measured the percentage of B cells that survived. BAFF does not induce B cell proliferation in vitro (42), so the effects observed must have been due to the response of the B cells in
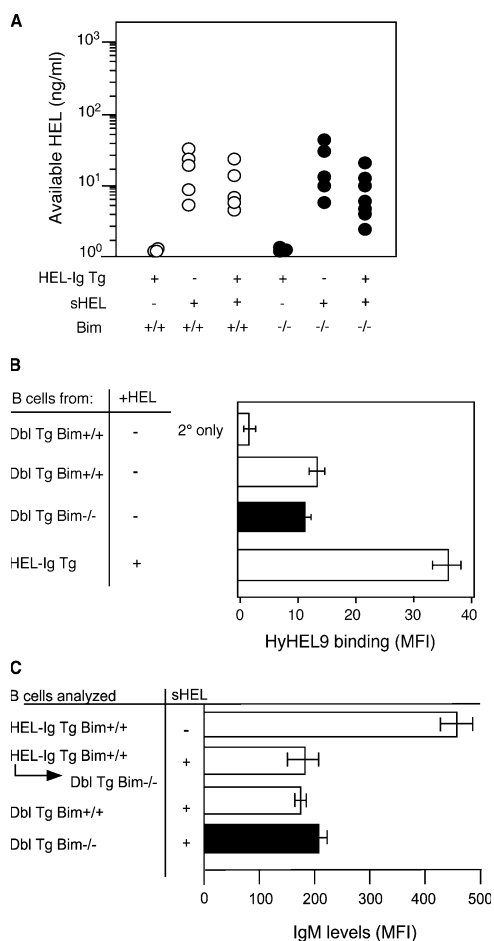
**Figure 6. Dbl Tg Bim$^{+/+}$ and Bim$^{-/-}$ mice contain similar amounts of HEL in their serum.** (A) Sera from 8–24-wk-old mice were analyzed by ELISA for levels of available HEL. The level of reactivity for each sample was compared with a titration of HEL (of known concentration) analyzed within the same plate. Closed circles represent Bim$^{-/-}$ mice, and open circles represent Bim$^{+/+}$ animals. Each dot reflects an individual mouse. (B) The B cells were incubated with biotinylated HyHEL9, a monoclonal anti-HEL antibody that binds to HEL on a different site than the Tg HEL-Ig receptor. HyHEL binding was detected with streptavidin-PE. Fluorescence observed after staining was used to measure background fluorescence. The staining of HEL-Ig Tg B cells with HEL added ex vivo is shown for comparison. (C) IgM levels were measured on B cells isolated from the various genotypes as well as on HEL-Ig Tg Bim$^{+/+}$ B cells that were transferred 24 h before analysis into a Dbl Tg Bim$^{-/-}$ mouse. (B and C) Mice were 8–10 wk of age at the time of analysis. Error bars represent the standard deviations of the results from three mice per genotype.



**Figure 7. Bim$^{-/-}$ B cells do not depend upon BAFF for survival.** (A–D) B cells were isolated from mice of the indicated genotypes and cultured for 4 d in the presence of various concentrations of BAFF. Survival was determined by forward and side scatter analysis using flow cytometry. (B and D) B220$^{+}$ B cells were isolated from mice of the indicated genotypes and cultured in the presence or absence of 100 ng/ml HEL as well as in increasing concentrations of BAFF. Data represent the average of cells from three mice of each genotype. Error bars show standard deviations from the mean.

the cultures directly to BAFF and not to preferential expansion of a particular B cell subset.

Some Bim$^{+/+}$ HEL-Ig B cells survived the 4 d of culture, but their survival was considerably improved by culture with increasing concentrations of BAFF. The survival of the B cells was compromised if they had been exposed to HEL in mice because barely any Dbl Tg B cells survived the culture, and very few of these cells were rescued by even high concentrations of BAFF (Fig. 7 A). These effects

were more pronounced if the cells were cultured with HEL (Fig. 7 B).

In the absence of Bim, many more B cells survived in culture. B cells from Bim$^{-/-}$ Dbl Tg mice survived almost as well as B cells from HEL-Tg animals (Fig. 7 C), so prior exposure to antigen had little effect on the life expectancy of these B cells. Likewise, exposure to antigen in vitro had little effect on the survival of the cells (Fig. 7 D). BAFF did not increase survival of the cells (Fig. 7, C and D).

Thus, as expected, exposure to antigen in the absence of helper factors such as BAFF did indeed cause the rapid death of wild-type cells and even more rapid death of anergic cells. As predicted by others, anergic B cells do indeed require higher concentrations of BAFF to survive than normal B cells do. Bim must play a key role in this antigen-mediated killing because Bim$^{-/-}$ B cells die slowly, regardless of antigen signaling.

**Bim$^{-/-}$ Dbl Tg B cells do not mature in the absence of BAFF**
We examined the maturation status, as measured by CD21 and CD93 levels, of the B cells that survived in the cultures described above. Culture with BAFF resulted in higher levels of CD21 on all the populations of surviving cells (Fig. 8, A–D). However, anergic Bim$^{+/+}$ B cells from Dbl Tg mice needed much more BAFF to mature into high expression of CD21 than nonanergic Bim$^{+/+}$ B cells, and this effect was also manifest by the addition of HEL to the cultures (Fig. 8, A and B). This was less obviously true for Bim$^{-/-}$ B cells. In
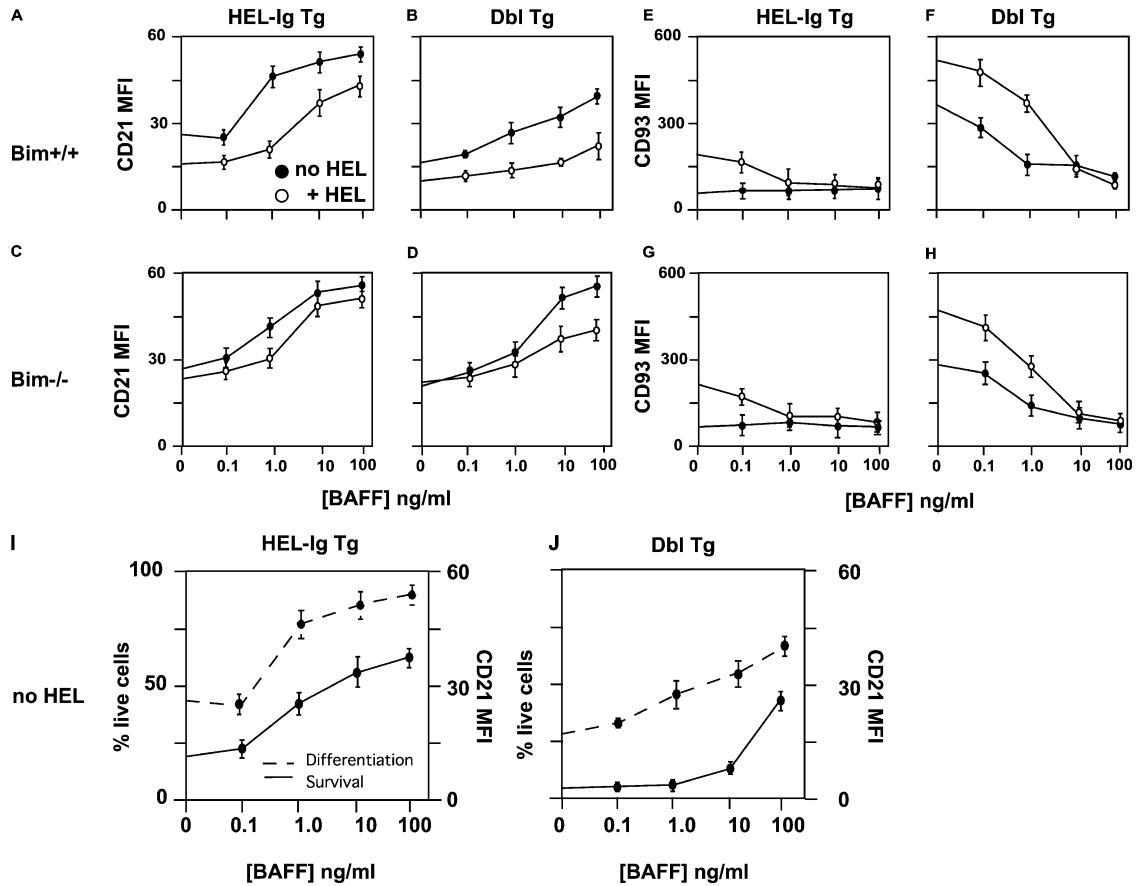
**Figure 8. Bim$^{-/-}$ B cells require BAFF to differentiate.** (A–H) B cells were isolated from mice of the indicated genotypes and cultured for 4 d in the presence of increasing concentrations of BAFF. CD21 or CD93 expression was determined by flow cytometry and plotted using the mean fluorescence intensity of each sample. Open circles represent 100 ng/ml HEL added at the beginning of each culture. Closed circles indicate that no HEL was added. (I and J) The samples described above were plotted differently to allow for comparison of the BAFF dose response for both survival and differentiation (CD21 expression) of the Bim$^{+/+}$ B cells. Data represent the average of three samples from mice of each genotype. Error bars show standard deviations from the mean.

the absence of HEL in the cultures, Bim$^{-/-}$ B cells matured efficiently in response to BAFF, whether or not the cells had been exposed to HEL in vivo (Fig. 8 C). On the other hand, the addition of HEL to their culture medium caused the Bim$^{-/-}$ B cells to require more BAFF to mature, and this effect was somewhat more pronounced for cells from Dbl Tg mice (Fig. 8 D).

Culture of cells in BAFF had little effect on expression of CD93 (AA4.1, 493, and C1qRp), a phenotypic marker of immature B cells (42, 43), on the anti-HEL Tg B cells from mice that lacked HEL (Fig. 8, E and G). This is because these cells were already too mature to express CD93. On the other hand, cells from Dbl Tg animals expressed high levels of CD93, which fell during culture in BAFF (Fig. 8, F and H). Culture of anti-HEL Tg B cells in the presence of HEL increased CD93 levels even further (Fig. 8, E–H). The addition of BAFF to the cultures abrogated this. Thus, exposure to BAFF can change the phenotype of anergic cells, allowing them to lose expression of immature markers, like CD93, and gain expression of mature B cell markers, such as CD21.

In these experiments, Bim$^{-/-}$ Dbl Tg B cells responded to lower levels of BAFF than the Bim$^{+/+}$ Dbl Tg control cells. The maturation of Bim$^{-/-}$ Dbl Tg B cells in response to low levels of BAFF could be explained in two ways. It might suggest that Bim affects maturation as well as survival of the B cells. On the other hand, this observation could be the result of reduced antigen exposure in vivo. The high levels of circulating antibody observed in these animals could bind sHEL and thus effectively lower the amount of antigen available to bind the B cells (as shown in Fig. 6 D). Thus, unlike their wild-type counterparts from Dbl Tg mice, these Dbl Tg B cells from Bim$^{-/-}$ animals are not anergic and therefore respond to BAFF just like normal naive cells do.

### Anergic B cells need higher concentrations of BAFF to survive than differentiate

The data from Figs. 7 and 8 (A–H) were plotted in a different way to allow comparison of the BAFF dose response for survival versus differentiation of Bim$^{+/+}$ B cells. Nonanergic B cells from HEL-Tg mice had about the same dose response

to BAFF for survival and maturation, with a midpoint between 1 and 10 ng/ml BAFF for both functions (Fig. 8 I). Anergic B cells from Dbl Tg mice showed evidence of differentiation at similarly low levels of BAFF, with significant differentiation at 1 ng/ml BAFF. However, the survival of these cells required much higher concentrations of BAFF, with virtually no effect seen until BAFF levels were above 10 ng/ml (Fig. 8 J).

Collectively, these results show that anergic B cells require about the same concentrations of BAFF for maturation as wild-type B cells do. However, for survival, anergic B cells need more BAFF than their wild-type equivalents do. Bim$^{-/-}$ B cells do not need BAFF at any level for survival; therefore, their only requirement for BAFF is as a maturation factor, a role BAFF can satisfy at quite low levels. Thus, these results account for the fact that lack of Bim allows B cells to escape anergy. Bim$^{-/-}$ B cells do not need BAFF for survival and need only relatively low levels of BAFF to mature.

## DISCUSSION

Anergic B cells have several properties that prevent them from producing autoantibodies. They respond poorly to antigen, as defined by relatively small increases in intracellular calcium, fail to enter cell cycle, and are unable to secrete antibody. They also have shorter half-lives in vivo than normal B cells. They do not fully mature and their development is arrested at the T2 stage of B cell maturation. They do not migrate properly and cannot make their way to the sites where B cells normally reside, splenic follicles and MZs (21). It is not clear which of these features contributes most to the absence of autoantibodies in healthy animals, and it is also not clear whether these properties are controlled by the same or different phenomena within the anergic cells.

To find out which properties are crucial for the maintenance of anergy, investigators have examined each separately for its effects. In almost all cases, B cells expressing high levels of the antiapoptotic proteins Bcl-2 or Bcl-xl have lengthened life spans but are still anergic in the presence of chronic self-antigen (22, 23, 44). In one model system, in which B cells express the transgenes for an insulin-specific Ig, the B cells are anergic even though they fully mature and migrate to follicles and MZs in spleens (14). In another set of experiments, investigators forced anergic B cells to migrate abnormally by adjusting their expression of CXCR5 and CCR7, yet the B cells remained short lived and anergic (21). Thus, anergy is not controlled in B cells by their shortened life expectancy, early maturational status, or abnormal location, at least as individual properties.

Of the several phenomena known to break B cell anergy (45–47), the most striking is overexposure to the cytokine BAFF. In normal animals, BAFF promotes the survival and maturation of B cells at and beyond the T2 stage (40, 41). Overexpression of BAFF causes mice to develop autoantibodies and allows B cells that would normally be anergic in Dbl Tg mice to mature, live longer, migrate to sites such as the MZ, and secrete autoantibodies (17, 24). However, aner-

gic B cells do not compete well with normal B cells for BAFF (24), which may account for the fact that in animals with normal levels of BAFF, anergic cells do not break tolerance. Collectively with the data discussed above, these results suggest that several features of anergic B cells prevent their maturation into autodestructive cells.

Here we show that absence of the proapoptotic protein Bim also allows B cells that would otherwise be anergic to mature and secrete autoantibodies. This seems to be allowed because of the different requirements of anergic cells for BAFF for survival versus maturation. Thus, normal B cells have about the same sensitivity to BAFF for survival and for maturation. However, anergic cells require more BAFF to mature than normal B cells do and, as suggested in the past, require even higher concentrations of BAFF to keep themselves alive. Normal and anergic B cells lacking Bim no longer need BAFF to keep themselves alive. They do still need BAFF to differentiate, but, for Bim$^{-/-}$ cells, the dose response to BAFF for maturation of anergic and normal cells is about the same. Thus, lack of Bim promotes loss of anergy because Bim-deficient B cells no longer need BAFF to survive and have about the same sensitivity to BAFF for maturation as normal cells do.

With these results in mind, it is interesting to wonder why overexpression of the antiapoptotic protein Bcl-2 does not similarly always interrupt B cell anergy. In the model of B cell tolerance in which B cell Ig is specific for class I MHC, overexpression of Bcl-2 prevents the deletion of the autoreactive cells and, depending on the site at which the class I is expressed, allows antibody production (23). In mice in which B cells are specific for soluble-stranded DNA, high levels of Bcl-2 cause anergic B cells to mature but not secrete autoantibody (44). In a model in which B cells are normally deleted in the bone marrow, heightened expression of Bcl-2 allows B cells to mature, only to become anergic (22). Of course overexpression of Bcl-2 is not quantitatively equivalent to complete absence of Bim. Even as a transgene, the Bcl-2 may not be overexpressed in quantities sufficient to overcome the action of normal levels of Bim. Thus, lack of Bim may shift the life expectancy and dose response to BAFF of anergic B cells more toward normality than overexpression of Bcl-2 does.

## MATERIALS AND METHODS

**Mice.** Homozygous Bim$^{-/-}$ mice were bred from mice provided by A. Strasser (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia; reference 29). TCRα$^{-/-}$ mice were originally obtained from The Jackson Laboratory (48). HEL-Ig Tg and sHEL Tg mice were also purchased from The Jackson Laboratory (10). All mice were backcrossed at least seven generations to C57BL/6 mice (The Jackson Laboratory). All mice were maintained under pathogen-free conditions in the Biological Resource Center, National Jewish Medical and Research Center, and were 8–16 wk old at the beginning of each experiment unless otherwise noted. The protocols used were in accordance with the guidelines drafted by National Jewish Medical and Research Center and Institutional Animal Care and Use Committee.

**Cell sorting and flow cytometry analysis.** For surface staining, cell suspensions were incubated for more than 20 min on ice with anti–mouse FcR-γ2, 2.4G2 to prevent nonspecific binding of antibodies to the Fc

receptors on cells. Cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide and incubated with an optimal amount of labeled antibodies as noted. Alternatively, cells were incubated for 30 min with antibodies directly diluted in 2.4G2. Antibodies used for these experiments included anti-CD21/CD35-FITC, anti–CD23-PE, anti-CD138, anti–IgM-APC, anti–IgM$^a$-PE, anti-IgD$^a$-PE, anti-IgM$^b$, and anti–B220-APC or anti-Cy-Chrome (all from BD Biosciences). The monoclonal hamster anti–mouse anti-TCRβ coupled to Oregon Green, Ham-597, was described previously (49). Biotinylated anti-CD93 was originally described elsewhere (43). After incubation with antibodies, cells were sorted using a MoFlo machine (DakoCytomation) or analyzed on a FACSCalibur machine. Data analysis was performed using CELLQuest (BD Biosciences) and FloJo (TreeStar) software.

**Cell transfer.** $5 \times 10^6$ B220$^+$ B cells purified by MoFlo cell sorting (DakoCytomation) were labeled with CFSE. In brief, 10$^7$ cells were incubated at 37° for 10 min in 2.5 μM CFSE and then washed. Cells were then transferred into the tail vein of recipient mice.

**Calcium mobilization.** To measure free intracellular calcium concentration ([Ca$^{2+}$]i), cells were loaded with Indo-1AM (Invitrogen), labeled with anti–B220-APC and CD21-Fitc, resuspended at 10$^6$ cells/ml in DMEM, and analyzed using flow cytometry. After the baseline was established, cells were stimulated with 1 μg/ml HEL. Mean [Ca$^{2+}$]i was evaluated over time using the LSR cytometer (Becton Dickinson) and analyzed using FloJo software (TreeStar).

**Cell culture.** Cells were isolated from spleens and a single cell suspension was prepared, depleted of erythrocytes using buffered ammonium chloride, and washed twice in DMEM. B220$^+$ B cells were isolated by MoFlo cell sorting (DakoCytomation) and then incubated in DMEM containing 10% FCS (Oatlanta Biologicals) as well as 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM 2-ME.

**HEL binding assay.** To analyze the amount of HEL bound to BCR on the cell surface, spleen cells were isolated and incubated on ice with a biotinylated HyHEL9 anti-HEL antibody diluted in 2.4G2. Cells were washed and then incubated in streptavidin-PE. As a positive control for HEL binding, HEL-Ig Tg spleen cells were incubated with 100 ng/ml HEL before analysis.

**ELISA.** Sera were collected from mice and analyzed for HEL-specific antibody titers or available HEL using ELISA. To measure HEL-specific antibodies, plates were coated with 5 μg/well HEL diluted in PBS, washed five times, and then blocked with 30% FBS in PBS for at least 1 h at room temperature. Plates were then washed and serial dilutions of sera and standards were added. Plates were incubated for 1 h at room temperature and then washed. The assays were incubated for 1 h at room temperature with HRP-labeled rat anti–mouse IgM. To measure available HEL, plates were coated with HyHEL9 and then blocked using 30% FCS. The HyHEL9 antibody recognizes HEL on a different epitope than that seen by the anti-HEL Tg BCR (37). Each serum was serially diluted and added to plate. After incubation with serum, plates were washed and then biotinylated HyHEL10 antibody was added. The HyHEL10 antibody recognizes the same epitope on HEL as the anti-HEL Tg BCR (35). Antibody binding was detected using streptavidin-HRP. To quantify the amount of available HEL, HEL was titrated in the absence of serum and detected using the method described above. Samples were compared with readings from this known standard. Plates were developed with PNPP for colorimetric detection.

**Histology and immunohistochemistry.** Mice were killed and lymph nodes and spleens were embedded in Tissue-Tek O.C.T. (Fisher Scientific), frozen in dry ice–cooled 2-methylbutane, and stored at −70°C until sectioning. Spleens were cut on a cryostat and successive sections of 6–8 mm were thaw-mounted on slides. Sections were air-dried, acetone-fixed for 5 min, and stored at −70°C. After thawing, slides were rehydrated with 5%

normal goat serum in PBS for 10 min, and the spleens were stained for 30 min at room temperature. The reagents used were anti–mouse MadCam-1 (BD Biosciences), anti-Ig (H+L), and anti-TCRβ.

For histology, spleens and lymph nodes were fixed in 4% paraformaldehyde, paraffin embedded, and stained with hematoxylin and eosin.

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