Immunity, Vol. 20, 441–453, April, 2004, Copyright ©2004 by Cell Press

Reduced Competitiveness of Autoantigen-Engaged B Cells due to Increased Dependence on BAFF

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Summary

Peripheral autoantigen binding B cells are poorly competitive with naive B cells for survival and undergo rapid cell death. However, in monoclonal Ig-transgenic mice lacking competitor B cells, autoantigen binding B cells can survive for extended periods. The basis for competitive elimination of autoantigen binding B cells has been unknown. Here we demonstrate that autoantigen binding B cells have increased dependence on BAFF for survival. In monoclonal Ig-transgenic mice, each autoantigen binding B cell receives elevated amounts of BAFF, exhibiting increased levels of NFκB p52 and of the prosurvival kinase Pim2. When placed in a diverse B cell compartment, BAFF receptor engagement and signaling are reduced and the autoantigen binding cells are unable to protect themselves from Bim and possibly other death-promoting factors induced by chronic BCR signaling. These findings indicate that under conditions where BAFF levels are elevated, autoantigen-engaged cells will be rescued from rapid competitive elimination, predisposing to the development of autoimmune disease.

Introduction

Autoreactive B cells are negatively selected by mechanisms of receptor editing and deletion in the bone marrow (Goodnow, 2001; Nemazee, 2000). However, some autoreactive B cells escape these early checkpoints and emigrate to the periphery. Using immunoglobulin (Ig) transgenic models, it has been established that peripheral B cells chronically exposed to weakly crosslinking autoantigens downregulate their surface IgM and partially uncouple the remaining B cell receptors (BCR) from downstream signaling pathways (Goodnow, 2001). This has been observed with B cells specific for the neo-self-antigen, soluble hen egg lysozyme (HEL), for B cells reactive with dsDNA, and for arsonate-specific B cells that are also reactive with an unknown autoantigen (Benschop et al., 2001; Goodnow et al., 1988; Lang and Nemazee, 2000; Mandik-Nayak et al., 1997).

In mice that carry only the heavy chain (Hc) of the anti-HEL immunoglobulin antibody, about 1% of the peripheral B cells rearrange a light chain permitting HEL binding (Cyster et al., 1994). However, this population of HEL binding B cells is not detectable in the periphery of mice that also express soluble HEL-autoantigen (Cyster et al., 1994). This contrasts with the situation in mice carrying anti-HEL heavy and light chain transgenes (hereafter referred to as Ig-transgenic mice) and the soluble HEL transgene. In these mice all of the peripheral B cells bind HEL-autoantigen, yet they are still present within 2- to 3-fold of the numbers present in Ig-transgenic mice lacking autoantigen (Mason et al., 1992). B cell lifespan measurements in the Ig/HEL double transgenic mice indicated a 50% turnover time of 1 week, and showed that as many as 40% of the cells lived considerably longer than this (Cook et al., 1997; Cyster and Goodnow, 1995; Fulcher and Basten, 1994). However, when small numbers of Ig/HEL-double transgenic B cells were transferred into a mouse with a diverse B cell repertoire—mimicking the situation in Hc-only transgenic mice—the autoantigen binding cells underwent cell death within 2 to 3 days (Cyster and Goodnow; Cyster et al., 1994). These findings indicated that in the presence of a diverse repertoire of competitor B cells, soluble HEL autoantigen binding B cells are rapidly eliminated. Early studies indicated that autoantigen binding B cells were blocked from entering follicles prior to their elimination, leading to a model where B cells compete for a trophic factor present within follicles (Cyster et al., 1994). However, recent experiments with CCR7-deficient cells have indicated that autoantigen binding B cells undergo competitive elimination even when they are not excluded from follicles (Ekland et al., 2004), suggesting competition for a more widely distributed factor. Competitive elimination is independent of T cells or Fas and can be substantially delayed by Bcl2 overexpression (Cyster et al., 1994; Mandik-Nayak et al., 2000; Schmidt and Cyster, 1999), but the nature of the proapoptotic signals transmitted by the chronically engaged BCR has not been defined. Recent studies in T cells have highlighted a role for the proapoptotic BH3-only molecule, Bim, in the death of autoreactive cells (Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002), and a role for Bim in B cells is indicated by the marked increase in B cell numbers and production of autoantibodies that occurs in Bim-deficient mice (Bouillet et al., 1999).

BAFF (also known as BLyS, TALL-1, THANK, zTNF4, or TNFS13b), a member of the TNF family of cytokines, has emerged as a critical B cell survival factor (Mackay et al., 2003). BAFF expression has been detected predominantly in lymphoid tissues and in peripheral blood mononuclear cells (Moore et al., 1999; Mukhopadhyay et al., 1999; Schneider et al., 1999; Shu et al., 1999). In vivo studies have demonstrated that increased BAFF levels lead to increased peripheral B cell numbers (Batten et al., 2000; Gross et al., 2000; Khare et al., 2000;

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Mackay et al., 1999; Moore et al., 1999), whereas when BAFF is neutralized, peripheral B cell numbers decrease (Gross et al., 2000, 2001; Schneider et al., 2001; Thompson et al., 2000), indicating a tight relationship between BAFF levels and homeostatic B cell numbers. Furthermore, BAFF transgenic mice develop diseases resembling systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) (Batten et al., 2000; Groom et al., 2002; Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999), and SLE-prone NZB/W F1 mice have increased levels of BAFF in their serum (Gross et al., 2000; Kayagaki et al., 2002). BAFF levels are also elevated in humans with autoantibody-mediated diseases (Groom et al., 2002; Mackay et al., 2002; Mariette et al., 2003).

Three BAFF binding receptors have been identified, BAFF receptor (BAFF-R, also called BR3), B cell maturation antigen (BCMA), and transmembrane activator and CAML interactor (TACI) (Mackay et al., 2003). Studies examining A/WySnJ mice that harbor a spontaneous mutation in the BAFF-R gene and in BAFF knockout mice have demonstrated that BAFF-R is the key receptor involved in promoting B cell survival (Gross et al., 2001; Lentz et al., 1996, 1998; Miller and Hayes, 1991; Schiemann et al., 2001). By contrast, BCMA-deficient mice have a normal B cell compartment and TACI-deficient mice exhibit exaggerated B cell activity, suggesting a possible role for TACI as a negative regulatory molecule (von Bulow et al., 2001; Xu and Lam, 2001; Yan et al., 2001b). Expression analysis indicates that BAFF-R is upregulated during B cell maturation and is expressed at highest levels on peripheral B cells (Avery et al., 2003; Gorelik et al., 2004; Smith and Cancro, 2003). By contrast, TACI and BCMA mRNA are not upregulated during B cell maturation, and in a recent study these receptors were not detected on resting human B cells by flow cytometry (Avery et al., 2003; Smith and Cancro, 2003). While both TACI and BCMA can bind a cytokine closely related to BAFF, known as APRIL, BAFF-R binds BAFF exclusively (Thompson et al., 2001; Yan et al., 2001a) and APRIL does not appear to have a role in B cell homeostasis (Varfolomeev et al., 2004). BAFF-R, but not TACI or BCMA, activate NFκB-inducing kinase (NIK) and the alternative NFκB pathway, leading to p100 degradation and generation of p52 (Claudio et al., 2002; Kayagaki et al., 2002). Consistent with a critical role for this pathway in B cell survival, mice lacking NIK or p52 show severe deficiencies in peripheral B cells (Claudio et al., 2002; Miosge et al., 2002). BAFF-R, as well as TACI and BCMA, also activate the classical NF-κB pathway (Hatada et al., 2003; Mukhopadhyay et al., 1999). The mechanism by which BAFF and the alternative pathway of NFκB activation leads to enhanced B cell survival is unclear. Although activation of the BAFF pathway promotes small increases in Bcl-xL, A1, and possibly Bcl-2 expression (Claudio et al., 2002; Do et al., 2000; Hatada et al., 2003; Hsu et al., 2002), there is as yet no consensus on whether these or other molecules are the key mediators of BAFF's strong prosurvival effects.

In this study we set out to understand the molecular mechanisms regulating the rapid competitive elimination of autoantigen binding B cells in a diverse B cell repertoire. We demonstrate that HEL-autoantigen binding B cells have elevated levels of Bim mRNA and protein and that they are dependent on higher levels of BAFF than non-autoantigen binding B cells for survival. We provide evidence that homeostatic BAFF production is independent of B cell numbers and in Ig/HEL-double transgenic mice the number of B cells is reduced such that each cell is engaged by higher amounts of BAFF. Within a diverse B cell compartment, the competition between B cells for the available BAFF is such that autoantigen binding B cells are unable to bind the high levels of BAFF that they require for survival and they are rapidly eliminated.

Results

Increased BAFF Dependence of HEL-Autoantigen Binding B Cells

To test the dependence of HEL autoantigen binding B cells on BAFF for survival, we used an adoptive transfer approach where mature non- or Ig/HEL-double transgenic B cells were transferred to matched recipients that were either left untreated or treated with the BAFF antagonist, BCMA-Ig. Consistent with other findings (Gross et al., 2000; Harless et al., 2001; Pelletier et al., 2003), we found that mature nontransgenic B cells were dependent on constant BAFF exposure, with their numbers in BCMA-Ig-treated mice declining to half of the control numbers by 3 days and to 25% of the control by 7 days (Figure 1A), whereas cotransferred T cells were unaffected by BAFF antagonism (Figure 1A). Compared to the nontransgenic B cells, the HEL-autoantigen binding B cells underwent a more marked reduction in cell number such that after 2.5 days, less than 15% of the transferred anergic B cells remained in mice treated with BCMA-Ig (Figure 1B). This rapid elimination of autoantigen binding B cells is comparable to the competitive elimination seen when Ig/HEL double transgenic B cells are transferred into a HEL-expressing mouse with a diverse B cell repertoire (Figure 1B) (Cyster and Goodnow, 1995). To test whether HEL-autoantigen binding B cells are also more sensitive to partial reductions in BAFF levels, Ig/HEL-double transgenic B cells and nontransgenic B cells were cotransferred into Ig/HEL-double transgenic mice that were then treated with 30 or 100 μg doses of BCMA-Ig or with human IgG as a control. As a further control, naive Ig-transgenic B cells were transferred into nontransgenic recipients and these animals were also treated with BCMA-Ig or human IgG. At the 30 μg dose of BCMA-Ig, the HEL-autoantigen binding B cells were reduced by more than 80% at day 3 whereas the naive nontransgenic and Ig-transgenic B cells were only marginally reduced in number (Figure 1C). Similar results were seen in lymph nodes and blood (not shown). These observations indicate that HEL autoantigen binding B cells are more sensitive to BAFF depletion than naive B cells and they die more rapidly when BAFF is removed.

Increased BAFF per B Cell in Ig/HEL Double Transgenic Mice

The above findings led us to consider the possibility that Ig/HEL double transgenic B cells may be dependent on higher levels of BAFF for survival compared to naive B cells. To test this possibility, we first examined the level of BAFF mRNA expression in various mouse tis-

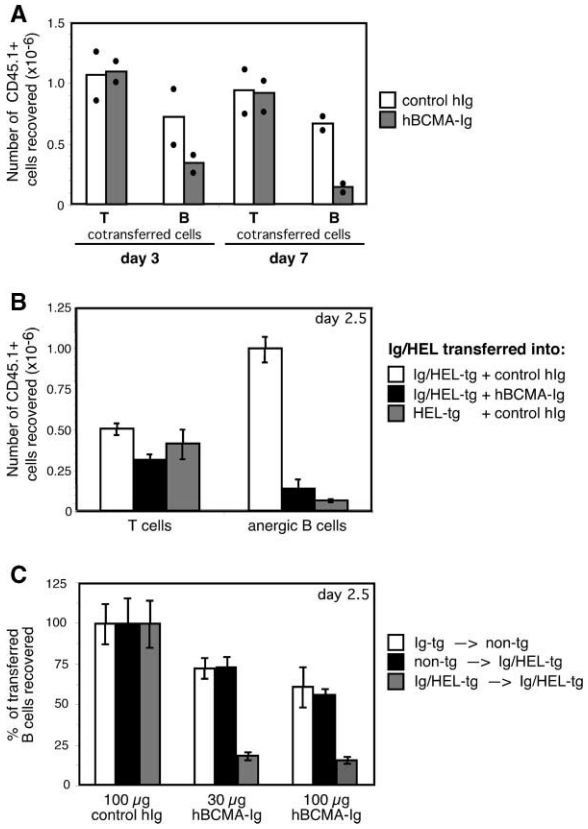

Figure 1. Autoreactive B Cells Have Increased Dependence on BAFF for Survival

(A) BAFF dependence of mature nontransgenic B cells. Mature lymph node B and T cells from CD45.1$^+$ mice were adoptively transferred into congenic CD45.2$^+$ recipients. Recipient mice were treated with 100 μg of control huIgG or huBCMA-Ig for 3 or 7 days, at which time CD45.1$^+$ T and B cells in the spleen were quantitated. Each point represents an individual animal and bars represent the mean. Data are representative of two similar experiments.

(B and C) Elevated dependence of autoantigen binding B cells on BAFF. In (B), spleen cells from CD45.1$^+$ Ig/HEL-double transgenic mice were transferred into CD45.2$^+$Ig/HEL-double transgenic or HEL-transgenic recipients. In (C), spleen cells from CD45.1$^+$ Ig/HEL-double transgenic mice or CD45.1$^+$ Ig-single transgenic mice were cotransferred with CD45.1$^+$ nontransgenic spleen cells into the indicated CD45.2$^+$ recipients. Recipient mice were treated with 100 μg (B) or the indicated amounts (C) of control huIgG or huBCMA-Ig at the time of cell transfer. After 2.5 days, transferred (CD45.1$^+$) B and T cells were quantitated in the recipient spleens. In (C), Ig-transgenic B cells were distinguished from cotransferred nontransgenic B cells by IgD^a and HEL/HyHEL9 staining and the nontransgenic B cell data shown are for the cells cotransferred with the Ig/HEL cells. In control treated mice, transferred Ig/HEL-, Ig-, and nontransgenic B cells survived similarly, as in previous studies (Cyster and Goodnow, 1995), and 3–9 $\times 10^5$ transferred cells were present per spleen. Data are shown as mean ± sd of three mice per group and are representative of three experiments.

sues by quantitative PCR. High levels of BAFF mRNA were detected in secondary lymphoid tissues and within blood cells (Figure 2A), consistent with previous reports (Moore et al., 1999; Mukhopadhyay et al., 1999; Schneider et al., 1999; Shu et al., 1999; Tribouley et al., 1999). High levels were also detected in peritoneal lavage cells and within bone marrow (Figure 2A). Comparison of BAFF mRNA levels in spleen cell supension and "stromal" fractions revealed strong enrichment for BAFF in the stromal fraction (Figure 2A). Splenic BAFF mRNA levels were not substantially affected by deficiency in LTα, LTβ, or TNF, consistent with the lack of evidence for B cell deficiencies in these mice (Fu and Chaplin, 1999), but also suggesting that LT- and TNF-dependent follicular dendritic cells (FDC) are not a major source of BAFF (Figure 2B). CSF1-deficient op/op spleens also contained amounts of BAFF message that were within the normal range, suggesting marginal zone macrophages are not a major source of this cytokine (Figure 2B). Importantly, BAFF expression was not reduced in Rag1$^{-/-}$ and μMT spleens, indicating that the amount of BAFF mRNA produced in the spleen in homeostasis is largely independent of the presence or absence of B cells (Figure 2B).

As Ig/HEL-double transgenic mice have approximately 2- to 3-fold fewer B cells than Ig- and nontransgenic mice, we considered the possibility that more BAFF may be available per HEL-autoantigen binding B cell in an Ig/HEL-double transgenic mouse than in a nontransgenic mouse. To measure this, we quantitated BAFF mRNA in lymphoid tissues from Ig/HEL-double transgenic or nontransgenic mice, and also CD19 mRNA as a measure of the B cell content of the tissues (Figure 2C). By flow cytometric analysis, CD19 levels on HEL-autoantigen binding and nontransgenic B cells were identical (data not shown). As a proportion of total RNA, levels of BAFF mRNA in the secondary lymphoid organs of the two types of mice were similar, whereas the amount of CD19 mRNA was lower in the Ig/HEL-double transgenic organs, reflecting the lower B cell numbers (Figure 2C). Therefore, when the ratio of BAFF mRNA to CD19 mRNA is considered, it is evident that there is more BAFF expressed per autoantigen binding Ig/HEL-double transgenic B cell than is expressed per nontransgenic B cell (Figure 2C). Serum ELISA analysis demonstrated that the difference in BAFF mRNA per B cell translated into a large difference in the abundance of serum BAFF, with Ig/HEL double transgenic mouse serum containing ~90 ng/ml compared to undetectable levels in the non- or HEL-transgenic mice (Figure 2D). Ig-transgenic mice, which have somewhat fewer B cells than nontransgenic mice, had slightly higher levels of serum BAFF than nontransgenics (Figure 2D), while B cell-deficient μMT mice showed significantly higher amounts of BAFF (833 ± 46 ng/ml, n = 3).

As a further approach to measure BAFF availability per B cell, we measured the amounts of BAFF protein bound to B cells in lymphoid organs by ex vivo flow cytometric analysis. In preliminary experiments, we observed that freshly isolated B cells can be stained with antibodies against the soluble portion of BAFF (Figure 3A). This staining was increased if cells were preincubated with further BAFF in vitro and diminished if mice were pretreated with BCMA-Ig to deplete endogeous BAFF, providing evidence of staining specificity (Figure 3A). Incubation of B cells at 37°C for up to 3 hr in the absence of added BAFF did not alter the amount of BAFF associated with the cells (data not shown), suggesting a long half-life for the BAFF-BAFF-R complex, consistent with studies indicating a high affinity (Kd ~0.1 nM) for this interaction and also with evidence that BAFF

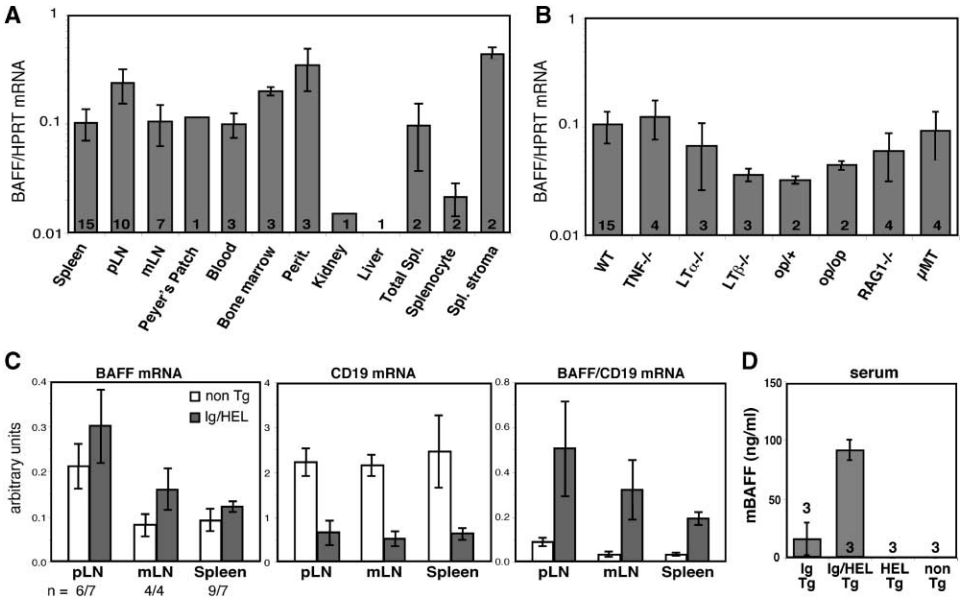

Figure 2. BAFF Expression Analysis and Elevated Levels of BAFF mRNA per B Cell in Ig/HEL Double Transgenic Mice

(A and B) Quantitative RT-PCR analysis of BAFF mRNA expression in (A) various tissues from wild-type mice and (B) in spleen tissue from mice of the indicated genotypes. Values were normalized to HPRT mRNA in each sample. PLN, peripheral lymph nodes (a mixture of axillary, brachial and inguinal); mLN, mesenteric lymph nodes; Perit., peritoneal lavage cells. All mice were on a C57Bl/6 background. The number of samples per group is shown in each bar and the bar represents mean ± sd.

(C) Estimation of BAFF mRNA levels per B cell in Ig/HEL double transgenic and nontransgenic mice. Quantitative RT-PCR was performed to measure BAFF mRNA (left panel) and CD19 mRNA (center panel) in lymphoid tissues of the indicated mice. The right panel shows the ratio of BAFF mRNA to CD19 mRNA as a measure of BAFF expression per B cell.

(D) ELISA analysis of serum BAFF levels in the indicated mice.

exists as a homotrimer and possibly a higher order complex (Kanakaraj et al., 2001; Kayagaki et al., 2002; Kim et al., 2003; Pelletier et al., 2003). To compare the amount of BAFF protein available per B cell in Ig/HEL-double transgenic mice and mice that have a full compartment of B cells, congenically marked nontransgenic B cells were transferred to Ig/HEL-double transgenic or HEL-single transgenic recipients. Six hours after transfer, significantly greater amounts of BAFF were bound to transferred B cells in Ig/HEL-double transgenic recipients than in HEL-transgenic recipients (Figure 3B, upper panels). Staining for BAFF-R at this 6 hr time point indicated that the levels were the same on the transferred cells in the two different recipients. Similar results were obtained if the donor B cells were autoantigen binding cells from Ig/HEL-double transgenic mice, with higher levels of BAFF being detected on cells transferred to double transgenic compared to HEL- or nontransgenic recipients (Figure 3B, lower panels). The similar level of BAFF on Ig/HEL-double transgenic B cells after transfer to either HEL- or nontransgenic recipients indicates that BAFF availability is not significantly different whether the cells are located at the B/T boundary (excluded) or in the follicle. In the course of this analysis we observed that Ig/HEL-double transgenic B cells have slightly lower levels of BAFF-R than nontransgenic B cells (Figure 3C, left panel). This was not a consequence of maturation differences as the majority (>65%) of double transgenic spleen B cells were mature (defined by lack of AA4 expression [Allman et al., 2001]) and the difference in BAFF-R level was also seen with lymph node B cells (data not shown). Initially we considered the possibility that the lower BAFF-R level might be due to the effects of chronic BCR signaling. However, when nontransgenic B cells were transferred to recipient Ig/HEL-double transgenic mice for 12 or more hr the level of BAFF-R was also found to be reduced (Figure 3C, middle panel), an effect that could not be attributed to autoantigen binding. Instead this observation suggested that the elevated levels of BAFF in Ig/HEL-double transgenic mice induced downmodulation of the receptor. Downregulation required 12 or more hr of in vivo exposure to elevated BAFF and was not seen in the 6 hr transfers used to measure amounts of BAFF that bound to the transferred cells (Figure 6B). In vitro incubation with saturating amounts of BAFF also led to receptor downregulation (Figure 3C, right panel). By Q-PCR we found similar levels of BAFF-R mRNA in Ig/HEL- and nontransgenic B cells (data not shown). TACI mRNA levels were also similar between the B cell populations while BCMA expression was low to undetectable (data not shown). Taken together, these observations indicate that the lower levels of BAFF-R on Ig/HEL-double transgenic B cells are most likely due to the higher levels of BAFF per B cell in these animals.

Constitutively Elevated BAFF Pathway Signaling in Ig/HEL Double Transgenic Mice

To determine whether the amount of BAFF signaling was greater for B cells in Ig/HEL-double transgenic mice compared to B cells in nontransgenic animals, we measured levels of NFκB2, an NFκB family member estab-

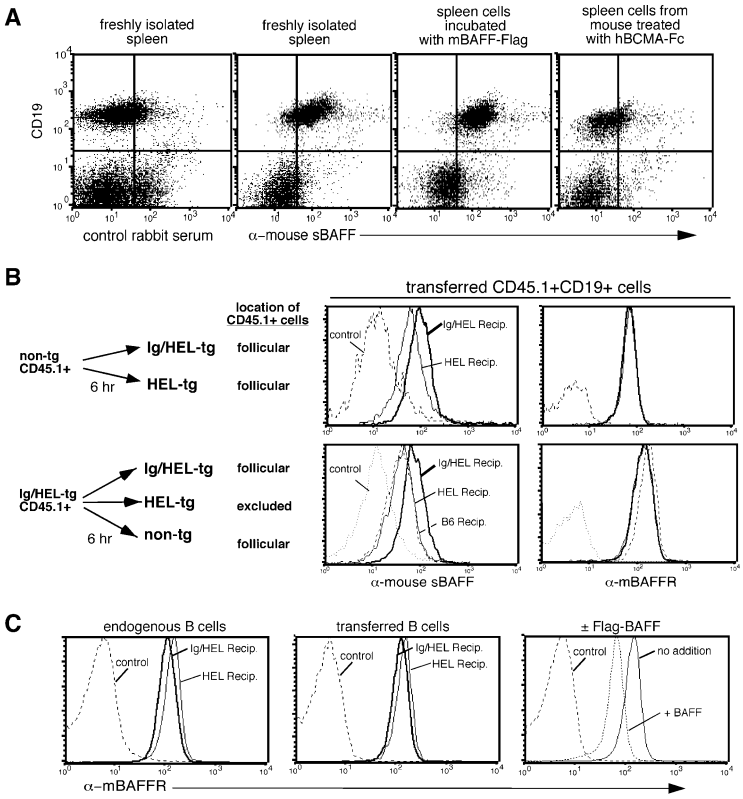

Figure 3. B Cells in Ig/HEL-Double Transgenic Mice Have More BAFF Bound per Cell than in Mice with a Diverse B Cell Repertoire

(A) Detection of levels of BAFF bound per B cell using a rabbit anti-BAFF serum. Freshly isolated spleen cells from untreated mice (two left panels), spleen cells incubated in vitro for 3 hr at 37°C with saturating amounts of mBAFF-Flag (center right panel), or spleen cells from an animal treated with 100 μg of BCMA-Ig for 2.5 days (right panel) were stained using rabbit anti-mouse BAFF serum or a control rabbit serum as indicated.

(B) Levels of BAFF bound per B cell in Ig/HEL-, HEL-, and nontransgenic mice. CD45.1$^+$ nontransgenic (upper panels) or CD45.1$^+$ Ig/HEL-double-transgenic (lower panel) spleen cells were transferred into Ig/HEL-double-transgenic, HEL-transgenic, or nontransgenic recipients, as indicated, and 6 hr later spleens were harvested for FACS analysis. Left histograms show anti-mouse BAFF staining and right histograms show anti-mouse BAFF-R staining on CD45.1$^+$CD19$^+$ cells in each type of recipient as indicated. Dotted line, staining with a control rabbit antiserum. The distribution of the transferred cells in recipient spleens (follicular or excluded from follicles) is indicated. The data in the upper and lower panels are from experiments performed on different days and therefore the staining intensity cannot be directly compared.

(C) Reduced BAFF-R on B cells in Ig/HEL-double transgenic mice and cells incubated in vitro with mBAFF-Flag. Left and center panels: CD45.1$^+$ nontransgenic spleen cells were transferred into Ig/HEL-double-transgenic or HEL-transgenic recipients and 12 hr later spleens were harvested for FACS analysis. The left panel shows anti-mouse BAFF-R staining on endogenous B cells (CD19$^+$CD45.1$^-$) and the middle panel shows anti-mouse BAFF-R staining on the transferred B cells (CD19$^+$CD45.1$^+$). The right panel shows the level of BAFF-R staining on B cells after incubation for 3 hr at 37°C in the presence or absence of mBAFF-Flag (0.5 μg/ml).

lished to function downstream of the BAFF-R (Claudio et al., 2002; Kayagaki et al., 2002). Degradation of NFκB2 p100 to the active p52 form was measured in follicular B cells sorted from the spleens of Ig/HEL-, non-, or Ig-transgenic mice (Figure 4A). Autoreactive B cells from Ig/HEL-double transgenic mice consistently showed higher p52 levels than detected in naive non- or Ig-transgenic B cells. As BCR stimulation is not thought to promote p100 processing (Claudio et al., 2002), and other studies have indicated reduced activation of the classical NFκB pathway downstream of the BCR in anergic B cells (Healy et al., 1997), it is unlikely that the increased p52 is due to the BCR stimulation. We also measured mRNA levels of the BAFF responsive gene, *Pim-2* (Xu et al., 2002), that encodes a kinase recently established to have prosurvival functions (Fox et al., 2003; Yan et al., 2003). A consistent ~2-fold elevation in Pim-2 mRNA levels was detected in Ig/HEL-double transgenic B cells (Figure 4B, left panel). These observations are consistent with the conclusion that there is more BAFF available per B cell in Ig/HEL-double transgenic mice than in animals with a diverse B cell compartment.

Autoantigen Binding B Cells Competing with Naive Cells Have Reduced BAFF Signaling

The analysis of BAFF levels and BAFF-R occupancy of B cells in HEL- and nontransgenic mice showed that there is less BAFF available to bind B cells in the competitive environment than in the noncompetitive Ig/HEL-double transgenic environment (Figure 3B). To determine whether BAFF signaling was also reduced, p100 and p52 levels were measured in autoantigen binding cells taken from a competitive environment. Large numbers of CD45.1$^+$ Ig/HEL-double transgenic cells were injected into HEL-transgenic mice and 18 hr later the recipient spleens were harvested and CD45.1$^+$ autoantigen binding B cells isolated by FACS sorting. Consistent with the hypothesis that autoantigen binding B cells receive less BAFF signal in the competitive environment, we no longer saw an increase in p52 levels in autoreactive Ig/HEL-double transgenic B cells as compared to the endogenous follicular B cells (Figure 4A). Simlarly, we no longer detected an increase in Pim-2 mRNA in competing autoantigen binding B cells compared to the naive B cells isolated from the same animals (Figure 4B). p52 protein and Pim-2 mRNA also returned to endogenous B cell levels when Ig/HEL-double transgenic B cells were transferred to nontransgenic recipients (Figures 4A and 4B, rightmost panels), conditions under which they experience reduced BCR engagment and migrate into follicles (Cyster and Goodnow, 1995). Therefore, the reduction in p52 and Pim2 in HEL-transgenic recipients cannot be explained by increased BCR engagement or by follicular exclusion of the cells. Instead, these observations favor the conclusion that

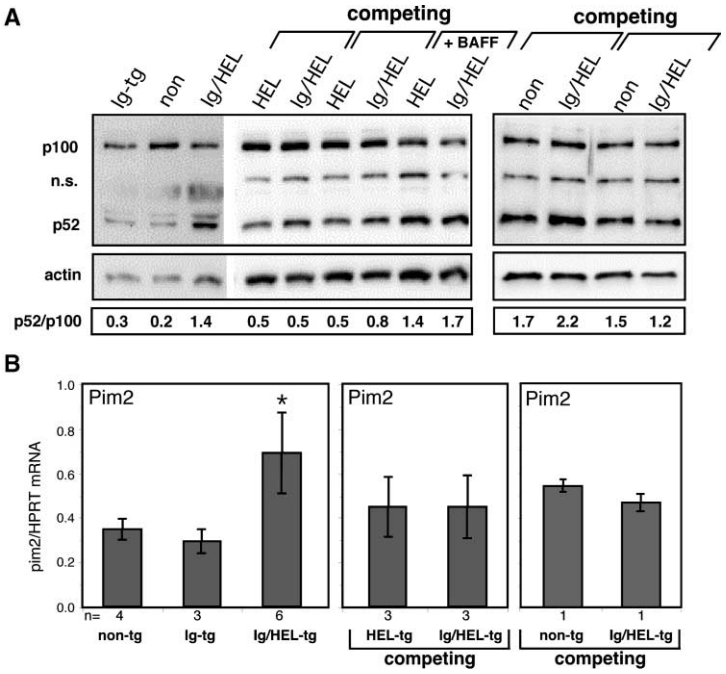

Figure 4. Ig/HEL Double Transgenic B Cells Have Constitutively Elevated BAFF Signaling but Show Reduced Signaling in the Presence of Competitor Cells

(A) Western blot analysis of NFκB p100 and p52 levels in autoantigen binding B cells in the absence and presence of a diverse competitor B cell repertoire. For B cells in the absence of competition (left panel, lanes 1–3), $B220^+CD23^+CD21$intermediate follicular B cells were sorted from the spleens of Ig-, non-, or Ig/HEL-transgenic mice. For competing B cells, $2–4 \times 10^7$ $CD45.1^+$ Ig/HEL-transgenic B cells were adoptively transferred into $CD45.2^+$ nontransgenic mice injected with 1 mg of HEL prior to cell transfer (lanes 4 and 5 of left panel); $CD45.2^+$ sHEL-transgenic mice (lanes 6–9 of left panel), or $CD45.2^+$ nontrangenic mice (lanes 1–4 right panel). 18 hr later, $B220^+$ CD21intermediate $CD45.1^+$ (Ig/HEL-double transgenic) or $B220^+$CD21intermediate $CD45.1^-$ (nontransgenic endogenous) B cells were sorted from the recipient spleens. In the lanes indicated +BAFF, the recipient mice were treated with 50 μg of huBAFF-HIS at 0 and 13 hr after transfer. n.s., nonspecific band detected with antiserum. Numbers shown below panels represent the ratio of p52 to p100 for each sample. In different Western blot analyses, the large p100 protein was found to transfer with different efficiencies and therefore the values obtained for the p52/p100 ratios can only be compared for samples run on the same gel. The left panel represents a single gel (lanes 1–3 were exposed for longer than lanes 4–9) and the right panel is a separate gel. Data for noncompeting samples are representative of two experiments with sorted cells and two experiments with autoMACS purified cells.

(B) Pim-2 mRNA levels in autoantigen binding B cells in the absence and presence of competitor B cells. Cells sorted as in (A) were used to prepare mRNA and the amount of Pim-2 mRNA was measured by quantitative RT-PCR. HPRT message was used as a control for equal mRNA loading. Panel shows the mean and standard deviation from three Q-PCR runs for the indicated number of independent samples. *$p < 0.001$ for Ig/HEL-tg compared to non-tg or Ig-tg using student's T test.

when double-transgenic B cells are placed in competition with a full compartment of naive cells, they no longer receive elevated levels of BAFF and are no longer able to maintain the exaggerated p52 or Pim-2 levels.

Excess BAFF Can Rescue Autoantigen Binding B Cells from Competitive Elimination

If autoreactive B cells require more BAFF for survival and do not have enough BAFF available to them when they are in a compartment with a diverse repertoire of competitor B cells, it follows that they may be rescued from competitive elimination by excess BAFF. To test this, Ig/HEL-double transgenic B cells were transferred into HEL-transgenic recipients that were treated with saline or BAFF (Figure 5A). While Ig/HEL-double transgenic B cells were almost completely eliminated from HEL-transgenic spleens within 2 to 3 days, daily BAFF treatment was able to partially rescue autoreactive B cells from competitive elimination (Figure 5A). Western blot analysis of B cells sorted from BAFF-treated mice showed that p52 levels were elevated (Figure 4A). During the treatment period, host B cell numbers also increased, although the survival effects of BAFF were greater on the HEL-autoantigen binding B cells (~5-fold increase) than on the endogenous host B cells (less than 2-fold increase) (Figure 5A). Analysis of B cell distribution within the spleen at day 3 after transfer demonstrated that BAFF-rescued autoantigen binding B cells, like the few autoantigen binding cells remaining in untreated mice, were excluded from B cell follicles (Figure 5B). To further explore the relationship between competitive elimination and BAFF dependence, we tested whether increased levels of BAFF could rescue autoantigen binding cells developing within the polyclonal repertoire of mixed bone marrow chimeras. Under these conditons, about half of the HEL autoantigen binding cells in the spleen are in an immature or transitional state (Figure 5C). However, as observed in the transfer studies, 3 days of BAFF treatment was able to rescue autoantigen binding cells, increasing their numbers to a greater extent than its effect on non-autoantigen binding B cells (Figures 5C and 5D). This effect was evident in both the immature $AA4^+$ and mature $AA4^-$ populations (Figures 5C and 5D). Increased numbers of autoantigen binding B cells were also detected in lymph nodes, and these cells were predominantly (>95%) $AA4^-$ mature cells (Figure 5D).

Elevated Bim Levels in Autoantigen Binding B Cells

The increased dependence of HEL-autoantigen binding B cells on BAFF for survival appeared likely to be due to increased activity of proapoptotic molecules induced by chronic autoantigen-mediated BCR stimulation in these cells. We considered the possibility that the BH3-only protein, Bim, may have a role in promoting elimination of Ig/HEL autoantigen binding B cells, as Bim-defi-

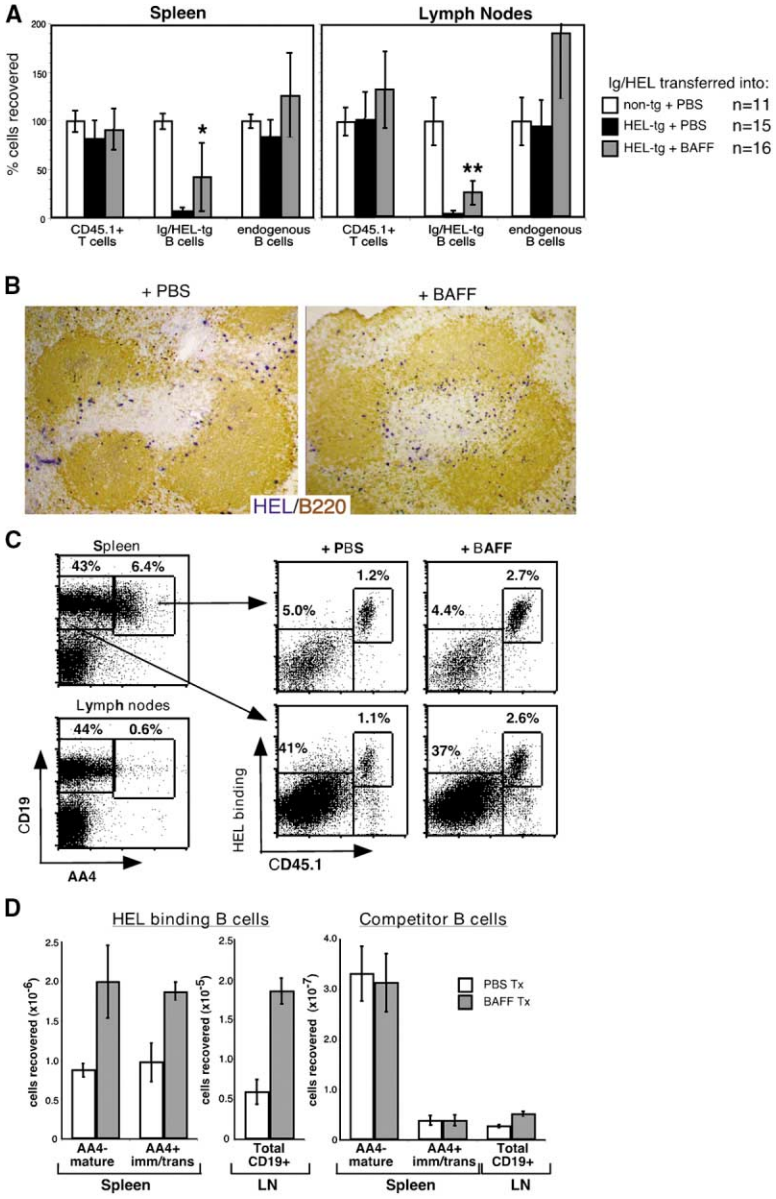

Figure 5. Excess BAFF Can Rescue Autoantigen Binding B Cells from Competitive Elimination

(A) CD45.1$^+$ Ig/HEL-double-transgenic spleen cells were adoptively transferred into CD45.1-HEL-transgenic mice and recipients were treated with PBS or hBAFF-HIS (50 μg) twice daily. As a control for maximal rescue of autoantigen binding cells, Ig/HEL-transgenic spleen cells were also transferred into nontransgenic recipients. Donor T cell numbers were monitored as a cell transfer control. After 2 or 3 days CD45.1$^+$CD19$^+$ or CD45.1+HEL binding cells and CD45.1$^+$CD3$^+$ from recipient spleen and lymph nodes were quantitated. Data shown are the mean ($\pm$sd) from 11–16 mice per group, pooled from four experiments after 2 or 3 days of transfer. Data are normalized to the nontransgenic recipient (set at 100%) from each experiment to account for the different number of B cells transferred in the separate experiments. *$p < 0.001$, **$p < 0.00001$ (student's T test).

(B) Distribution of transferred HEL-autoantigen binding B cells in mice treated for 3 days with PBS or BAFF. Frozen spleen sections were stained to detect total B cells (B220, brown) and transferred HEL binding B cells (blue). In the experiment shown, BAFF caused a 3-fold rescue of Ig-transgenic B cells as determined by FACS quantitation.

(C and D) Rescue of autoreactive B cells in mixed bone marrow chimeras. HEL-transgenic mice reconstituted with a mixture of CD45.1$^+$ Ig-transgenic and CD45.1$^-$ nontransgenic bone marrow (see Experimental Procedures) were treated with PBS or hBAFF-HIS (100 μg) twice daily for 2.5 days. In (C), spleen and LN cells were stained to detect AA4, CD19, CD45.1, and HEL binding. Left panels show total lymphocyte-size gated cells with numbers showing percentage of total cells that were CD19$^+$AA4$^+$ immature/transitional cells or CD19$^+$AA4$^-$ mature cells. Right panels show the CD19$^+$AA4$^+$ (upper panels) or CD19$^+$AA4$^-$ (lower panels) spleen cells from PBS- or BAFF-treated mice and numbers show percentage of total cells within the indicated gates. In (D) the left and center panels show the number of HEL binding CD45.1$^+$ CD19$^+$ AA4$^-$ or AA4$^+$ spleen cells or CD45.1$^+$ CD19$^+$ LN cells and the right panel shows the non-HEL binding CD45.1$^-$ CD19$^+$ AA4$^-$ or AA4$^+$ spleen cells and CD45.1$^-$ CD19$^+$ LN cells as indicated. Data are for three mice per group (mean $\pm$ sd) and are representative of two experiments.

cient mice show significantly increased numbers of B cells and B cells from these mice survive for prolonged periods in vitro (Bouillet et al., 1999). Bim has also been demonstrated to promote the death of antigen-stimulated peripheral T cells (Davey et al., 2002; Hildeman et al., 2002). While our manuscript was in preparation, Strasser and coworkers reported that Bim functions in the peripheral elimination of autoantigen binding B cells (Enders et al., 2003). Measurements of Bim mRNA and protein in HEL-autoantigen binding and naive B cells revealed that B cells from Ig/HEL-double transgenic mice had increased Bim mRNA (Figure 6A) and protein (Figure 6B) compared to B cells from Ig-transgenic or nontransgenic mice. Bim mRNA and protein were further increased in competing anergic B cells sorted from HEL transgenic recipients (Figures 6A and 6B, right panels).

Discussion

The above findings demonstrate that mature autoreactive B cells have a greater dependence on BAFF for survival than naive B cells. When in a host with a diverse repertoire of B cells, autoantigen binding cells receive insufficient amounts of BAFF to be protected from BCR-induced death signals and they are competitively eliminated. Bim protein levels are elevated in autoantigen binding B cells indicating that BAFF must act at least in part by antagonizing Bim function (Figure 7A). These

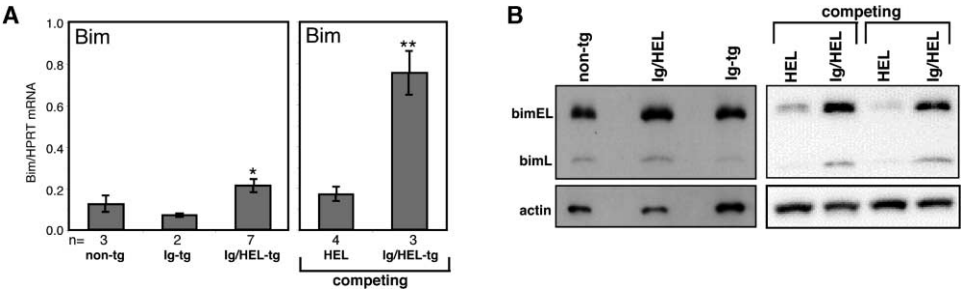

Figure 6. Elevated BIM Levels in HEL Autoantigen Binding B Cells

(A and B) Analysis of Bim mRNA and protein levels in autoantigen binding B cells in the absence and presence of competitor non-autoantigen binding B cells. Transfers were performed and samples prepared as described in Figure 4. In (A), data shown are the average and standard deviation from one Q-PCR anlaysis of Bim mRNA relative to HPRT mRNA of the indicated number of sorted B cell samples. *$p < 0.05$ for comparison of Ig/HEL-tg and non-tg, **$p < 0.01$ for comparison of competiting Ig/HEL-tg and non-tg cells (student's T test). In (B), Bim protein was detected by Western blot in the indicated samples using anti-bim polyclonal antibody. Protein levels were determined with anti-β-actin antibodies. The left panel is representative of two experiments with FACS sorted cells.

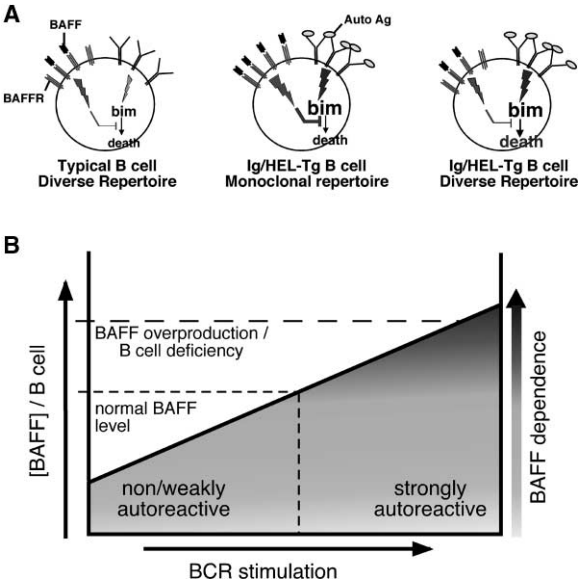

Figure 7. Proposed Model to Explain Elimination of Autoantigen Binding B Cells from a Diverse B Cell Repertoire

(A) The typical B cell in a diverse repertoire receives minimal death signals through the BCR. With a constant amount of BAFF, an equilibrium B cell number is achieved where each B cell receives enough BAFF to survive many weeks. In a monoclonal Ig/HEL-transgenic repertoire the autoantigen binding B cells receive a stronger death signal, involving increased levels of Bim expression, due to chronic BCR stimulation and these cells are more dependent on BAFF for survival. Under these conditions the equilibrium is shifted such that there are fewer B cells in the compartment and more BAFF per B cell. When autoantigen binding B cells are present in a diverse repertoire, the BAFF per B cell ratio is insufficient for the autoantigen binding B cells to be protected from the death signal and they are rapidly eliminated.

(B) Suggested relationship between autoantigen-induced BCR signal strength, BAFF levels, and BAFF dependence for survival. Under normal conditions, BAFF levels are fixed, B cell production is in excess, and only the non- or weakly autoreactive cells that have a low or intermediate dependence on BAFF can survive. Under conditions where BAFF production is elevated or B cell production is reduced, BAFF levels per B cell are increased allowing strongly autoreactive B cells that have a higher BAFF dependence to survive.

observations suggest that under conditions where BAFF levels per B cell are elevated there will be an increased accumulation of autoantigen binding cells. Reciprocally, the greater BAFF dependence of autoantigen binding B cells suggests that treatments that cause partial reductions in BAFF may selectively deplete the most strongly autoreactive cells.

A long-running question in the Ig/HEL-transgenic B cell tolerance model has been to understand why B cells that are chronically engaged by soluble HEL-autoantigen can survive for more than 1 week in Ig/HEL-double transgenic mice, where all the B cells are HEL binding, but survive less than 3 days in mice with a diverse B cell repertoire (Cook et al., 1997; Cyster and Goodnow, 1995; Cyster et al., 1994; Fulcher and Basten, 1994). Although differences in antigen-receptor occupancy are observed under these conditions, with occupancy being greater in the competitive situation due to the smaller number of HEL binding B cells, these differences have not provided a sufficient explanation for the differences in survival (Cyster, 1997; Phan et al., 2003). In particular, in Ig/HEL double transgenic mice carrying the highly expressed albumin promoter-HEL transgene (AL3 line) rather than the metallothionein promoter-HEL transgene (ML5 line) (Adelstein et al., 1991), the BCR is fully occupied yet the cells are not rapidly eliminated (Phan et al., 2003; unpublished data). Furthermore, when autoantigen binding cells were transferred in equal numbers to HEL-containing B cell-deficient or wild-type recipients, the cells survived in the B cell-deficient recipients while being rapidly eliminated in the wild-type recipients (Schmidt and Cyster, 1999). Our findings here indicate that a key difference between the noncompetitive and competitive situations relates to the amount of BAFF available per B cell (Figure 7B). We demonstrate that homeostatic BAFF production occurs in a manner that is largely independent of the number of B cells in the host, with spleen and lymph nodes from B cell-deficient mice containing similar amounts of BAFF mRNA as spleen and lymph nodes from wild-type mice. In Ig/HEL-double transgenic mice, the B cell compartment size has been reset to about half to one-third the size of Ig-single transgenic mice, such that a new equilibrium is achieved between B cell numbers and the amount of

BAFF per B cell. Our results indicate that this increased level of BAFF per B cell, and associated increase in BAFF-R signaling, is critical for the ability of the autoantigen-engaged cells to survive for more than a few days. Reciprocally, the greater dependence of HEL-autoantigen binding B cells on BAFF causes them to be poor competitors relative to non-autoantigen binding B cells. When in a diverse B cell compartment they cannot maintain the higher levels of BAFF-R signaling required to overcome the BCR-induced death signal and they are deleted (Figure 7).

In earlier studies, a correlation was observed between exclusion of autoantigen binding B cells from lymphoid follicles and rapid elimination of the cells (Cyster et al., 1994). To examine whether this might reflect BAFF being present at higher levels in follicles than in the T zone or other sites, we attempted to determine the distribution of BAFF within secondary lymphoid tissues. BAFF mRNA was detected at highest levels in lymphoid tissue stromal preparations and was not significantly diminished in CSF1-deficient mice that lack many macrophages, consistent with a recent report demonstrating that BAFF is made predominantly by radiation-resistant cells (Gorelik et al., 2003). We also examined expression levels in the spleen of LTα/β or TNF-deficient mice as these animals are deficient in lymphoid stromal cells, particularly FDCs (Fu and Chaplin, 1999). The spleen tissues from these animals maintained levels of BAFF mRNA that were not significantly different from wild-type controls. Thus, although the quantitative PCR approach does not exclude the possibility of a reduction of up to 30%–40% in BAFF expression, it would appear that mature FDC are not a major source of BAFF. Attempts to more precisely determine the location of BAFF-producing cells using rabbit antibodies raised against both the extracellular and cytoplasmic domains of BAFF have so far been unsuccessful (not shown). The finding that levels of BAFF bound to transferred Ig/HEL-double transgenic B cells was equivalent whether the cells were excluded from follicles (in HEL-transgenic recipients) or localized within follicles (in nontransgenic recipients) suggests that follicular access is not a major factor determining the extent of access to BAFF. Consistent with the notion that the B cell competition for BAFF is not dependent on compartmental segregation, we have recently found that CCR7$^{-/-}$ Ig/HEL-double transgenic B cells are not excluded from the B cell follicle, yet still undergo rapid cell death when in a diverse B cell repertoire (Ekland et al., 2004).

While BAFF expression in the spleen was not strongly dependent on LTα or β, the requirement of this cytokine for lymph node and Peyer's patch development means that a fraction of the BAFF produced in the body is absent in mice lacking LTα/β. We speculate that this reduces total BAFF levels in the body and therefore that there is a corresponding reduction in total body B cells. In addition to expression in secondary lymphoid organs, we found it notable that BAFF was expressed at high levels within the bone marrow. Expression has also been detected in human bone marrow (Moore et al., 1999). However, BAFF is not thought to be required for B cell development in the bone marrow (Schiemann et al., 2001). Instead bone marrow BAFF most likely plays an important role in the survival of long-lived plasma cells (Avery et al., 2003; O'Connor et al., 2004) and perhaps also for recirculating bone marrow B cells. Bone marrow expression is likely to contribute to systemic levels of BAFF and thereby influence the size of the mature B cell compartment.

The mechanism by which BCR signaling promotes B cell death is not fully defined, but many studies have indicated that Bcl-2 overexpression can provide some protection from BCR-induced apoptosis. The proapoptotic molecule, Bim, has been established to play a role in elimination of antigen- and autoantigen- binding T cells (Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002) and to function in promoting B cell homeostasis (Bouillet et al., 1999). In work that appeared while our study was in preparation, Enders et al. demonstrated that Bim is involved in elimination of in vitro anti-IgM-stimulated cells and that it is required for in vivo elimination of HEL-autoantigen binding B cells (Enders et al., 2003). This study also demonstrated that anti-IgM-stimulated cells exhibited a small increase in Bim protein levels (Enders et al., 2003). Our finding that Bim mRNA and protein levels are elevated in freshly isolated HEL-autoantigen binding B cells is in agreement with these observations. Based on these combined data, we consider that the most straightforward explanation for how BAFF protects HEL-autoantigen binding B cells from elimination is by antagonizing Bim function. In vitro studies have shown that prosurvival Bcl-2 family members Bcl-xL, A1, and possibly Bcl-2 are upregulated by BAFF (Claudio et al., 2002; Hatada et al., 2003), although it has been less clear whether these molecules are regulated in vivo by BAFF. During our study we measured mRNA levels of Bcl-2, Bcl-xL, A1, and Mcl-1 in cells exposed to increased or decreased amounts of BAFF. Although we observed upregulation of Bcl-2, Bcl-xL, and A1 mRNA by quantitative PCR analysis after 6 hr in vitro culture with BAFF (not shown), increased expression of these genes was not maintained upon longer (24 hr) incubation. Moreover, in in vivo studies where mice were treated with BAFF to augment signaling or with BCMA-Ig to inhibit BAFF signaling, we did not observe any reproducible effects on levels of Bcl-2, Bcl-xL, A1, or Mcl-1 mRNAs in purified B cells (not shown). However, we were able to detect increases in the levels of mRNA for Pim-2, a ser/thr kinase that has recently been shown to function as a prosurvival molecule downstream of hematopoetic growth factors (Fox et al., 2003; Yan et al., 2003), and to interact with the BH3-only molecule, Bad (Yan et al., 2003). Pim-2 was also identified as an NFκB regulated gene but it is not yet known if it is induced by p52 complexes (Li et al., 2001). A full characterization of Pim-2-deficient mice has not been reported, although it has been stated that these animals are phenotypically normal (Allen and Berns, 1996). It will be important in future studies to determine whether autoantigen binding B cells are more readily eliminated in these animals.

While our studies indicate that differential dependence on BAFF contributes to the competitive elimination of autoantigen binding B cells, they do not exclude the possiblity that B cells compete for additional prosurvival activities. In this regard, we previously found that autoantigen binding B cell survival was augmented in an antigen nonspecific manner by the presence of T cells

(Schmidt and Cyster, 1999). Survival of Btk-deficient B cells is also augmented by T cells (Karagogeos et al., 1986). As naive T cells are not a source of BAFF and we have not observed reduced BAFF levels in spleens from T cell-deficient mice (not shown), this effect most likely involves another B cell trophic activity. There is also the possibility that proapoptotic signaling increases when autoantigen binding B cells are competing with other B cells. The increased Bim levels in competing compared to noncompeting autoantigen binding B cells (Figure 5) is consistent with this possibility. We examined whether the rise in Bim levels might be secondary to the reduced BAFF availability but have so far not observed an effect of increasing or decreasing BAFF in vivo on B cell Bim mRNA levels (R.L., Y. X., and J.G.C., unpublished data). Therefore, in addition to the competition for BAFF demonstrated here, competitive elimination of B cells may involve elevated proapoptotic signaling and possibly competition for other trophic factors.

Several reports have demonstrated a relationship between BAFF levels and autoantibody-mediated disease. Transgenic mice overexpressing BAFF develop rheumatoid factor, anti-DNA, and anti-nuclear antibodies (Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999), and some animals show features of Sjögren's syndrome (SS) (Groom et al., 2002). Furthermore, serum BAFF levels and B cell p52 levels are elevated in lupus prone NZB/W F1 mice (Gross et al., 2000; Kayagaki et al., 2002) and BAFF levels are high in a fraction of human lupus, RA, and SS patients (Groom et al., 2002; Zhang et al., 2001). Our findings indicate that elevated BAFF levels may predispose to autoimmunity by increasing the threshold amount of chronic BCR signaling that is required to cause B cell deletion in the periphery (Figure 7B). Therefore, individuals with high BAFF levels might have a higher burden of autoantigen binding B cells in the periphery. The recent demonstration that many of the B cells that emerge into the periphery in humans are autoreactive and that a fraction of these specificities are normally purged prior to full maturation highlights how shifts in BAFF levels might readily affect the fraction of the mature B cell repertoire that is autoreactive (Wardemann et al., 2003). Moreover, the association between B cell deficiency and the development of systemic autoimmune diseases such as autoimmune hemolytic anemia (August and Hathaway, 1989) might be a consequence of the higher BAFF levels per B cell in immunodeficient states. We consider it less likely that BAFF overproduction rescues cells from deletion in the bone marrow as immature bone marrow B cells express little if any BAFF-R (Gorelik et al., 2004; Hsu et al., 2002; and unpublished data) and addition of BAFF to bone marrow B cells in an in vitro B cell tolerance model failed to rescue the BCR engaged cells (Rolink et al., 2002). Conversely, it seems likely that the absence of BAFF-R on immature cells helps ensure that they are deleted when persistently engaged by multivalent self-antigen. Finally, the greater dependence of chronically engaged peripheral B cells on BAFF suggests that treatments that cause only partial blocking of BAFF, and thus have minimal effects on naive B cell numbers, may be effective in eliminating autoreactive B cells without disrupting B cell immunity. Thus, even partial BAFF antagonism may have therapeutic benefit in patients prone to developing autoantibody-mediated diseases.

Experimental Procedures

Reagents

Human BCMA-Ig and human IgG control were produced as described (Thompson et al., 2000). Hamster anti-mBAFF-R (B9C11) was prepared as described (Gorelik et al., 2004). Human BCMA-Ig neutralizes both mBAFF and mAPRIL (Schneider et al., 2001). Human soluble HIS-BAFF (aa 134-285) used for in vivo BAFF treatments was produced as described (Liu et al., 2002). In one experiment with mixed bone marrow chimeras, human soluble myc-BAFF (Karpusas et al., 2002) was used with similar results. To generate antibodies against mouse BAFF, the extracellular region (aa 127-310) with an NH_2-terminal HIS-tag was expressed in the pET expression system in BL21 bacteria. The band corresponding to mouse HIS-BAFF was cut from an SDS-PAGE gel and used as an immunogen to generate rabbit polyclonal anti-mouse BAFF serum. To generate antibodies against the mouse BAFF cytoplasmic domain, amino acids 1-45 were expressed as a GST fusion protein and the affinity purified protein was used as an immunogen in rabbits. The anti-cytoplasmic domain antibody was used as a staining control in some flow cytometric experiments. Rabbit polyclonal anti-p52 (raised against aa 1-399) was a kind gift of U. Siebenlist (Laboratory of Immunoregulation, NIAID). Mouse soluble BAFF-Flag was produced by transiently transfecting 293T cells with a pEF vector containing mouse BAFF amino acids 127-310 with an NH_2-terminal prolactin signal sequence and FLAG-tag (vector as described in Gunn et al. [1998]) or purchased from Alexis Biochemicals.

Animals

Ig/HEL double transgenic mice were generated by crossing HEL-specific MD4 Ig transgenic mice (Goodnow et al., 1988) to ML5 sHEL Tg mice (Adelstein et al., 1991) or by making radiation chimeras as described (Cyster and Goodnow, 1995) using MD4 Ig transgenic bone marrow to repopulate lethally irradiated (1100 Rads) ML5 sHEL transgenic mice. $Rag1^{-/-}$, μMT mice were purchased from Jackson Laboratories. Tissues from $TNF^{-/-}$, LTα, LTβ, op/+, and op/op mice were prepared as previously described (Ngo et al., 1999). Mixed bone marrow chimeras were produced by reconstituting ML5 homozygous transgenic mice with 80% CD45.1 Ig-transgenic bone marrow and 20% CD45.2 nontransgenic bone marrow as previously (Cyster et al., 1994). Analysis of the immature bone marrow B cell compartment of reconstituted mixed chimeras revealed that HEL binding B cells represented about 20%–30% of the cells, whereas only 2%–5% of peripheral B cells were HEL binding, as in previous studies (Cyster et al., 1994).

Adoptive Transfer

Adoptive transfers were intravenous, and unless otherwise noted, intraperitoneal treatments with BAFF or BCMA-Ig were begun at the time of cell transfer. All donors and recipients were sex matched. In most cases, T cells in the donor spleen or LN preparations were used to confirm uniformity of cell transfer numbers in each animal. To study competition, Ig/HEL double transgenic spleen cells were injected intravenously into HEL transgenic recipients. Spleen cells were injected such that each recipient received 5–10 $\times$ 10^6 Ig/HEL double transgenic B cells.

Flow Cytometry

Single cell suspensions from spleen, mesenteric, and brachial lymph nodes, or blood (RBC lysed) were incubated with various antibodies for four-color flow cytometry on a Becton Dickinson FACS Caliber. Monoclonal antibodies to CD19, CD45.1, CD3, CD4, CD8, CD19, B220, IgD^a, IgM^a, CD21, and CD23 were from BD Pharmingen (San Diego, CA), as was polyclonal goat anti-Rabbit biotin. HyHEL9-tricolor and HyHEL9-PECy5.5 were from custom conjugations performed by Caltag laboratories. AA4-PE was a gift of D. Allman. Anti-Flag-biotin (M2) was from Sigma and was preadsorbed with 4% normal rat and mouse serum to prevent background staining. Streptavidin-APC was from Molecular Probes (Eugene, OR). Follicular $B220^+$ CD21intermediate $CD23^+$ B cells were isolated on a MoFlo

FACS sorter (Cytomation). Competing autoantigen binding ($CD45.1^+$) and endogenous ($CD45.1^-$) B cells were sorted on the basis of CD45.1 positive or negative, CD21 intermediate, and $B220^+$ staining, excluding $CD21^{high}$ marginal zone B cells. All sorted B cells were >98% pure. For BAFF occupancy staining, $CD45.1^+$ nontransgenic or Ig/HEL-double transgenic cells were transferred i.v. into Ig/HEL-double transgenic non-, or sHEL-transgenic recipients. 6 or 12 hr later recipient spleens were harvested on ice for FACS analysis. After FcR blocking, cells were incubated with rabbit anti-mouse BAFF-ectodomain serum at a 1:1000 dilution, followed by goat anti-rabbit-biotin and then SA-APC along with anti-CD45.1-FITC and anti-CD19-PE. In some experiments, unconjugated anti-HEL (clone HyHEL9) was included with the anti-FcR to block any nonspecific binding of antibodies to the HEL bound to the B cells. Results were identical whether HyHEL9 was included or not.

Taqman Real-Time PCR Analysis

Primer pairs and probes, including their specificity, orientation (forward, F; reverse, R), and sequence were as follows: HPRT(F-AGG TTGCAAGCTTGCTGGT, R-TGAAGTACTCATTATAGTCAAGGGCA, probe-TGTTGGATACAGGCCAGACTTTGTTGGAT), BAFF (F-CAGG AACAGACGCGCTTTC, R-GTTGAGAATGGCGGCATCC, probe-AGG GACCAGAGGAAACAGAACAAGATGTAGACCT), CD19 (F-CCATC GAGAGGCACGTGAA, R-GACTATCCATCCACCAGTTCTCAAC, probe-CATTGCAAGGTCAGCAGTGTGGCTCT), Bim (F-CGGATCGGAGAC GAGTTCA, R-GTCTTCAGCCTCGCGGTAAT, probe-CGAAACTTACAC AAGGAGGGTGTTTGCAA), and Pim2 (F-TCCTGGGTAAGGGAGGC TTT, R-CCTAGCACACGGTTCCGG, probe-CGGATAGACGTCAGG TGGCCATCAA). Quantitative RT-PCR was performed on an ABI7700 sequence detection instrument (Taqman; PE Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Tissues were taken from C57BL/6 mice as indicated. Total WT spleen was compared to splenocyte and stromal fractions of spleen that were prepared by mashing the spleen through a 70 μm nylon filter. The single cell suspension that passed through the filter into the media is termed "splenocytes." The white material that did not dissociate into a cell suspension was retrieved from the top of the filter and termed the splenic "stromal" fraction. This fraction probably contains fibroblasts, follicular dendritic cells, other B zone stromal cells, T zone stromal cells, dendritic cells, marginal zone, and red-pulp macrophages, and connective tissue.

Western Blot

For Western blot of sorted B cells, 1–10 $\times 10^6$ B cells were washed free of serum and lysed. 6–15 μg of whole protein was run on 12% Tris-HCl acrylamide Ready Gels (BioRad), transferred to Immobilon-P PVDF transfer membrane, and probed with the following antibodies: rabbit polyclonal anti-p52 (aa.1-399) (a kind gift of U. Siebenlist), rabbit polyclonal anti-Bim (BD Pharmingen), and rabbit polyclonal anti-actin (Sigma). Anti-rabbit HRP and ECL^+ were from Amersham. Blots were visualized on Kodak film or using a Kodak ImageStation and bands were quantitated using 1D ImageStation software. For comparisons between levels of p100 and p52, it was necessary to compare samples that were analyzed on the same gel since the extent of transfer of the large p100 protein varied considerably between different blots.

Immunohistochemistry

9×10^6 $CD45.1^+$ congenic Ig-transgenic splenic B cells were transferred intravenously into sex matched sHEL-transgenic recipients treated with PBS or human sBAFF-HIS (50 μg twice daily) starting approximately 8 hr after cell transfer. Three days later, recipient spleens were harvested for FACS and IHC. HEL binding cells in 7 μm sections were detected by incubating the section in 1 μg/ml HEL followed by incubation with polyclonal rabbit anti-HEL-biotin (Rockland), then with streptavidin-AP, and detected with Fast Blue. B zones were detected by staining with Rat anti-B220 (RA3-6B2) (BD Pharmingen) followed by donkey anti-Rat-HRP (Jackson ImmunoResearch Labs) and detected with DAB.

ELISA

Mouse BAFF ELISA analysis was as in Gorelik et al. (2003). The calculations were done using SoftMax Pro software from Molecular devices.

Acknowledgments

We thank C. Low for excellent assistance with the mouse colony, U. Siebenlist for anti-p52 serum, D. Allman for antibodies to AA4, S. Jiang and C. McArthur for cell sorting, M. Lesneski for technical assistance, and E. Ekland and N. Haynes for comments on the manuscript. R.L. was supported in part by the UCSF Biomedical Sciences (BMS) Graduate Program. J.G.C. is a HHMI Assistant Investigator. This work was supported by National Institutes of Health grant AI-40098.

Received: November 12, 2003
Revised: February 14, 2004
Accepted: February 25, 2004
Published: April 20, 2004

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