Immunity, Vol. 20, 785–798, June, 2004, Copyright ©2004 by Cell Press

Excess BAFF Rescues Self-Reactive B Cells from Peripheral Deletion and Allows Them to Enter Forbidden Follicular and Marginal Zone Niches

Marilyn Thien,[1] Tri Giang Phan,[1] Sandra Gardam,[1] Michelle Amesbury,[1] Antony Basten,[1] Fabienne Mackay,[2] and Robert Brink[1,*]

[1]Centenary Institute of Cancer Medicine and Cell Biology
Locked Bag Number 6
Newtown, New South Wales 2042

[2]Garvan Institute of Medical Research
Department of Arthritis and Inflammation
384 Victoria Street
Darlinghurst, New South Wales 2030
Australia

Summary

The role of BAFF in B cell self tolerance was examined by tracking the fate of anti-HEL self-reactive B cells in BAFF transgenic mice using four different models of self-reactive B cell deletion. BAFF overexpression did not affect the development of self-reactive B cells normally deleted in the bone marrow or during the early stages of peripheral development. By contrast, self-reactive B cells normally deleted around the late T2 stage of peripheral development were rescued from deletion, matured, and colonized the splenic follicle. Furthermore, self-reactive B cells normally selectively deleted from the marginal zone repopulated this compartment when excess BAFF was present. Self-reactive B cells rescued by excess BAFF were not anergic. BAFF overexpression therefore rescued only self-reactive B cells normally deleted with relatively low stringency and facilitated their migration into otherwise forbidden microenvironments. This partial subversion of B cell self tolerance is likely to underlie the autoimmunity associated with BAFF overexpression.

Introduction

Stringent controls over the development of B cells expressing anti-self BCRs are essential for preventing the onset of antibody-mediated autoimmune diseases (Goodnow et al., 1990). Studies using transgenic (Tg) mice expressing BCRs directed against various forms of self-antigens have demonstrated that B cells which encounter high-avidity self-antigen in the bone marrow (BM) fail to progress past the most immature stage of B cell development and die before they can migrate to the periphery (Hartley et al., 1991; Nemazee and Buerki, 1989). By contrast, self-reactive B cells recognizing soluble self-antigens are able to colonize peripheral lymphoid tissues, as exemplified in the anti-hen egg lysozyme (HEL) x soluble HEL (sHEL) double Tg model (Goodnow et al., 1988; Phan et al., 2003). In this case, peripheral self-reactive B cells are anergic provided that their interaction with sHEL self-antigen exceeds a critical threshold (Goodnow et al., 1989). In the presence of competing non-self-reactive B cells, the lifespan and migration of anergic B cells in the periphery are limited. Thus, within a diverse B cell repertoire, anergic B cells fail to mature, have a half-life of only 2 to 3 days, and are excluded from the splenic follicle (FO) and marginal zone (MZ) (Cyster and Goodnow, 1995; Cyster et al., 1994; Phan et al., 2003).

Disruption of the mechanisms underlying normal elimination of self-reactive B cells is likely to be a significant factor contributing to the onset of autoantibody-mediated diseases. Tg mice overexpressing the tumor necrosis factor (TNF) superfamily member BAFF (TALL-1, THANK, BLyS, zTNF4, TNFSF13b) exhibit systemic autoimmunity accompanied by elevated levels of circulating autoantibodies and immune complex-mediated glomerulonephritis (Khare et al., 2000; Mackay et al., 1999). These mice also contain increased numbers of mature B cells, in particular MZ B cells, due to the activity of BAFF as a potent B cell survival factor (Batten et al., 2000; Rolink et al., 2002). Older BAFF transgenic mice demonstrate hallmarks of Sjögren's syndrome (SS), such as severe sialadenitis and decreased saliva production (Groom et al., 2002). Significantly elevated levels of BAFF are found in serum of patients with SS, suggesting a role for dysregulated expression of BAFF in this and potentially other systemic autoimmune diseases (Groom et al., 2002). Although the means by which excess BAFF precipitates autoimmunity remains unclear, it has been proposed that the normal mechanisms restricting the survival of self-reactive B cells may be subverted under such conditions (Groom et al., 2002; Mackay et al., 2003). While this is an attractive proposition, the influence of excess BAFF on the development of self-reactive B cells has yet to be directly tested.

BAFF-mediated B cell survival signals are transduced via BAFF-R, a member of the TNFR superfamily (Schiemann et al., 2001; Thompson et al., 2001). Responsiveness to BAFF is not a property of all B cells but is acquired during B cell maturation. Thus, immature BM B cells and splenic transitional type 1 (T1) B cells (CD21/35^{lo} CD23^{lo}) in spleen express low levels of BAFF-R (Hsu et al., 2002), are not expanded in BAFF transgenic mice (Batten et al., 2000) nor in mice administered exogenous BAFF (Hsu et al., 2002), and are largely unaffected in *baff*$^{-/-}$ mice (Gross et al., 2001; Schiemann et al., 2001). Maturation of T1 B cells to the T2 stage is marked by the upregulation of CD23 (Allman et al., 2001), increased BAFF-R expression, and the onset of responsiveness to BAFF-mediated survival signals (Hsu et al., 2002). Progression of B cell development past the T2 stage is dependent on BAFF (Gross et al., 2001; Schiemann et al., 2001). Acquisition of both responsiveness to and dependence on BAFF therefore corresponds closely to the point during B cell development at which the majority of peripheral B cells die (Forster and Rajewsky, 1990; Hsu et al., 2002), suggesting that B cell homeostasis may be controlled by the limiting amounts of BAFF present in vivo. In particular, the ability of competing non-self-reactive B cells to eliminate anergic B cells before they can mature (Phan et al., 2003) raises the possibility that

*Correspondence: r.brink@centenary.usyd.edu.au

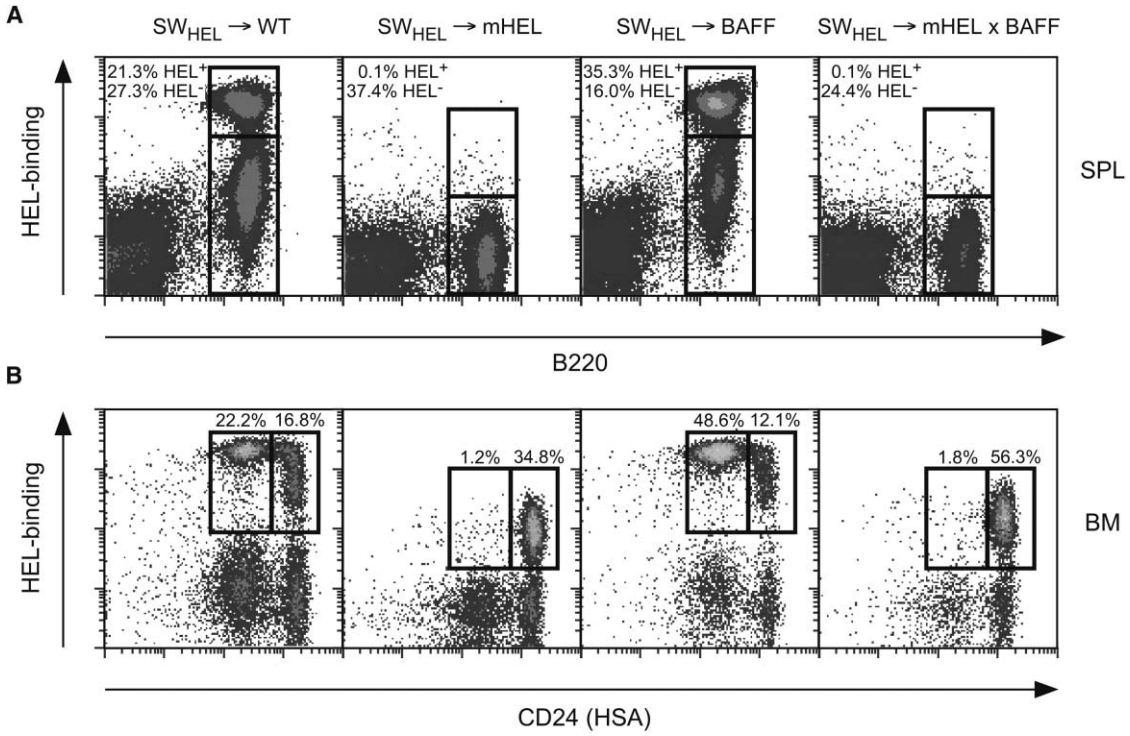


Figure 1. Excess BAFF Does Not Rescue Self-Reactive B Cells from Deletion by mHEL Self-Antigen in the BM

wt, mHEL Tg, BAFF Tg, and mHEL x BAFF Tg mice were lethally irradiated and reconstituted with BM from a SW$_{HEL}$ donor heterozygous for the CD45.1 allele. Spleen (A) and BM (B) cells were harvested from the BM chimeras 8 weeks later and stained with anti-B220-PerCP, anti-CD45.1-FITC, anti-CD24(HSA)-PE, and HEL + HyHEL5-Alexa Fluor® 647. Plots depict donor-derived (CD45.1$^+$) lymphocytes (A) or donor-derived B220$^+$ cells (B). Numbers show the proportion of gated events in the indicated windows. The low levels of HEL-staining on the HEL$^-$ B cell populations in the spleens of wt and BAFF Tg chimeras is not due to the expression of anti-HEL Ig (Phan et al., 2003) but may represent "cytophilic" antibody derived from the HEL$^+$ B cells (Mason et al., 1992). Less than 1% of B cells stain as HEL$^+$ in wt and BAFF Tg mice (Phan et al., 2003) (data not shown).

self-reactive B cells are poor competitors for limiting BAFF survival signals.

To clarify the role of BAFF in autoimmunity and B cell self tolerance, we studied the fate of HEL$^+$ self-reactive B cells in BAFF Tg mice. By using four different models of self-reactive B cell elimination, we demonstrate that overexpression of BAFF affects the development of some but not all self-reactive B cells. Thus, the development of self-reactive B cells upon which deletion is most stringently enforced is unaffected by excess BAFF. In contrast, self-reactive B cells deleted during later stages of maturation can be rescued by BAFF overexpression and migrate to anatomical microenvironments from which they are usually excluded. This partial subversion of B cell self tolerance is likely to be the mechanism by which excess production of BAFF drives systemic autoantibody-mediated disease.

Results

BAFF Overexpression Does Not Rescue High-Affinity Self-Reactive B Cells from Deletion in the BM

The effect of BAFF overexpression on B cell self tolerance was examined initially using a model in which high-affinity self-reactive B cells recognizing a high-avidity self-antigen are deleted in the BM. In this model, lethally irradiated Tg mice expressing membrane HEL (mHEL) (Hartley et al., 1991) are reconstituted with BM from Ig Tg mice capable of generating high-affinity anti-HEL (HEL$^+$) B cells. HEL$^+$ B cells produced in the BM of the resulting chimeras undergo developmental arrest and die in situ before they can migrate to peripheral lymphoid tissues (Hartley et al., 1993; Phan et al., 2003).

Lethally irradiated wild-type (wt), mHEL Tg, BAFF Tg, and mHEL x BAFF Tg were reconstituted with BM from Tg mice that produce HEL$^+$ B cells capable of undergoing Ig class switch recombination (SW$_{HEL}$) (Phan et al., 2003). In wt recipients reconstituted with SW$_{HEL}$ BM, 40%–60% of peripheral B cells were HEL$^+$ (Figure 1A), a similar proportion to that observed in intact SW$_{HEL}$ mice (Phan et al., 2003). By contrast, mHEL Tg recipients of SW$_{HEL}$ BM lacked peripheral HEL$^+$ B cells (Figure 1A) and contained only immature CD24^{hi} HEL$^+$ B cells in BM (Figure 1B). BAFF Tg recipients contained increased frequencies of mature HEL$^+$ B cells in spleen and BM (Figures 1A and 1B) as well as an expanded splenic MZ HEL$^+$ B cell compartment (data not shown), thereby confirming that secretion of BAFF from the liver of BAFF Tg mice is maintained following lethal irradiation and reconstitution. When the recipients expressed both excess BAFF and mHEL, the fate of HEL$^+$ B cells did not differ from that observed in mice Tg for mHEL alone (Figures 1A and 1B). Thus, overexpression of BAFF failed

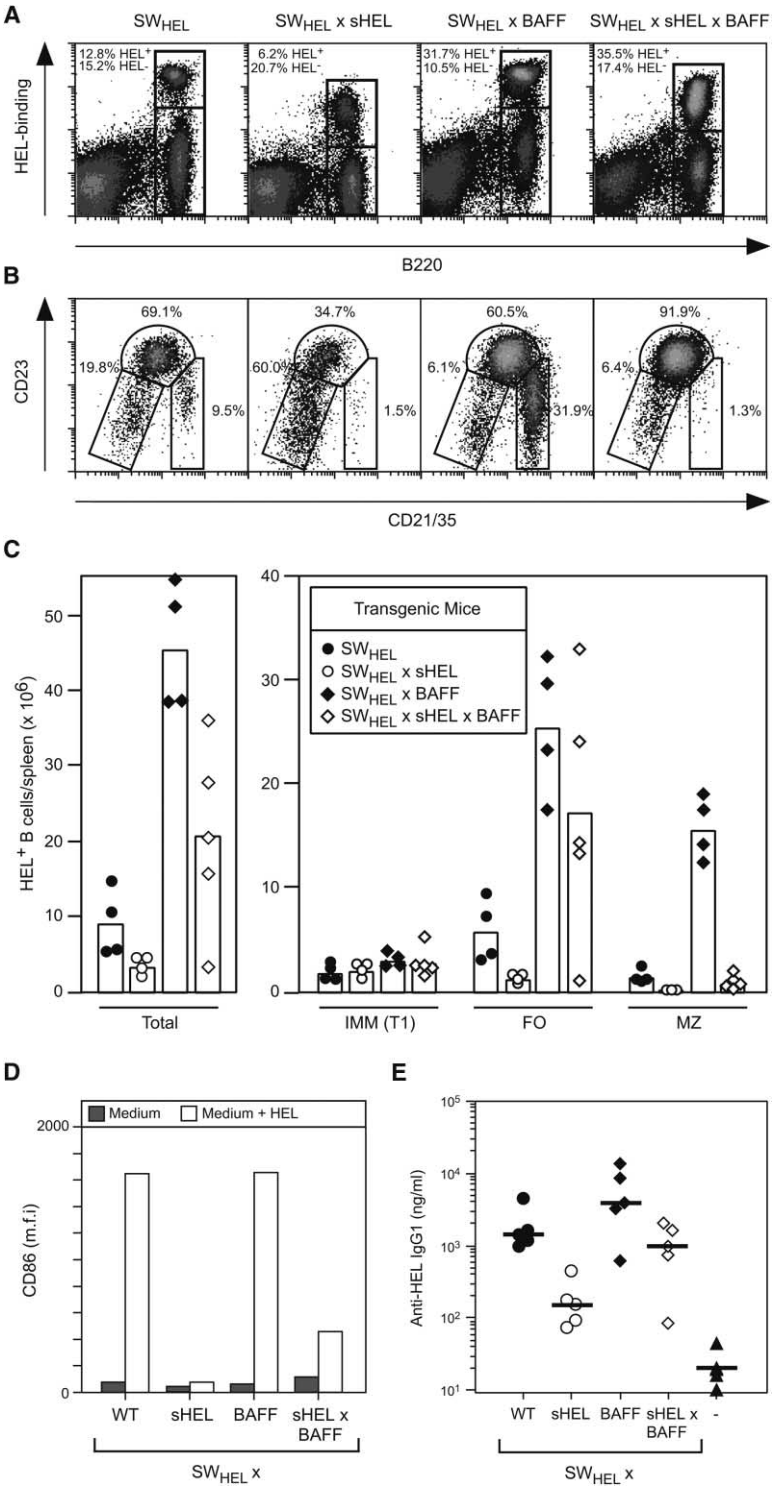


Figure 2. Self-Reactive B Cells Exposed to sHEL Self-Antigen Are Rescued from Deletion in the Spleen of Intact Mice Overexpressing BAFF and Are Not Anergic

Spleen cells from mice of the indicated genotypes were stained with anti-B220-PerCP, anti-CD21/35-FITC, anti-CD23-PE, and HEL + HyHEL5-Alexa Fluor® 647.

(A) FACS data showing proportion of HEL binding and non-HEL binding B cells in each indicated mouse genotype.

(B) CD21/35 and CD23 profiles of the HEL^+ B cells from (A). Numbers in (A) and (B) represent the proportion of gated events in the indicated windows, including the immature T1 ($CD21/35^{lo}$ $CD23^{lo}$), FO ($CD21/35^{int}$ $CD23^{hi}$), and MZ ($CD21/35^{hi}$ $CD23^{lo}$) B cell populations.

(C) Numbers of HEL^+ B cells per spleen were calculated by multiplying the proportion of HEL^+ B cells in each mouse (A) by the total number of spleen cells. Numbers of immature T1 (IMM), FO, and MZ HEL^+ B cells were calculated by multiplying the proportion of HEL^+ B cells that exhibited the relevant CD21/35 versus CD23 phenotype (B) by the total number of HEL^+ B cells. Points represent data from individual mice with the mean value in each group indicated by the columns.

(D) Spleen cells were incubated with or without HEL and stained for B220, HEL binding, and CD86 as described in the Experimental Procedures. The geometric mean of CD86 fluorescence intensity was calculated after gating on HEL^+ B cells. Data is representative of three independent experiments.

(E) Recipient wt mice were injected with an inoculum of 10^4 HEL^+ B cells from the indicated mouse genotype plus 2×10^8 HEL-SRBC in an adoptive transfer assay as described in the Experimental Procedures. Sera were collected from recipients after 7 days and anti-HEL IgG1 antibodies measured by ELISA. Points show data from individual recipients and the bar represents the geometric mean. Recipients receiving 10^4 HEL^+ B cells plus mock-conjugated SRBC contained less than 20 ng/ml anti-HEL IgG1 regardless of donor genotype. Data are representative of two independent experiments.

to rescue self-reactive B cells destined for deletion in the BM.

BAFF Overexpression Can Rescue High-Affinity Self-Reactive B Cells Normally Deleted in the Periphery

The role of BAFF in peripheral as opposed to central deletion of self-reactive B cells was examined by testing the effects of BAFF overexpression on B cells encountering HEL as a soluble self-antigen (sHEL). In SW_{HEL} x sHEL Tg mice, self-reactive HEL^+ B cells are present at reduced frequencies in the spleen (Figure 2A) with the majority of them (60%–70%) exhibiting an immature T1 phenotype ($CD21/35^{lo}$ $CD23^{lo}$) and the remainder an immature T2 phenotype ($CD21/35^{lo}$ $CD23^{hi}$) (Figure 2B). The failure of these self-reactive HEL^+ B cells to mature

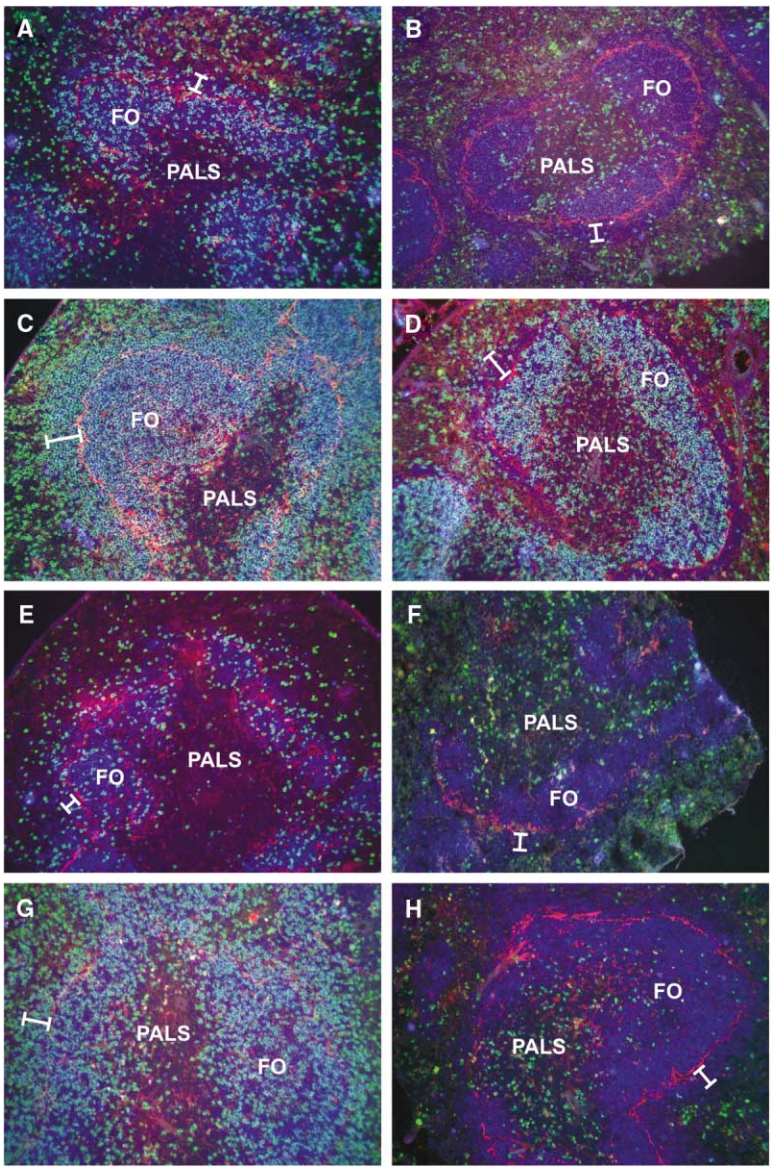


Figure 3. Self-Reactive B Cells Rescued by BAFF Overexpression Colonize the Splenic FO but Not the MZ

Immunohistological stains illustrating the localization of HEL^+ B cells in the spleens of 9- to 12-week-old intact Tg mice (A–D) and SW_{HEL} + wt mixed BM chimeras analyzed 10 weeks after lethal irradiation and reconstitution (E–H) (see Figure 5). Sections of snap-frozen spleens were fixed and stained for B cells (B220; blue), HEL binding receptors (green), and the marginal sinus (MadCAM-1; red) to distinguish the MZ (crossbar) from the follicle (FO). The T cell-rich PALS area inside the FO and the extrafollicular red pulp show minimal B cell staining. HEL binding B cells are cyan blue due to the costaining of blue ($B220^+$) and green (HEL^+). Immature HEL^+ B cells are $B220^{lo}$ and therefore appear greener than mature B cells. Spleen sections from intact Tg mice are as follows. (A) SW_{HEL} – HEL^+ B cells in the FO and MZ. (B) SW_{HEL} x sHEL; self-reactive HEL^+ B cells are mainly in the PALS and red pulp. (C) SW_{HEL} x BAFF – HEL^+ B cells expanded and dominate the FO and MZ. (D) SW_{HEL} x sHEL x BAFF; self-reactive HEL^+ B cells localize in FO but not the MZ. Spleen sections of SW_{HEL} + wt mixed BM chimera recipients: (E) wt, (F) sHEL Tg, (G) BAFF Tg, and (H) BAFF x sHEL Tg. Results were similar to the corresponding intact Tg mice except in the case of BAFF x sHEL Tg chimeras (H) where, unlike in SW_{HEL} x sHEL x BAFF Tg mice (D), BAFF overexpression did not alter the localization of the self-reactive HEL^+ B cells to the PALS and red pulp.

is due to the presence of competing non-HEL binding (HEL^-) B cells, which leads to death around the T2 stage of maturation (Phan et al., 2003). Self-reactive HEL^+ B cells in SW_{HEL} x sHEL Tg mice are excluded from the splenic FO and MZ, residing primarily in the red pulp and the T cell-rich PALS (Figures 3A and 3B).

To test the effects of BAFF overexpression in this system, SW_{HEL} x sHEL Tg mice were mated with BAFF Tg mice to produce SW_{HEL} x BAFF and SW_{HEL} x sHEL x BAFF Tg animals. The phenotype of HEL^+ splenic B cells in control SW_{HEL} x BAFF Tg mice mirrored that observed previously in BAFF Tg mice reconstituted with SW_{HEL} BM (Figure 1). Thus, overall HEL^+ B cell numbers were increased 5.4-fold compared to SW_{HEL} mice (Figures 2A, 2C, and 3C), with the MZ ($CD21/35^{hi}$ $CD23^{lo}$) B cell compartment being preferentially expanded (12.4-fold) (Figures 2B, 2C, and 3C). Examination of SW_{HEL} x sHEL x BAFF Tg mice revealed that overexpression of BAFF had a striking effect on the development of self-reactive HEL^+ B cells. Unlike the case in SW_{HEL} x sHEL Tg mice, the HEL^+ B cells from all but one of the SW_{HEL} x sHEL x BAFF Tg mice examined progressed efficiently past the immature T1 stage to form a prominent population of cells exhibiting the mature FO B cell phenotype ($CD21/35^{int}$ $CD23^{hi}$) (Figure 2B) that was similar in size to the analogous population in SW_{HEL} x BAFF Tg mice (Figure 2C). On the other hand, self-reactive HEL^+ B cells exposed to excess BAFF still failed to populate the MZ compartment in SW_{HEL} x sHEL x BAFF Tg mice (Figures 2B and 2C). Immunohistological analysis of spleen sections was consistent with the results obtained by flow cytometry, demonstrating colonization of the FO by self-reactive HEL^+ B cells in SW_{HEL} x sHEL x BAFF Tg mice with only HEL^- B cells being detected in the MZ (Figure 3D).

High-Affinity Self-Reactive B Cells Rescued by BAFF Overexpression Are Not Anergic

Anergic FO self-reactive HEL^+ B cells express inactive BCR molecules lacking the capacity to signal upregula-

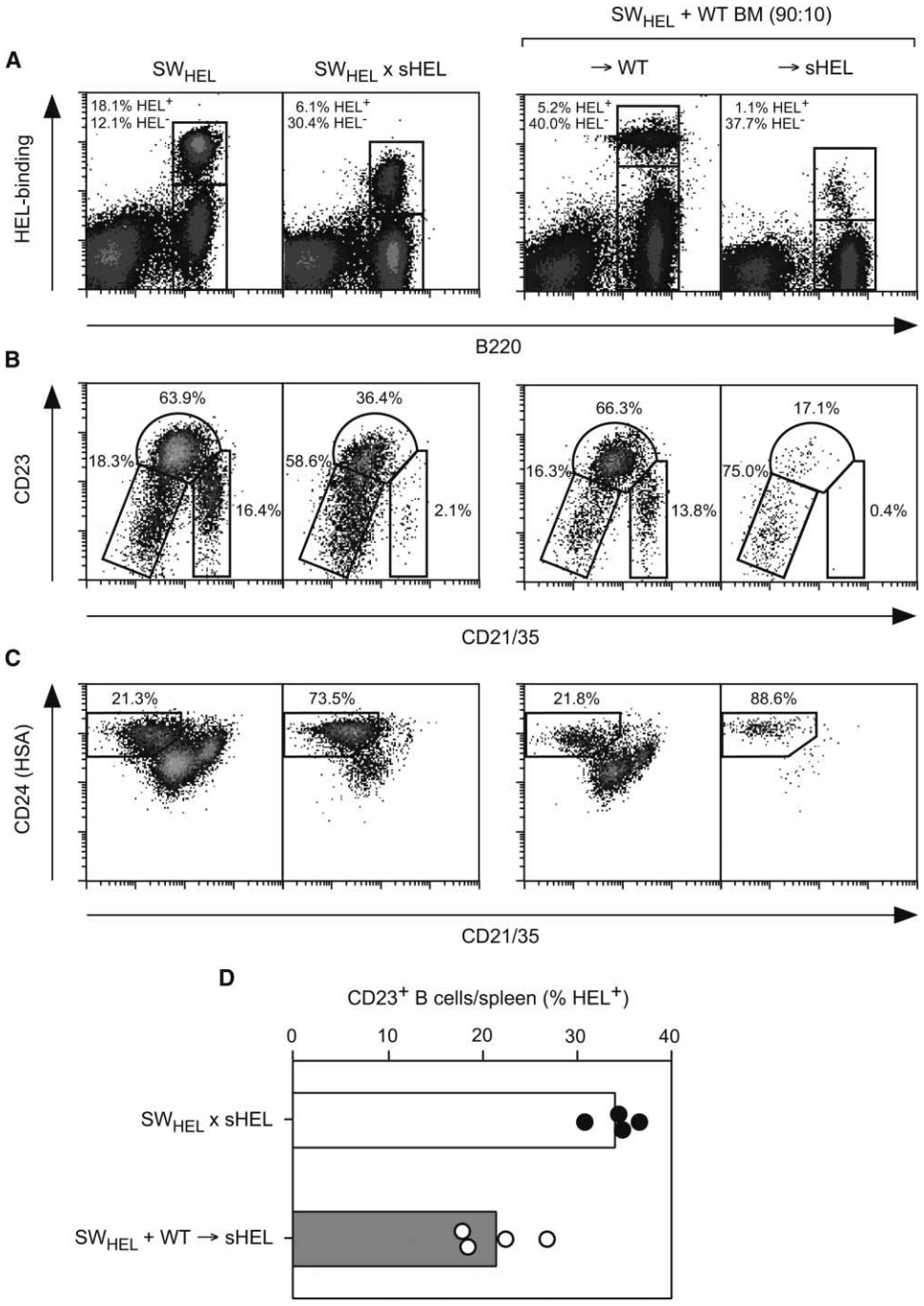


Figure 4. Increased Competition in SW$_{HEL}$ + wt Mixed BM Chimeras Compared to Intact SW$_{HEL}$ Mice Results in More Stringent Deletion of Self-Reactive B Cells

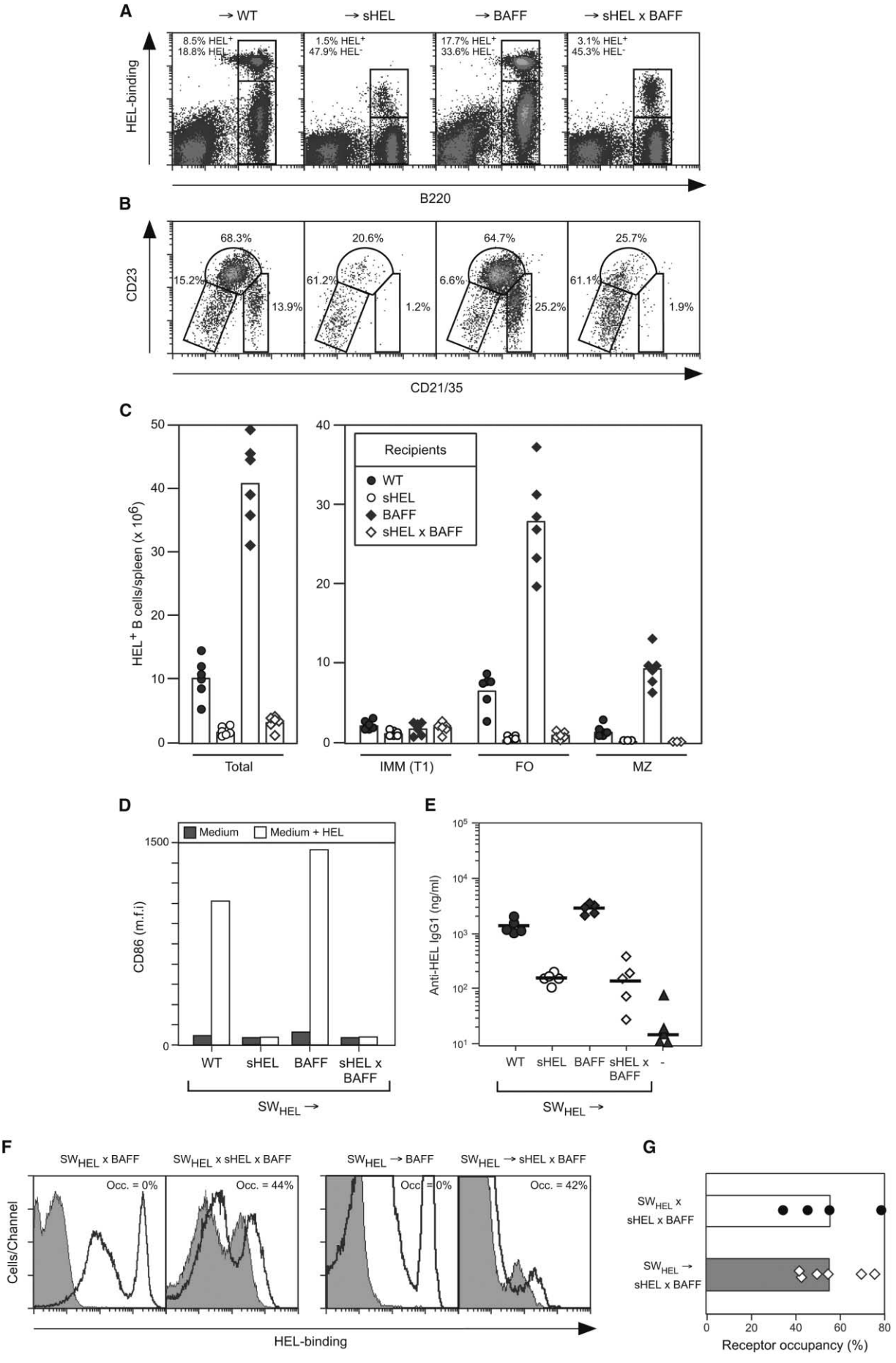
Mixed BM chimeras were produced by reconstituting lethally irradiated wt and sHEL recipients with a 90:10 mixture of SW$_{HEL}$ and wt BM cells.

(A) Splenocytes from intact SW$_{HEL}$ and SW$_{HEL}$ x sHEL Tg mice or wt and sHEL Tg mixed BM chimeras were stained and analyzed by FACS as described in Figure 2.

(B) CD21/35 and CD23 expression profiles of HEL$^+$ B cells using the gates shown in (A). Windows indicate immature T1 (CD21/35^{lo} CD23^{lo}), FO (CD21/35^{int} CD23^{hi}), and MZ (CD21/35^{hi} CD23^{lo}) B cells, and figures are the frequency of gated cells in the respective populations.

(C) Splenocytes from the same mice were also stained for CD24 (HSA) and its expression on HEL$^+$ B cells plotted against CD21/35. B cells exhibiting the immature CD21/35^{lo} CD24^{hi} phenotype and the proportion of gated cells in this population are indicated.

(D) The proportion of self-reactive HEL$^+$ B cells exhibiting the more mature CD23^{hi} phenotype was calculated in intact SW$_{HEL}$ x sHEL Tg mice and sHEL Tg recipients of SW$_{HEL}$ plus wt BM cells using the gates shown in (B). Points are from individual mice while the bars indicate the mean values obtained.


A
→ WT
→ sHEL
→ BAFF
→ sHEL x BAFF
8.5% HEL+
18.8% HEL-
1.5% HEL+
47.9% HEL-
17.7% HEL+
33.6% HEL-
3.1% HEL+
45.3% HEL-
HEL-binding
B220
B
CD23
68.3%
15.2%
13.9%
20.6%
61.2%
1.2%
64.7%
6.6%
25.2%
25.7%
61.1%
1.9%
CD21/35
C
HEL+ B cells/spleen (x 10^6)
Recipients
WT
sHEL
BAFF
sHEL x BAFF
Total
IMM (T1)
FO
MZ
D
Medium
Medium + HEL
CD86 (m.f.i.)
WT
sHEL
BAFF
sHEL x BAFF
SWHEL →
E
Anti-HEL IgG1 (ng/ml)
WT
sHEL
BAFF
sHEL x BAFF
-
SWHEL →
F
SWHEL x BAFF
SWHEL x sHEL x BAFF
SWHEL → BAFF
SWHEL → sHEL x BAFF
Occ. = 0%
Occ. = 44%
Occ. = 0%
Occ. = 42%
Cells/Channel
HEL-binding
G
SWHEL x sHEL x BAFF
SWHEL → sHEL x BAFF
Receptor occupancy (%)

tion of the costimulatory molecule CD86 in response to antigen (Cooke et al., 1994; Phan et al., 2003; Rathmell et al., 1998) and produce 10-fold less antibody than non-self-reactive HEL^+ B cells when challenged with HEL conjugated to sheep red blood cells (HEL-SRBC) in vivo (Goodnow et al., 1988). To test whether the FO self-reactive HEL^+ B cells rescued by overexpression of BAFF remained anergic, we first measured their ability to upregulate CD86 in response to HEL in vitro. Non-self-reactive (SW_{HEL} and SW_{HEL} x BAFF Tg) HEL^+ B cells underwent efficient CD86 upregulation in response to HEL, whereas the anergic self-reactive HEL^+ B cells from SW_{HEL} x sHEL Tg mice did not (Figure 2D). In contrast, the self-reactive HEL^+ B cells rescued by BAFF overexpression in SW_{HEL} x sHEL x BAFF Tg mice underwent partial upregulation of CD86 (Figure 2D), indicating that these cells are not fully anergic.

We next tested whether the partial responsiveness of BCRs on self-reactive B cells rescued by excess BAFF was sufficient to facilitate autoantibody production in response to antigenic stimulation in vivo. For this purpose, equal numbers of HEL^+ B cells from the various Tg mice were challenged with HEL-SRBC in an adoptive transfer assay. A week after challenge, non-self-reactive HEL^+ B cells from SW_{HEL} and SW_{HEL} x BAFF Tg donors generated robust antibody responses comprising both anti-HEL IgG1 (Figure 2E) and IgM (data not shown) antibodies. By contrast, sera from recipients of anergic HEL^+ B cells from SW_{HEL} x sHEL Tg donors contained 10-fold lower levels of anti-HEL antibodies. Transferred self-reactive B cells from SW_{HEL} x sHEL x BAFF Tg mice produced comparable amounts of anti-HEL IgG1 (Figure 2E) and IgM (data not shown) antibodies to those secreted by non-self-reactive (i.e., SW_{HEL} and SW_{HEL} x BAFF Tg) HEL^+ B cells. Thus, overexpression of BAFF reversed the anergic state of self-reactive B cells in SW_{HEL} x sHEL x BAFF Tg mice sufficiently to enable them to produce both switched and unswitched autoantibodies in response to specific antigen in vivo.

Increased Intercellular Competition Results in Earlier Peripheral Deletion of High-Affinity Self-Reactive B Cells

The results obtained thus far suggested that overexpression of BAFF can rescue self-reactive B cells normally destined to undergo peripheral deletion but not those B cells that undergo deletion in the BM. However, full responsiveness to BAFF does not occur until some point during the T2 phase of B cell maturation (Gross et al., 2001; Hsu et al., 2002; Schiemann et al., 2001). Hence, it remained possible that self-reactive B cells normally deleted in the periphery before reaching this critical maturational window may not be rescued by excess amounts of BAFF. We therefore established an alternative model of peripheral B cell deletion in which self-reactive HEL^+ B cells are eliminated earlier during B cell maturation than occurs in intact SW_{HEL} x sHEL Tg mice.

To do this, we first tested whether the introduction of extra competition from polyclonal HEL^- B cells would further restrict the maturation of self-reactive B cells. Mixed BM chimeras were therefore produced in which lethally irradiated wt and sHEL Tg mice were reconstituted with a 90:10 mixture of SW_{HEL} and wt BM cells. The presence of prerearranged Ig transgenes is known to greatly reduce the expansion of B lineage precursors in the BM (Cook et al., 1997; Mason et al., 1992). Thus, the inclusion of only 10% wt cells in the BM inoculum resulted in an increase in the ratio of HEL^- to HEL^+ B cells in the spleen from less than 1:1 in the intact SW_{HEL} mice to an average of 6:1 in wt mice reconstituted with a 90:10 mixture of SW_{HEL} and wt BM cells (Figure 4A and data not shown).

In the absence of sHEL expression, the additional competition from HEL^- B cells did not affect the maturation of nontolerant HEL^+ B cells to FO and MZ phenotypes (Figures 4B and 4C; SW_{HEL} versus SW_{HEL} + wt → wt chimera). However, comparison of the peripheral HEL^+ B cells obtained from SW_{HEL} x sHEL Tg mice versus SW_{HEL} + wt → sHEL Tg mixed BM chimeras revealed that the development of self-reactive B cells was indeed affected by this additional competition. Thus, the proportion of HEL^+ self-reactive B cells exhibiting the more mature $CD23^{hi}$ phenotype was lower in sHEL Tg mixed chimeras compared to intact SW_{HEL} x sHEL Tg mice (Figures 4B and 4D). The more immature phenotype of the self-reactive B cells in the mixed chimeras was confirmed by the finding of higher frequencies of immature $CD24^{hi}$ B cells in these mice (Figure 4C). These data indicate that the additional competition from HEL^- B cells in the mixed BM chimeras results in more stringent deletion of self-reactive HEL^+ B cells at an earlier time point during maturation.

Figure 5. BAFF Overexpression Does Not Rescue Self-Reactive B Cells from Deletion or Anergy in Mixed BM Chimeras

(A and B) wt, sHEL Tg, BAFF Tg, and sHEL x BAFF Tg mice were lethally irradiated and reconstituted with a mixture of BM from SW_{HEL} and wt donors (90:10 ratio). Spleen cells were harvested 10 weeks later and analyzed as for Figure 2. Data from total splenic lymphocytes are shown in (A), while (B) portrays data from HEL^+ B cells using the windows shown in (A). (C) Spleen cells from mixed BM chimeras were stained as for (A). Total numbers of HEL^+ B cells/spleen and those in the immature T1 (IMM), FO, or MZ compartments were calculated as for Figure 2. The data points represent individual mice with the mean value in each group indicated by the columns. (D) Spleen cells were incubated with or without HEL and stained for B220, HEL binding, and CD86 as for Figure 2D. The geometric mean of CD86 fluorescence intensity was calculated after gating on HEL^+ B cells. Data is representative of three independent experiments. (E) Recipient wt mice were injected with 10^4 HEL^+ B cells from the indicated mixed BM chimeras plus 2×10^8 HEL-SRBC in an adoptive transfer assay and serum antibody in recipients analyzed as for Figure 2E. Recipients receiving 10^4 HEL^+ B cells plus mock-conjugated SRBC contained less than 40 ng/ml anti-HEL IgG1 regardless of donor genotype. Data are representative of two independent experiments. (F) Spleen cells from SW_{HEL} x BAFF and SW_{HEL} x sHEL x BAFF Tg mice (Figure 2) and BAFF and sHEL x BAFF Tg chimeras reconstituted with mixed SW_{HEL} + wt BM (Figure 5) were stained with HyHEL5-Alexa Fluor® 647 with (open curve) and without (shaded curve) prior addition of saturating HEL. Profiles represent $B220^+$ cells. The percentage of BCR on HEL^+ B cells occupied by endogenous sHEL (BCR occupancy) was calculated as described (Phan et al., 2003). (G) Comparison of receptor occupancy in SW_{HEL} x sHEL x BAFF Tg mice and sHEL x BAFF Tg chimeras reconstituted with mixed SW_{HEL} + wt BM. Points represent data from individual mice and bars indicate the mean value.

Overexpression of BAFF Does Not Rescue High-Affinity Self-Reactive B Cells from Peripheral Deletion in the Presence of High Levels of Intercellular Competition

We next determined whether additional intercellular competition influenced the ability of excess BAFF to rescue self-reactive B cells from peripheral deletion. For this purpose, mixed BM chimeras were produced by reconstituting lethally irradiated wt, sHEL Tg, BAFF Tg, and sHEL x BAFF Tg recipients with SW_{HEL} plus wt BM at a 90:10 ratio.

Analysis of BAFF Tg mixed chimeras again showed the typical expansion of non-self-reactive HEL^+ B cells exposed to excess BAFF in vivo, with a greater than 4-fold rise in total numbers as well as a preferential increase in MZ B cell numbers (Figures 5B and 5C). In contrast to intact SW_{HEL} x sHEL x BAFF Tg mice, however, self-reactive HEL^+ B cells from SW_{HEL} + wt → sHEL x BAFF Tg mixed BM chimeras exhibited an almost identical phenotype to those present in recipients expressing sHEL alone. Thus, despite a small increase in cell numbers (Figure 5C) and CD21/35 expression (Figure 5B), few self-reactive HEL^+ B cells from the SW_{HEL} + wt → sHEL x BAFF Tg mixed BM chimeras progressed past the immature T1 ($CD21/35^{lo}$ $CD23^{lo}$) stage (Figures 5B and 5C). Self-reactive B cells were also excluded from the splenic FO and retained in the PALS and red pulp of sHEL x BAFF Tg mixed BM chimeras (Figure 3H). Functional comparison of B cells from the various recipients revealed that the self-reactive HEL^+ B cells from sHEL x BAFF Tg mixed BM chimeras were completely anergic. These cells therefore failed to upregulate CD86 in response to HEL in vitro (Figure 5D) and responded as poorly as HEL^+ B cells from sHEL Tg recipients when tested for anti-HEL IgG1 (Figure 5E) and IgM (data not shown) production in response to HEL-SRBC in vivo. Thus, in the higher stringency mixed chimera model, overexpression of BAFF failed to rescue self-reactive B cells from either peripheral deletion or anergy.

The observation that peripheral deletion of anergic self-reactive B cells in SW_{HEL} x sHEL Tg mice is circumvented by excess BAFF (Figure 2) whereas the additional competition from HEL^- B cells in the mixed BM chimeras prevents BAFF-mediated rescue (Figure 5) could be explained by a difference in the degree of interaction with sHEL self-antigen between the two systems. This possibility was excluded by demonstrating that the degree of BCR occupancy by endogenous sHEL did not differ between B cells from SW_{HEL} x sHEL x BAFF Tg mice and sHEL x BAFF Tg mixed BM chimeras (Figures 5F and 5G).

Intermediate Affinity Self-Reactive B Cells Developing in the Presence of High Levels of Intercellular Competition Are Preferentially Deleted from the MZ Compartment

In the mixed BM chimera model described above, the increased intercellular competition prevented high-affinity HEL^+ self-reactive B cells from being rescued by excess BAFF (Figure 5). One interpretation of this result is that excess BAFF is unlikely to rescue self-reactive B cells from deletion under physiological conditions since high intercellular competition exists naturally. However, it remained possible that self-reactive B cells possessing a lower affinity for self-antigen are deleted with lower stringency and may therefore be susceptible to rescue from deletion by excess BAFF.

To test this possibility, we took advantage of the fact that mice expressing the rearranged anti-HEL heavy chain variable gene alone produce HEL^+ B cells at low but detectable frequencies and with varying affinities for HEL (Hartley and Goodnow, 1994). In mice carrying the SW_{HEL} heavy chain without the light chain transgene [$SW_{HEL(H)}$ mice], the proportion of spleen cells readily detectable as HEL^+ B cells was only 0.1%–0.2% when stained with low (4.1 ng/ml) concentrations of HEL (Figure 6A). These cells have a high affinity for HEL, as demonstrated by their similar HEL binding kinetics to HEL^+ B cells from SW_{HEL} (heavy plus light chain) mice (Figure 6A and data not shown). A second, larger (0.2%–0.4%) population of HEL^+ B cells was detected when $SW_{HEL(H)}$ spleen cells were stained with higher concentrations (e.g., 37 ng/ml) of HEL (Figure 6A). Titration of HEL binding indicated that this population includes B cells that bind HEL with an intermediate affinity in the range of 10- to 50-fold lower than that of the high-affinity population (data not shown).

As would be predicted, the high-affinity HEL^+ B cell population was efficiently deleted from the peripheral B cell pool of $SW_{HEL(H)}$ x sHEL Tg mice (Figures 6A and 6B). On the other hand, only 70% of the intermediate affinity population was deleted, demonstrating a direct relationship between the affinity of self-reactive B cells and deletion stringency. Further examination revealed that deletion of intermediate affinity HEL^+ self-reactive B cells was only partial (64%) within the FO population but almost complete (92%) in the MZ compartment (Figures 7A and 7B). Measurement of surface CD86 levels following in vitro stimulation with HEL demonstrated that the majority of the HEL^+ self-reactive B cells from $SW_{HEL(H)}$ x sHEL Tg mice were not anergic (Figure 6C). This, in combination with the fact that these B cells almost exclusively exhibited a mature FO phenotype (Figure 7A and data not shown) indicates that the HEL^+ B cells remaining in $SW_{HEL(H)}$ x sHEL Tg mice are not tolerized, presumably because their affinity for HEL falls below the threshold required for tolerance induction.

Overexpression of BAFF Leads to Accumulation of Intermediate Affinity Self-Reactive B Cells in the MZ Compartment

To assess the effects of excess BAFF on the deletion of HEL^+ self-reactive B cells in the $SW_{HEL(H)}$ variable affinity system, spleen cells from $SW_{HEL(H)}$ x sHEL x BAFF Tg mice were stained with varying concentrations of HEL. Consistent with the results in the mixed BM chimera system (Figure 5), BAFF overexpression failed to rescue high-affinity HEL^+ self-reactive B cells from deletion (Figures 6A and 6B). By contrast, the population of B cells with intermediate affinity for HEL was expanded 4.7-fold in $SW_{HEL(H)}$ x sHEL x BAFF relative to $SW_{HEL(H)}$ x sHEL Tg mice (Figures 6A and 6B). The effect of excess BAFF on intermediate affinity HEL^+ self-reactive B cells was most striking in the MZ compartment. Thus, the number of MZ phenotype intermediate affinity HEL^+ B cells was increased 24-fold in $SW_{HEL(H)}$ x sHEL x BAFF

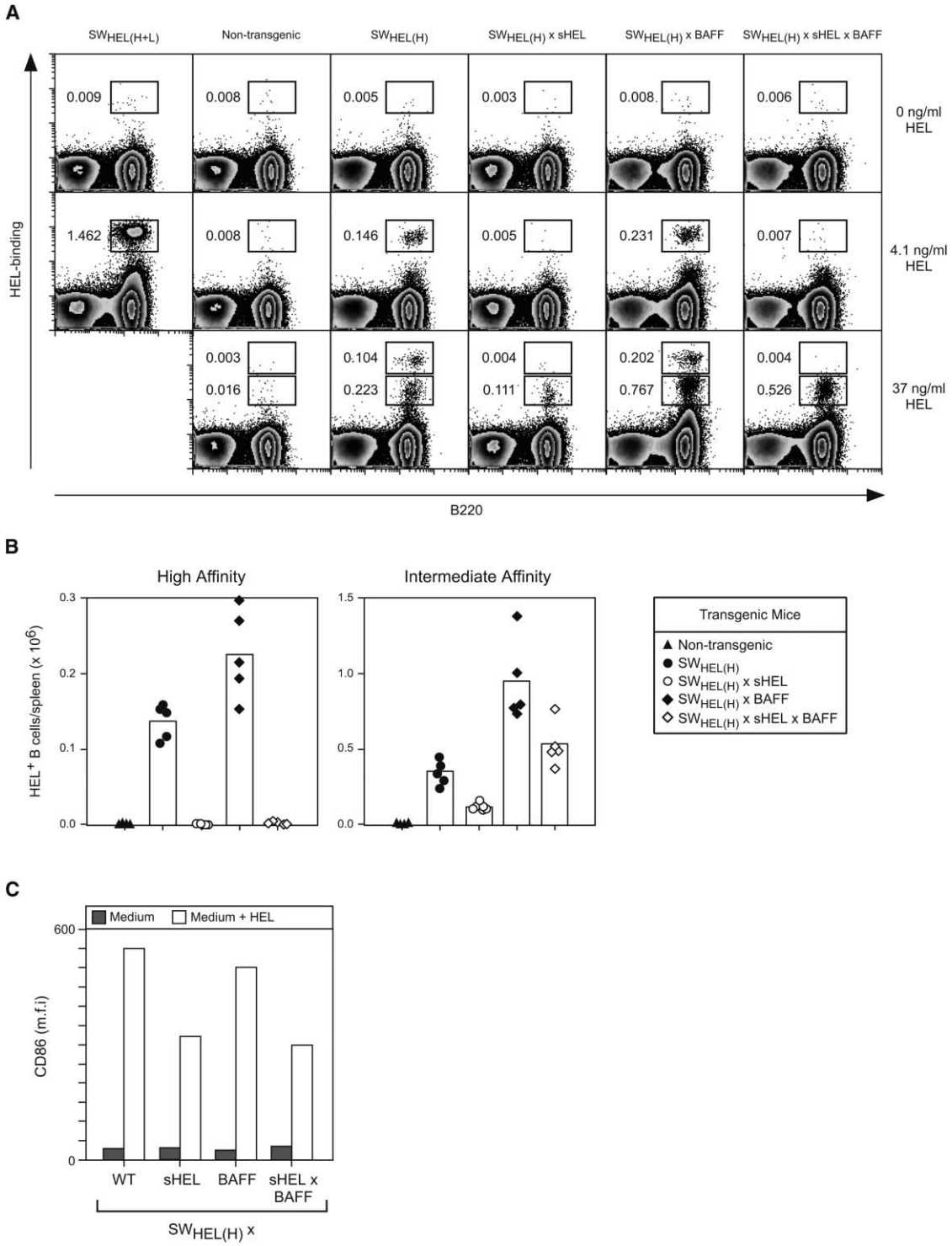


Figure 6. Excess BAFF Expands Intermediate Affinity Self-Reactive HEL$^+$ B Cells

(A) Spleen cells from the indicated mice were stained with anti-B220-PerCP and HEL (at 0, 4.1, or 37 ng/ml) + HyHEL5-Alexa Fluor® 647. Note that $SW_{HEL(H+L)}$ spleen cells were diluted 1:10 in nontransgenic spleen cells prior to staining so that the frequency of HEL$^+$ B cells was comparable to that in $SW_{HEL(H)}$ mice. Numbers represent the proportion of gated events within the windows.

(B) Spleen cells were stained as for (A) using 37 ng/ml HEL. The numbers of high affinity and intermediate affinity HEL$^+$ B cells/spleen were calculated using the gates shown in (A). Points represent data from individual mice of the indicated genotype while the columns represent the mean.

(C) Spleen cells were incubated with or without HEL-FITC (1 μg/ml) and stained for B220, HEL binding, and CD86 as described in Figure 2D. The geometric mean of CD86 fluorescence intensity was calculated after gating on HEL$^+$ B cells. Data is representative of two independent experiments.

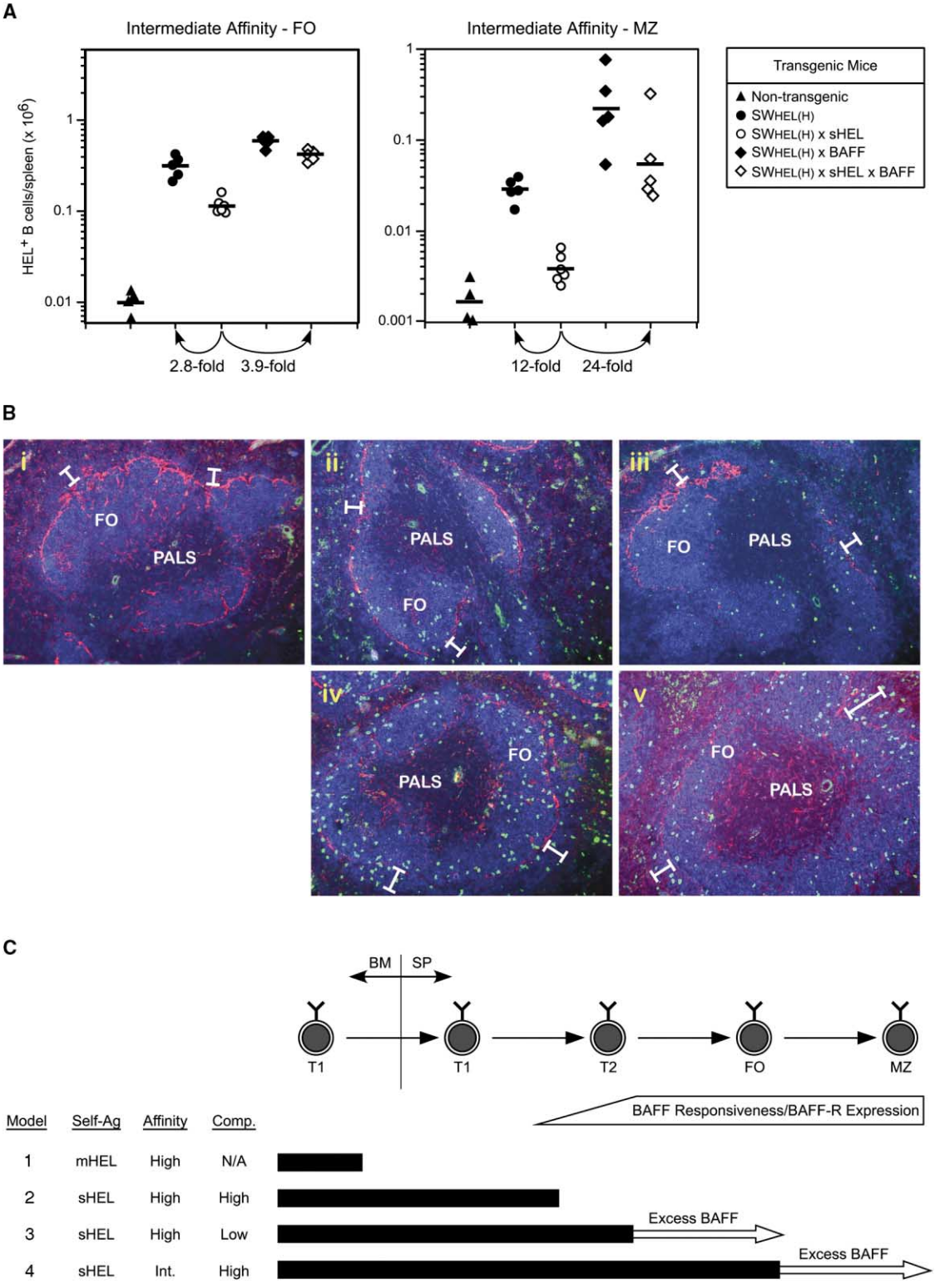


Figure 7. Excess BAFF Promotes Intermediate Affinity Self-Reactive HEL$^+$ B Cells into the MZ Compartment

(A) Spleen cells from the indicated mice were stained with anti-B220-PerCP, anti-CD21/35-FITC, anti-CD23-PE, and 37 ng/ml HEL + HyHEL5-Alexa Fluor® 647. Numbers of intermediate affinity HEL$^+$ B cells in the splenic FO and MZ compartments were calculated using gates as for Figures 2B and 6A.

(B) Sections of snap-frozen spleens were fixed and stained as for Figure 3. B cells (B220; blue), HEL binding receptors (green), and the marginal sinus (MadCAM-1; red) are stained and the MZ (crossbar), FO, and PALS indicated. (i) Non-Tg C57BL/6—negative control for HEL$^+$ B cells; (ii) $SW_{HEL(H)}$ − HEL$^+$ B cells in the FO and MZ; (iii) $SW_{HEL(H)}$ x sHEL − HEL$^+$ B cells in FO only; (iv) $SW_{HEL(H)}$ x BAFF − HEL$^+$ B cells in FO and MZ; (v) $SW_{HEL(H)}$ x sHEL x BAFF − HEL$^+$ B cells in MZ as well as FO.

compared to $SW_{HEL(H)}$ x sHEL Tg mice (Figure 7A). Histological analysis confirmed the appearance of self-reactive HEL^+ B cells in the MZ of $SW_{HEL(H)}$ x sHEL x BAFF Tg mice (Figure 7Bv compared to 7Biii). HEL^+ self-reactive B cells from $SW_{HEL(H)}$ x sHEL x BAFF Tg mice were not anergic (Figure 6C) although it was not possible to identify the individual responses of the MZ versus FO subsets.

Discussion

The importance of BAFF as a survival factor in peripheral B cell development has been well documented in studies of both BAFF deficient (Schiemann et al., 2001; Thompson et al., 2001) and BAFF Tg (Khare et al., 2000; Mackay et al., 1999) mice. In this study, we characterized the specific role played by BAFF in the regulation of self-reactive B cells. The effects of BAFF overexpression were examined in four different scenarios involving self-reactive B cells with (1) high affinity for membrane-bound self-antigen (Figure 1), (2) high affinity for soluble antigen with extensive (physiological) intercellular competition (Figures 3 and 5), (3) high affinity for soluble antigen with limited intercellular competition (Figures 2 and 3), and (4) intermediate affinity for soluble antigen with extensive (physiological) intercellular competition (Figures 6 and 7). These models and the effects of BAFF overexpression on self-reactive B cell development in each case are summarized in Figure 7C.

Self-reactive B cells were deleted at different points during B cell development in each of the four models described above, ranging from complete elimination from the periphery (model 1) to specific deletion from the MZ alone (model 4) (Figure 7C). When the effects of BAFF overexpression in each model are considered together, it is apparent that the development of self-reactive B cells is only affected by excess BAFF when the developmental stage at which they are normally deleted occurs after a critical point around the T2 stage of B cell maturation (Figure 7C). Since full responsiveness to BAFF survival signals is not acquired until some time during the T2 stage (Gross et al., 2001; Hsu et al., 2002; Schiemann et al., 2001), this finding suggests that only those self-reactive B cells that mature sufficiently to acquire responsiveness to BAFF survival signals are rescued from deletion by excess BAFF.

The requirement for self-reactive B cells to pass a critical maturational "checkpoint" in order to be rescued from deletion by excess BAFF means that only those cells that are deleted with relatively low stringency can be rescued under such circumstances (e.g., models 3 and 4; Figure 7C). By contrast, self-reactive clones that prove the greatest threat to the host (e.g., high affinity for self-antigen, recognition of an autologous polyvalent molecule) are insulated from the effects of BAFF by virtue of their deletion early during B cell development (e.g., models 1 and 2; Figure 7C). Taken together, these findings establish a role for excess BAFF in subverting the normal mechanisms of B cell self tolerance, thereby providing a primary mechanism for the induction of autoimmunity associated with BAFF overexpression. Moreover, the limitation of the effects of BAFF overexpression to self-reactive B cells deleted with relatively low stringency provides an explanation for the gradual rather than acute onset of manifestations of autoimmunity in BAFF Tg mice (Groom et al., 2002; Khare et al., 2000; Mackay et al., 1999).

A role for BAFF overexpression in mediating autoimmunity receives additional support from the finding in this study that self-reactive B cells rescued by BAFF were positioned in splenic microenvironments from which they are typically excluded. In the first case, high-affinity self-reactive B cells normally excluded from the FO in tolerant SW_{HEL} x sHEL Tg mice efficiently colonized this area in SW_{HEL} x sHEL x BAFF Tg mice (model 3; Figure 3). Nevertheless, these cells failed to colonize the MZ, consistent with the high stringency of deletion of self-reactive B cells from this compartment as previously characterized in the anti-HEL x sHEL double Tg model (Mason et al., 1992, Phan et al., 2003). The high stringency of deletion from the MZ was also exemplified in the case of the intermediate affinity HEL^+ self-reactive B cells, which, in the absence of BAFF overexpression, were efficiently deleted from the MZ but only partially from the FO (model 4; Figures 7A and 7B). In this case, excess BAFF restored HEL^+ self-reactive B cells to the MZ compartment (Figures 7A and 7B). The restoration of self-reactive B cells to different splenic microenvironments in these two models probably reflects the different stringency of deletion imposed on the respective self-reactive B cell populations in the absence of excess BAFF. Thus, self-reactive B cells rescued by excess BAFF appear to be "promoted" to the next stage of development rather than being channeled into a specific microenvironment or phenotype (Figure 7C).

The promotion of self-reactive B cells to normally forbidden microenvironments within the spleen is likely to be as important in the induction of BAFF-driven autoimmunity as the rescue of such cells from deletion. According to the results presented here and elsewhere (Mason et al., 1992; Cyster et al., 1994; Phan et al., 2003), the immune system has evolved a hierarchical system in which self-reactive B cells are most stringently deleted from the MZ, followed by the FO, and then complete deletion from the periphery (Figure 7C). This hierarchical system progressively reduces the likelihood of the self-reactive B cells being activated by either antigen or

(C) Summary of self-reactive B cell deletion in the four models examined in this study and the influence of excess BAFF. B cell development is indicated as a maturational progression in which deletion of self-reactive B cells can occur at different stages depending on parameters, such as self-antigen avidity, affinity for self-antigen, and the degree of intercellular competition. Excess BAFF did not affect the development or localization of self-reactive B cells deleted early during development (models 1 and 2) but rescued self-reactive B cells normally deleted later during maturation (models 3 and 4). Responsiveness to excess BAFF coincided with development past a critical maturational window during the T2 stage at which BAFF responsiveness and BAFF-R expression are acquired. See text for details. Note that a direct progression from FO to MZ stages is indicated for convenience and to reflect the hierarchy of deletion of self-reactive B cells. The actual maturational relationship between these two compartments remains unclear.

polyclonal stimuli. The need to prevent B cells with significant autoreactivity from colonizing the MZ is paramount, since B cells in this area are most exposed to polyclonal B cell stimuli, such as LPS and CpG associated with blood-borne pathogens (Cyster, 2000). Moreover, MZ B cells produce greater and more sensitive responses to T dependent and T independent stimuli than FO B cells (Oliver et al., 1997, 1999) and are more potent costimulators of T helper cells (Attanavanich and Kearney, 2004). Removal of self-reactive B cells from the FO further reduces the likelihood of inappropriate activation of self-reactive B cells, since cells excluded from this compartment have a short half-life and cannot recirculate through the body (Cyster and Goodnow, 1995; Phan et al., 2003). In other words, the presence of excess BAFF is likely to precipitate autoimmunity not only by increasing the numbers of self-reactive B cells but also by promoting them into microenvironments where their location and associated phenotype increase their chances of inappropriate activation.

The likelihood of self-reactive B cells rescued by BAFF overexpression being activated in vivo is also increased by the fact that they are not anergic. Thus, in each of the cases where excess BAFF countered the deletion signal (models 3 and 4), self-reactive B cells acquired sufficient BCR responsiveness to upregulate CD86 and to mount T dependent antibody responses (Figures 2D, 2E, and 6C). A possible mechanism of action of BAFF in reversing anergy is suggested by the fact that BAFF is a potent activator of members of the NF-κB/Rel transcription factor family in B cells (Do et al., 2000). Since BCR-induced NF-κB activation is specifically blocked in anergic B cells (Healy et al., 1997), direct augmentation of cellular NF-κB activity therefore provides a plausible mechanism whereby the presence of excess BAFF may overcome the BCR signaling defect characteristic of anergic B cells. Interestingly, the self-reactive intermediate affinity HEL^+ B cells that matured to the FO stage and survived in $SW_{HEL(H)}$ x sHEL Tg mice in the absence of BAFF overexpression were fully responsive to antigen rather than anergic (Figure 6C). This result suggests that, within a normal polyclonal repertoire, anergic B cells probably do not enter the mature B cell pool at all but exist only transiently as immature B cells. Hence, the phenomena of anergy and peripheral deletion in the B lineage are likely to be tightly linked under normal circumstances.

The requirement for competition from non-self-reactive B cells for the peripheral deletion of high-affinity self-reactive B cells by soluble self-antigen (Cyster and Goodnow, 1995; Cyster et al., 1994; Phan et al., 2003) raises the possibility that the development of certain self-reactive B cells may be curtailed due to an inability to compete efficiently for essential survival signals. The finding here that self-reactive B cells can be rescued from deletion at the T2 stage by provision of excess BAFF is consistent with this possibility and suggests that these cells may be relatively poor at receiving BAFF signals in vivo. If exclusion from BAFF signals is important for the deletion of self-reactive B cells near the T2 stage, it cannot be explained on the basis of altered expression of receptors for BAFF, since self-reactive B cells from SW_{HEL} x sHEL Tg mice appear to express normal levels of both BAFF-R and TACI (data not shown). Alternatively, BAFF signaling pathways may be compromised in these cells in parallel with the inactivation of BCR signaling that characterizes B cell anergy. Either way, competition for BAFF as a mechanism for deletion of self-reactive B cells is likely to apply only in the case of cells deleted around the late T2 stage of development since selective purging of self-reactive B cells from the MZ compartment occurs independently of intercellular competition (Mason et al., 1992, Phan et al., 2003).

Experimental Procedures

Mice

The various mouse strains used were maintained on a C57BL/6 background in the Centenary Institute Animal Facility. All experimental mice were hemizygous for their respective transgenes. SW_{HEL} mice have been described previously (Phan et al., 2003) and carry the V_H10 anti-HEL heavy chain variable region coding exon targeted to the endogenous IgH allele plus a V_H10-κ anti-HEL light chain transgene. For the experiments described here, the loxP-flanked neomycin resistance cassette upstream of the targeted V_H10 exon was removed from the SW_{HEL} germline by crossing with the Zp3-Cre Tg line (Lewandoski et al., 1997). $SW_{HEL(H)}$ mice carried the targeted IgH allele alone without the light chain transgene. HEL Tg mice either expressed mHEL from the mouse H2-K^b promoter (KLK3 line) (Hartley et al., 1991) or sHEL from the mouse metallothionein promoter (ML5 line) (Goodnow et al., 1988). BAFF Tg mice that secrete high levels of murine BAFF under the influence of a liver-specific α_1-antitrypsin promoter have been previously described (Mackay et al., 1999). All mice were screened for the presence of transgenes by PCR amplification of genomic DNA prepared from peripheral blood leukocytes.

BM Chimeras

Recipient mice aged 8–14 weeks were lethally irradiated (950 rad) and rescued 6–8 hr later with an intravenous injection of 2×10^7 BM cells. BM cells were harvested from donor mice of the indicated genotypes by aspirating femurs and tibiae with medium (RPMI/10% FCS). Chimeras were analyzed 8–14 weeks after reconstitution.

Flow Cytometry

Four color flow cytometry was performed on a dual laser FACSCalibur™ flow cytometer (BD Biosciences) and analyzed with CELLQuest™ v.3.3 software (BD Biosciences). Splenocytes and BM cells were stained in 96-well round-bottom plates as described (Phan et al., 2003). Antibodies utilized in staining were obtained from BD Biosciences, including anti-CD21/35-FITC (clone 7G6), anti-CD23-PE (B3B4), anti-CD24(HSA)-PE (M1/69), anti-B220(CD45R)-PerCP (RA3-6B2), anti-CD45.1-FITC (A20), and anti-CD86-PE (GL1). HEL^+ B cells were identified by staining with a saturating concentration of HEL (Sigma-Aldrich) at 200 ng/ml and revealed by HyHEL5 mAb conjugated to either Alexa Fluor® 647 (Molecular Probes) or FITC. For detection of HEL^+ B cells of differing affinities, staining was also performed using HEL at 4.1 or 37 ng/ml. HyHEL5 mAb was purified from hybridoma supernatants and conjugated to FITC and Alexa Fluor® 647 as described (Phan et al., 2003). Receptor occupancy was determined by staining with and without the addition of HEL prior to staining with conjugated HyHEL5 and calculated as previously described (Phan et al., 2003).

Adoptive Transfer

Splenocytes from donor mice were prepared and the percentage of HEL binding B cells present determined by FACS. HEL-SRBC were prepared as previously described (Goodnow et al., 1988) and mock-conjugated SRBC produced without the addition of HEL. Non-irradiated recipients (8- to 14-week-old male C57BL/6 wt mice) received 200 μl of inoculum intravenously containing 10^4 HEL^+ B cells and 2×10^8 HEL-SRBC or mock-conjugated SRBC. Equivalent numbers of spleen cells were injected into each recipient by balancing donor cells with splenocytes from C57BL/6 wt mice. Serum was collected 7 days after injections and anti-HEL antibodies quantitated by ELISA.

ELISAs

A modified ELISA protocol (Goodnow et al., 1988; Phan et al., 2003) was used to measure the amount of anti-HEL antibodies present in the serum samples. In brief, 96-well polystyrene plates (Nunc) were coated with 10 μg/ml HEL and incubated at 4°C overnight. The wells were blocked with 5% skim milk/PBS at 37°C in a humidity chamber for 1 hr. Serial dilutions of sera and HyHEL10 anti-HEL IgM or IgG1 standards (Phan et al., 2003) were then added and anti-HEL Ig subsequently detected with either anti-mouse IgM-biotin (R6-60.2) or IgG1-biotin (A85-1) (BD Biosciences) followed by avidin-alkaline phosphatase conjugate (Roche) and revealed with the *p*-nitrophenyl phosphate substrate (ICN Biomedicals). Absorbance at 405 nm was read and the concentration of anti-HEL Igs calculated with reference to the appropriate HyHEL10 standard curve.

Immunohistology

Spleens were snap-frozen in liquid N_2 and 5–7 μm sections prepared and stained for B220, MadCAM-1, and HEL binding as described previously (Phan et al., 2003).

In Vitro Cultures

The ability of self-reactive B cells to upregulate CD86 was assayed by culturing 10^6 splenocytes overnight at 37°C with or without HEL (500 ng/ml) or HEL-FITC (1 μg/ml) in RPMI 1640 medium containing 10% heat-inactivated FCS (Life Technologies), 2 mM L glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-ME (all from Sigma-Aldrich). Cells were harvested after 16 hr, stained with anti-B220-PerCP, HEL + HyHEL5-FITC and anti-mouse CD86-PE (BD Biosciences), and analyzed by flow cytometry. Mean CD86 fluorescence intensity was calculated for HEL^+ $B220^+$ cells.

Acknowledgments

We thank Dr. Jenny Kingham and the staff of the Centenary Institute Animal Facility for animal husbandry; Tyani Chan and Christopher Brownlee for mouse screening; and Dr. Susan Kalled and Biogen Corporation for permission to use BAFF Tg mice. We also thank Dr. Stuart Tangye for his critical revision of the manuscript. This work was supported by a program grant from the National Health and Medical Research Council of Australia. M.T. and T.G.P. are both supported by National Health and Medical Research Council postgraduate research scholarships.

Received: October 14, 2003
Revised: April 2, 2004
Accepted: April 15, 2004
Published: June 15, 2004

References

Allman, D., Lindsley, R.C., DeMuth, W., Rudd, K., Shinton, S.A., and Hardy, R.R. (2001). Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. J. Immunol. *167*, 6834–6840.

Attanavanich, K., and Kearney, J.F. (2004). Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. J. Immunol. *172*, 803–811.

Batten, M., Groom, J., Cachero, T.G., Qian, F., Schneider, P., Tschopp, J., Browning, J.L., and Mackay, F. (2000). BAFF mediates survival of peripheral immature B lymphocytes. J. Exp. Med. *192*, 1453–1466.

Cook, M.C., Basten, A., and Fazekas de St Groth, B. (1997). Outer periarteriolar lymphoid sheath arrest and subsequent differentiation of both naive and tolerant immunoglobulin transgenic B cells is determined by B cell receptor occupancy. J. Exp. Med. *186*, 631–643.

Cooke, M.P., Heath, A.W., Shokat, K.M., Zeng, Y., Finkelman, F.D., Linsley, P.S., Howard, M., and Goodnow, C.C. (1994). Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. J. Exp. Med. *179*, 425–438.

Cyster, J.G. (2000). B cells on the front line. Nat. Immunol. *1*, 9–10.

Cyster, J.G., and Goodnow, C.C. (1995). Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. Immunity *3*, 691–701.

Cyster, J.G., Hartley, S.B., and Goodnow, C.C. (1994). Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. Nature *371*, 389–395.

Do, R.K., Hatada, E., Lee, H., Tourigny, M.R., Hilbert, D., and Chen-Kiang, S. (2000). Attenuation of apoptosis underlies B lymphocyte stimulator enhancement of humoral immune response. J. Exp. Med. *192*, 953–964.

Forster, I., and Rajewsky, K. (1990). The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. Proc. Natl. Acad. Sci. USA *87*, 4781–4784.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., and Raphael, K. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676–682.

Goodnow, C.C., Crosbie, J., Jorgensen, H., Brink, R.A., and Basten, A. (1989). Induction of self-tolerance in mature peripheral B lymphocytes. Nature *342*, 385–391.

Goodnow, C.C., Adelstein, S., and Basten, A. (1990). The need for central and peripheral tolerance in the B cell repertoire. Science *248*, 1373–1379.

Groom, J., Kalled, S.L., Cutler, A.H., Olson, C., Woodcock, S.A., Schneider, P., Tschopp, J., Cachero, T.G., Batten, M., Wheway, J., et al. (2002). Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J. Clin. Invest. *109*, 59–68.

Gross, J.A., Dillon, S.R., Mudri, S., Johnston, J., Littau, A., Roque, R., Rixon, M., Schou, O., Foley, K.P., Haugen, H., et al. (2001). TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. Impaired B cell maturation in mice lacking BLyS. Immunity *15*, 289–302.

Hartley, S.B., and Goodnow, C.C. (1994). Censoring of self-reactive B cells with a range of receptor affinities in transgenic mice expressing heavy chains for a lysozyme-specific antibody. Int. Immunol. *6*, 1417–1425.

Hartley, S.B., Crosbie, J., Brink, R., Kantor, A.B., Basten, A., and Goodnow, C.C. (1991). Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature *353*, 765–769.

Hartley, S.B., Cooke, M.P., Fulcher, D.A., Harris, A.W., Cory, S., Basten, A., and Goodnow, C.C. (1993). Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell *72*, 325–335.

Healy, J.I., Dolmetsch, R.E., Timmerman, L.A., Cyster, J.G., Thomas, M.L., Crabtree, G.R., Lewis, R.S., and Goodnow, C.C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. Immunity *6*, 419–428.

Hsu, B.L., Harless, S.M., Lindsley, R.C., Hilbert, D.M., and Cancro, M.P. (2002). Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators. J. Immunol. *168*, 5993–5996.

Khare, S.D., Sarosi, I., Xia, X.Z., McCabe, S., Miner, K., Solovyev, I., Hawkins, N., Kelley, M., Chang, D., Van, G., et al. (2000). Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. Proc. Natl. Acad. Sci. USA *97*, 3370–3375.

Lewandoski, M., Wassarman, K.M., and Martin, G.R. (1997). Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. Curr. Biol. *7*, 148–151.

Mackay, F., Woodcock, S.A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., Tschopp, J., and Browning, J.L. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J. Exp. Med. *190*, 1697–1710.

Mackay, F., Schneider, P., Rennert, P., and Browning, J. (2003).

BAFF AND APRIL: a tutorial on B cell survival. Annu. Rev. Immunol. *21*, 231–264.

Mason, D.Y., Jones, M., and Goodnow, C.C. (1992). Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. Int. Immunol. *4*, 163–175.

Nemazee, D., and Buerki, K. (1989). Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras. Proc. Natl. Acad. Sci. USA *86*, 8039–8043.

Oliver, A.M., Martin, F., Gartland, G.L., Carter, R.H., and Kearney, J.F. (1997). Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. Eur. J. Immunol. *27*, 2366–2374.

Oliver, A.M., Martin, F., and Kearney, J.F. (1999). IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J. Immunol. *162*, 7198–7207.

Phan, T.G., Amesbury, M., Gardam, S., Crosbie, J., Hasbold, J., Hodgkin, P.D., Basten, A., and Brink, R. (2003). B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. J. Exp. Med. *197*, 845–860.

Rathmell, J.C., Fournier, S., Weintraub, B.C., Allison, J.P., and Goodnow, C.C. (1998). Repression of B7.2 on self-reactive B cells is essential to prevent proliferation and allow Fas-mediated deletion by CD4(+) T cells. J. Exp. Med. *188*, 651–659.

Rolink, A.G., Tschopp, J., Schneider, P., and Melchers, F. (2002). BAFF is a survival and maturation factor for mouse B cells. Eur. J. Immunol. *32*, 2004–2010.

Schiemann, B., Gommerman, J.L., Vora, K., Cachero, T.G., Shulga-Morskaya, S., Dobles, M., Frew, E., and Scott, M.L. (2001). An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science *293*, 2111–2114.

Thompson, J.S., Bixler, S.A., Qian, F., Vora, K., Scott, M.L., Cachero, T.G., Hession, C., Schneider, P., Sizing, I.D., Mullen, C., et al. (2001). BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science *293*, 2108–2111.