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Alternative and Classical NF-κB Signaling Retain Autoreactive B Cells in the Splenic Marginal Zone and Result in Lupus-like Disease

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Summary

Expression of B cell-activating factor (BAFF), a critical B cell survival factor, is elevated in autoimmune and lymphoproliferative disorders. Mice overproducing BAFF develop systemic lupus erythematosus (SLE)-like disease and exhibit B cell activation of classical and alternative NF-κB-signaling pathways. We used a genetic approach and found that both NF-κB-signaling pathways contributed to disease development but act through distinct mechanisms. Whereas BAFF enhanced long-term B cell survival primarily through the alternative, but not the classical, NF-κB pathway, it promoted immunoglobulin class switching and generation of pathogenic antibodies through the classical pathway. Activation of the alternative NF-κB pathway resulted in integrin upregulation, thereby retaining autoreactive B cells in the splenic marginal zone, a compartment that contributes to their survival. Thus, both classical and alternative NF-κB signaling are important for development of lupus-like disease associated with BAFF overproduction. The same mechanisms may be involved in the pathogenesis of human SLE.

Introduction

Autoimmunity results from failed establishment and maintenance of tolerance to self-antigens (Lesage and Goodnow, 2001). Although many cell types contribute to autoimmune disorders, lymphocytes are key effectors in their initiation and propagation. During normal lymphopoiesis, a few self-reactive B lymphocytes emerge (Wardemann et al., 2003) but are normally silenced by at least three different mechanisms: clonal deletion, development of anergy, and B cell receptor (BCR) editing (Nemazee and Burki, 1989).

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B cell-activating factor (BAFF, also known as BLyS or TALL-1) is a fundamental B cell survival factor (Batten et al., 2000) produced by myeloid cells, T cells, and different stromal cells (Mackay and Tangye, 2004). Three BAFF receptors were identified: BCMA (B cell maturation antigen), TACI (transmembrane activator and CAML interactor), and BAFF-R (BAFF receptor or BR3) (Mackay et al., 2003). BCMA and TACI also bind APRIL (a proliferation-inducing ligand), whereas BAFF-R binds only BAFF (Mackay et al., 2003). Studies with mice harboring inactivating mutation of BAFF-R (*Tnfrsf13c*) demonstrated that BAFF-R is required for mature B cell survival in the periphery (Miller and Hayes, 1991). In contrast, BCMA-deficient ($Tnfrsf17^{-/-}$) mice have normal B cell numbers, whereas TACI-deficient ($Tnfrsf13b^{-/-}$) mice show a 2-fold increase in B cells (von Bulow et al., 2001). Mice overexpressing BAFF (BAFF-Tg mice) develop an autoimmune disease with hallmarks resembling human systemic lupus erythematosus (SLE), including autoantibodies against double-stranded (ds)-DNA, circulating immune complexes, and a severe membranoproliferative glomerulonephritis (Mackay et al., 1999).

The inhibitor of κB (IκB) kinase (IKK) complex (Rothwarf and Karin, 1999) activates via its two catalytic subunits, IKKβ and IKKα, the classical and alternative NF-κB-signaling pathways, respectively, that play important roles in innate and adaptive immunity (Bonizzi and Karin, 2004). The classical NF-κB pathway targets dimers composed of p65 (RelA), c-Rel, and NF-κB1 (p50), which are retained in the cytoplasm by IκB proteins (Ghosh et al., 1998). Activation of this pathway causes phosphorylation of IκBs and their subsequent degradation, allowing nuclear entry of liberated NF-κB dimers (Karin and Ben-Neriah, 2000). The alternative NF-κB pathway depends on IKKα dimers that phosphorylate the NF-κB2 (p100) precursor protein, leading to degradation of its C-terminal half and release of its N-terminal portion, p52, that enters the nucleus as a dimer with RelB (Senftleben et al., 2001a). Whereas all three BAFF receptors activate the classical NF-κB pathway, only BAFF-R activates the alternative NF-κB pathway (Claudio et al., 2002).

We examined which NF-κB pathway and which B cell type are involved in development of SLE-like autoimmune disease in BAFF-Tg mice. Given the known pathogenic role of high-affinity dsDNA autoantibodies in SLE, it was anticipated that the CD40-dependent germinal center (GC) reaction, in which B cells undergo affinity maturation and Ig class switching, would be essential for disease development (McHeyzer-Williams, 2003). However, we found that CD40-dependent GC formation was not required for development of SLE-like disease. In contrast, another splenic B cell compartment, the marginal zone (MZ), turned out to be critical. The enlarged MZ B cell pool of BAFF-Tg mice, in which B cell survival depends mainly on the alternative NF-κB pathway, harbors the majority of autoreactive B cells, and its elimination or reduction prevents or strongly diminishes the disease.

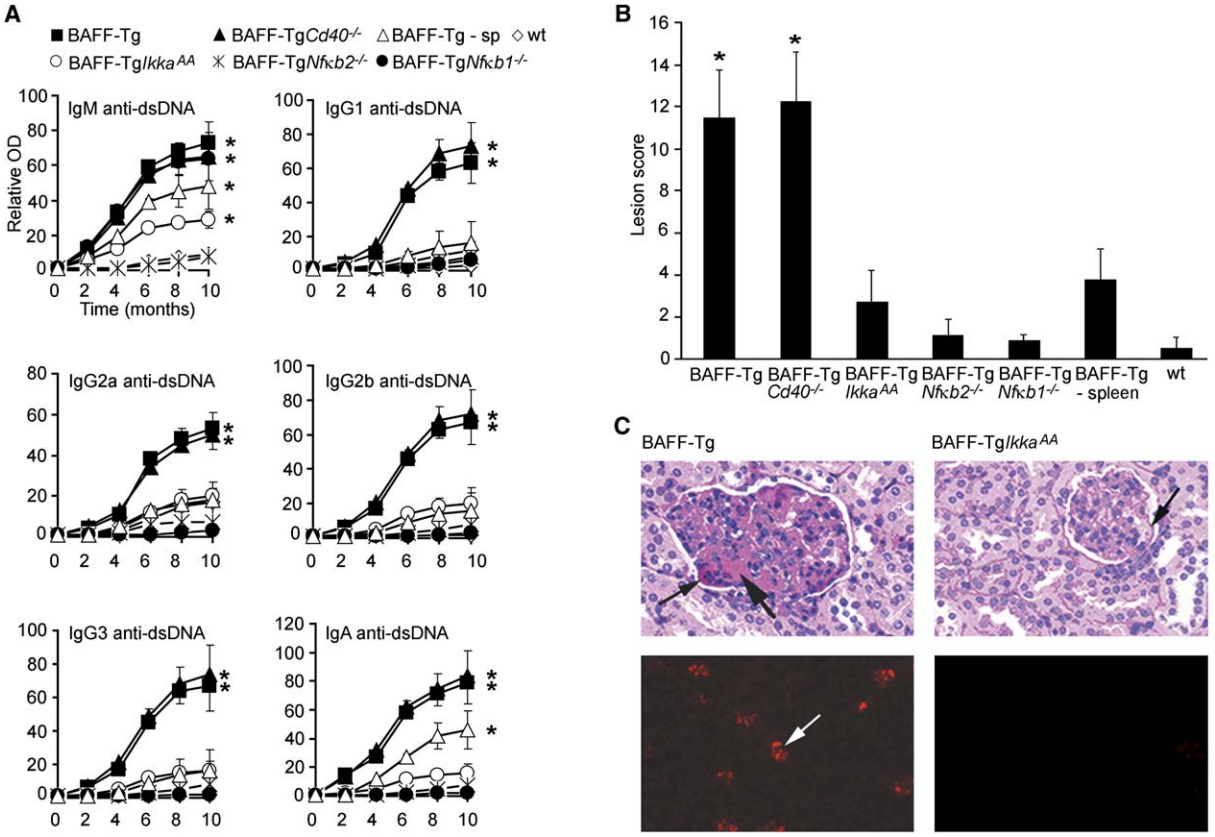

Figure 1. BAFF-Tg Mice Develop a SLE-like Autoimmune Disease Dependent on Both NF-κB Pathways

(A) dsDNA autoantibody titers monitored over a 10 month period in BAFF-Tg, BAFF-Tg compound mutants, and splenectomized (−sp) BAFF-Tg mice. Values represent relative mean ± SD. OD_{405} measurements of autoantibody titers at different ages in 8 mice per genotype or treatment. Statistically significant differences ($p < 0.01$) between mutants and wt controls are indicated by asterisks.

(B) Lesion scores mean ± SD of kidneys from 10-month-old mice (n = 8) of the indicated cohorts. Asterisks indicate significant changes ($p < 0.01$) from the wt.

(C) Top: Periodic acid Schiff-(PAS)-stained kidneys of BAFF-Tg and BAFF-Tg*Ikka*$^{AA/AA}$ mice, 20× magnification. Thin arrows point to basement membranes, thick arrow marks necrotic zone. Bottom: Immunohistology via a PE-labeled anti-IgG antibody to identify glomerular IgG deposits.

Results

Disruption of Either NF-κB Pathway or Splenectomy Prevents or Strongly Reduces Autoimmune Disease in BAFF-Tg Mice

The roles of the classical and alternative NF-κB pathways, as well as CD40, in development of SLE-like disease in mice that overexpress BAFF were examined. Because BAFF-Tg mice exhibit marked splenomegaly (Mackay et al., 1999), some mice were splenectomized at 3 weeks of age to evaluate the role of the spleen in disease development. In addition, we used cohorts of eight age-matched mice that were either wild-type (wt) or deficient in various components of the classical and alternative NF-κB pathways. All mice were monitored for dsDNA autoantibody titers of IgM, IgG subclasses, and IgA and for proteinuria (Balow, 2005).

Production of IgM dsDNA autoantibodies in BAFF-Tg mice required the alternative NF-κB pathway, because these titers were markedly reduced in BAFF-Tg*Ikka*$^{AA/AA}$ mice, which express a variant of IKKα that can not be activated, or were absent upon loss of NF-κB2 (Figure 1A). IgM autoantibody production, however, was not impaired by the absence of NF-κB1 or CD40. Interruption of either NF-κB1 or NF-κB2 signaling, but not loss of CD40, prevented generation of dsDNA autoantibodies of the IgG subclasses and IgA. Although early splenectomy had only a partial effect on concentrations of IgM and IgA autoantibodies, it markedly reduced IgG autoantibody production. dsDNA autoantibody titers did not differ by gender and were undetectable in mice without the transgene encoding BAFF. No nonspecific autoantibody binding to bovine serum albumin could be found, ruling out contribution of polyreactive background Igs (data not shown). Only mice with high IgG autoantibody titers developed a considerable proteinuria (Table 1).

At 10 months of age, mice were sacrificed and kidneys were analyzed. Only mice with high titers of IgG dsDNA autoantibodies exhibited severe kidney lesions, whereas kidney lesions were mild or absent in mice without IgG autoantibodies, regardless of IgM or IgA autoantibody concentrations (Figures 1B and 1C). Immunohistology of kidneys with severe lesions revealed numerous glomerular deposits of IgG (Figure 1C). Thus, the spleen and intact classical and alternative NF-κB pathways, but not CD40, are required for disease development.

Table 1. Proteinuria in 8-Month-Old Mice of Different Genotypes

Mouse	1	2	3	4	5	6	7	8
wt	—	—	—	(+)	—	—	+	(+)
BAFF-Tg	++	++	+++	+++	++	+	+++	++
BAFF-Tg*Cd40*$^{-/-}$	+++	+++	+++	++	++	+++	++	++
BAFF-Tg*Ikka*^{AA}	+	+	(+)	—	+	+	+	+
BAFF-Tg*Nfκb2*$^{-/-}$	—	—	(+)	—	—	—	+	—
BAFF-Tg*Nfκb1*$^{-/-}$	—	—	(+)	+	+	+	—	—
BAFF-Tg - spleen	+	+	+(+)	—	+	+	+	(+)

Quantification of proteinuria: + 30 mg/dl; ++ 100 mg/dl; +++ 300 mg/dl.

BAFF Controls B Cell Survival via Classical and Alternative NF-κB Pathways

Disruption of the alternative NF-κB pathway, as in splenic B cells isolated from *Ikka*$^{AA/AA}$, *Ikka*$^{-/-}$ transplanted wt, or *Nfκb2*$^{-/-}$ mice, and loss of BAFF-R reduced or abrogated the prosurvival effect of BAFF (Figure 2A). Also, BAFF stimulation of *Nfκb1*$^{-/-}$ B cells did not significantly enhance in vitro survival within the first 12 hr. When present for 24 hr or longer, however, BAFF enhanced the survival of *Nfκb1*$^{-/-}$ B cells almost as effectively as in wt B cells.

Whereas IKKα- or BAFF-R-deficient B cells were unable to process p100 upon BAFF stimulation, residual p100 processing to p52 was seen in *Ikka*$^{AA/AA}$ B cells (Figure 2B). The degree of p100 processing correlated with the survival advantage conferred by BAFF, not only in vitro, but also in vivo. In this regard, splenic B cell numbers were 70%–80% lower than normal in B cell-deficient BAFF-TgμMT mice, which were transplanted with fetal liver cells from IKKα-deficient embryos, or in BAFF-Tg*Nfκb2*$^{-/-}$ mice (wt → BAFF-TgμMT, splenic B cells: 65 ± 7.4 × 10^6; *Ikka*$^{-/-}$ fetal liver cells → BAFF-TgμMT: 13.5 ± 1.9 × 10^6; BAFF-Tg*Nfκb2*$^{-/-}$: 16.1 ± 2.7 × 10^6; n = 5). Congruent with their ability to produce some p52 in response to BAFF, BAFF-Tg*Ikka*$^{AA/AA}$ mice contained as many splenic B cells as wt mice, but much fewer B cells than BAFF-Tg mice (BAFF-Tg*Ikka*$^{AA/AA}$, splenic B cells: 34 ± 4.5 × 10^6; wt: 32 ± 2.8 × 10^6, BAFF-Tg: 76 ± 8.3 × 10^6; n = 5).

As expected by the partial requirement of NF-κB1 for BAFF-mediated short-term survival, BAFF activated the classical NF-κB pathway. Electroporetic mobility shift assays (EMSAs), as well as immunoblotting with an IκBα antibody, revealed activated classical NF-κB signaling within 3 hr after BAFF addition (Figure 2E, see Figure S1 in the Supplemental Data available online). Stimulation with BAFF not only induced p50:p65 dimers but also enhanced formation of p50 homodimers. Addition of BAFF-neutralizing BR3-F_c immunoadhesin completely prevented activation of the classical pathway, indicating that the response was due to BAFF rather than unknown contaminants. In agreement with previous observations (Bonizzi et al., 2004), BAFF activation of the classical NF-κB pathway was normal in *Ikka*$^{AA/AA}$ and *Nfκb2*$^{-/-}$ B cells. Earlier studies suggested that BAFF activates the classical NF-κB pathway through TACI and BCMA (Marsters et al., 2000). However, we found modest, but reproducible, reduction in NF-κB binding activity in B cells from BAFF-R-deficient mice. Thus, BAFF-R also contributes to activation of the classical pathway. This observation was substantiated by impaired IκBα degradation in BAFF-stimulated B cells from BAFF-R-deficient mice (Figure S1). BAFF-R-deficient B cells expressed BCMA and TACI at amounts similar to wt B cells (data not shown). Nonetheless, a much greater impairment of classical NF-κB signaling was found in B cells from NF-κB1- or TACI-deficient mice. DNA binding complexes in *Nfκb1*$^{-/-}$ B cells induced by BAFF primarily consisted of p52:RelB and p65:RelB dimers (Figure 2E).

Besides upregulation of the antiapoptotic proteins Bcl-X_L and A1/Bfl1, whose maximal induction required both NF-κB pathways (Hatada et al., 2003), BAFF-induced long-term survival correlated with upregulation of Pim-2, which depended only on the alternative pathway (Figures 2C and 2D). Pim-2 is a serine and threonine kinase that phosphorylates the proapoptotic protein Bad at Ser112, resulting in its inactivation (Fox et al., 2005). Correspondingly, BAFF-induced Bad Ser112 phosphorylation was dependent on the alternative pathway and correlated with Pim-2 amounts (Figure 2D). Moreover, the survival curves of BAFF-stimulated *Pim2*$^{-/-}$ or *Nfκb2*$^{-/-}$ B cells were very similar (Figure 2A). Defects in either NF-κB pathway had no effect on Pim-1 or Pim-3 expression (data not shown). Taken together, BAFF enhances short- and long-term survival of B cells primarily via the classical and alternative NF-κB signaling pathways, respectively.

BAFF Induces In Vivo Expansion of Transitional T1 and T2 B Cells

BM-derived B cell precursors pass through intermediate differentiation stages in the spleen, which can be distinguished as transitional (T) 1 (B220$^+$AA-4.1$^+$CD21^{lo}CD23^{hi}CD24^{hi}) and T2 (B220$^+$AA-4.1$^+$CD21^{hi}CD23^{hi}CD24^{hi}) (Goodyear et al., 2004; Loder et al., 1999). Precursors from either healthy individuals or SLE patients produce comparable amounts of antinuclear autoantibodies (ANAs), whereas mature naive B cells from SLE patients generate many more ANAs than B cells from normal individuals. It was therefore suggested that ANA-producing B cells in SLE patients are not eliminated at the splenic transitional stage (Yurasov et al., 2005).

Accordingly, we examined effects of BAFF and NF-κB signaling on survival of transitional B cells. In contrast to previous reports, we found that BAFF enhanced the representation not only of T2 cells (Batten et al., 2000) but also of T1 cells (Figure 3), without affecting cell-cycle distribution (S + G2 phase of B220$^+$AA-4.1$^+$CD24^{hi} B cells from wt and BAFF-Tg mice were 8.2% ± 0.3% versus 8.4% ± 0.3% of total; n = 3). This effect of BAFF on transitional B cells was dependent on the NF-κB2

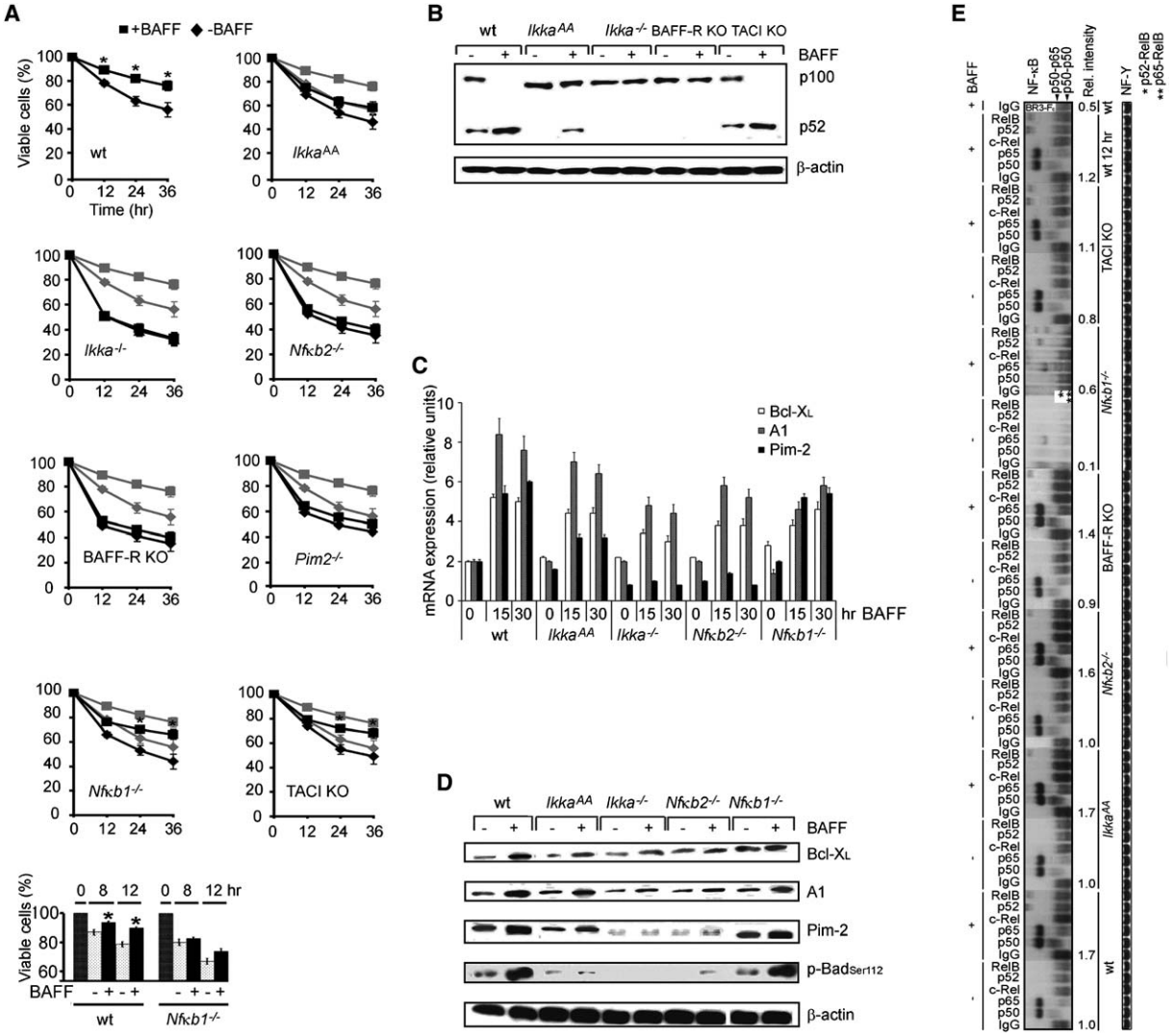

Figure 2. BAFF-Induced Long-Term Survival of CD3$^-$CD5$^-$CD43$^-$ Splenic B Cells Is Primarily Dependent on the Alternative NF-κB Pathway and BAFF-R

(A) Effect of BAFF on in vitro survival, determined by trypan blue exclusion, of purified B cells from the indicated strains. Black curves represent mean values ± SD for cells from three individual mice. Gray curves: wt B cells. Lower left: Bars represent survival of B cells from the indicated strains (n = 5) in the presence or absence of BAFF. Statistically significant effects ($p < 0.05$) on survival are indicated by asterisks.

(B) BAFF-induced NF-κB2 (p100) processing. B cells from different mouse strains (n = 3 mice per strain) were cultured for 12 hr with or without BAFF before cell extracts were prepared, and processing of NF-κB2 (p100) was analyzed by immunoblotting.

(C) Expression of mRNAs for antiapoptotic proteins. B cells from different mouse strains were incubated with BAFF, and total RNA was isolated at 0, 15, and 30 hr and analyzed for expression of different mRNAs as indicated by real-time PCR (mean ± SD; n = 3).

(D) Expression of antiapoptotic proteins. Cellular extracts obtained as in (C) after 15 hr in presence (+) or absence (−) of BAFF were subjected to immunoblot analysis with antibodies against the indicated proteins.

(E) Analysis of NF-κB binding activity. B cells (n = 3 mice per strain) were cultured for 3 hr, or as indicated, with or without BAFF. Nuclear extracts were prepared and EMSAs were performed in the presence of antibodies against the indicated NF-κB subunits or with isotype IgG control. NF-κB complexes as identified by supershift analysis are marked. Values underneath the panel represent mean relative signal intensities of total NF-κB DNA binding activity. BR3-F_c was used to inhibit BAFF. Extract quality was monitored by binding to a NF-Y probe.

component of the alternative pathway. The low amount of p52 generation in BAFF-Tg*Ikka*$^{AA/AA}$ mice seemed sufficient to produce a slightly enlarged T2 but not T1 B cell population. Analysis of transitional B cells from BAFF-Tg*Nfκb1*$^{-/-}$ mice showed a similar increase in T1 and T2 B cells as in BAFF-Tg mice. Thus, BAFF expands T1 and T2 transitional B cells mainly through activation of the alternative NF-κB signaling pathway.

BAFF Strongly Enhances Thymus-Independent Ig Class Switching, even without T Cells

Immunization of BAFF-Tg and BAFF-Tg*Cd40*$^{-/-}$ mice with the thymus-independent (TI) antigens DNP-Ficoll or DNP-LPS generated equally potent anti-DNP responses of the IgG1, IgG2a, IgG2b, IgG3, and IgA classes, which were significantly stronger than those in wt mice (Figures 4A and 4B). Anti-DNP responses of the IgG1, IgG3, and IgA classes were markedly reduced in

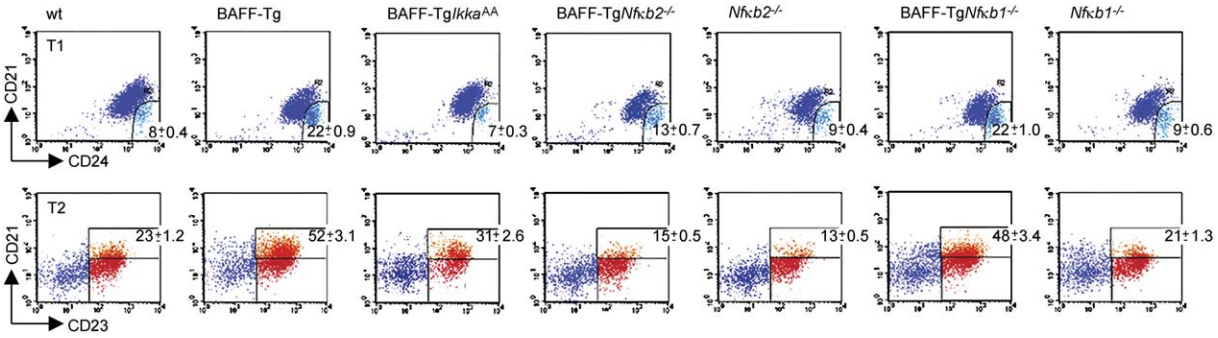

Figure 3. Analysis of Transitional T1 and T2 B Cells in Different Mouse Strains

Equal numbers of B220$^+$AA-4.1$^+$-sorted splenocytes from three mice per strain were analyzed by flow cytometry with different markers. T1 cells are CD21^{lo}CD24^{hi}. T2 cells are CD21^{hi}CD23^{hi}. The numbers represent percentages mean ± SD of gated cells compared to total B220$^+$AA-4.1$^+$ cells. One representative experiment is shown.

BAFF-Tg mice with defective alternative NF-κB signaling, such as BAFF-Tg*Nfκb2*$^{-/-}$ or BAFF-Tg*Ikka*$^{AA/AA}$ mice, while the IgM, IgG2a, and IgG2b responses were less affected. Conversely, interference with the classical NF-κB pathway in BAFF-Tg*Nfκb1*$^{-/-}$ mice significantly reduced the IgG2a and IgG2b responses and virtually abrogated the IgG3 and IgA responses, although titers of IgM antibodies in BAFF-Tg*Nfκb1*$^{-/-}$ were higher than in BAFF-Tg mice. Our observation that BAFF failed to promote in vitro class switching in NF-κB1-deficient B cells strongly suggested that activation of NF-κB1 by BAFF is, indeed, essential for class switching (Figure S2).

Similar to BAFF-Tg*Nfκb1*$^{-/-}$ mice, TI class switching was substantially, but not as strongly, reduced in BAFF-TgTACI-deficient mice (Figure S3A). TI class switching was also impaired in BAFF-R-deficient mice in the presence of excessive BAFF, indicating that BAFF-induced Ig class switching is likely mediated through both TACI and BAFF-R.

To test whether BAFF can promote TI class switching in the absence of T cells, B220$^+$-sorted B cells from wt mice were transferred into *Rag2*$^{-/-}$ and BAFF-Tg*Rag2*$^{-/-}$ mice. After 3 weeks, mice were immunized with DNP-Ficoll, and serum was analyzed (Figure S3B).

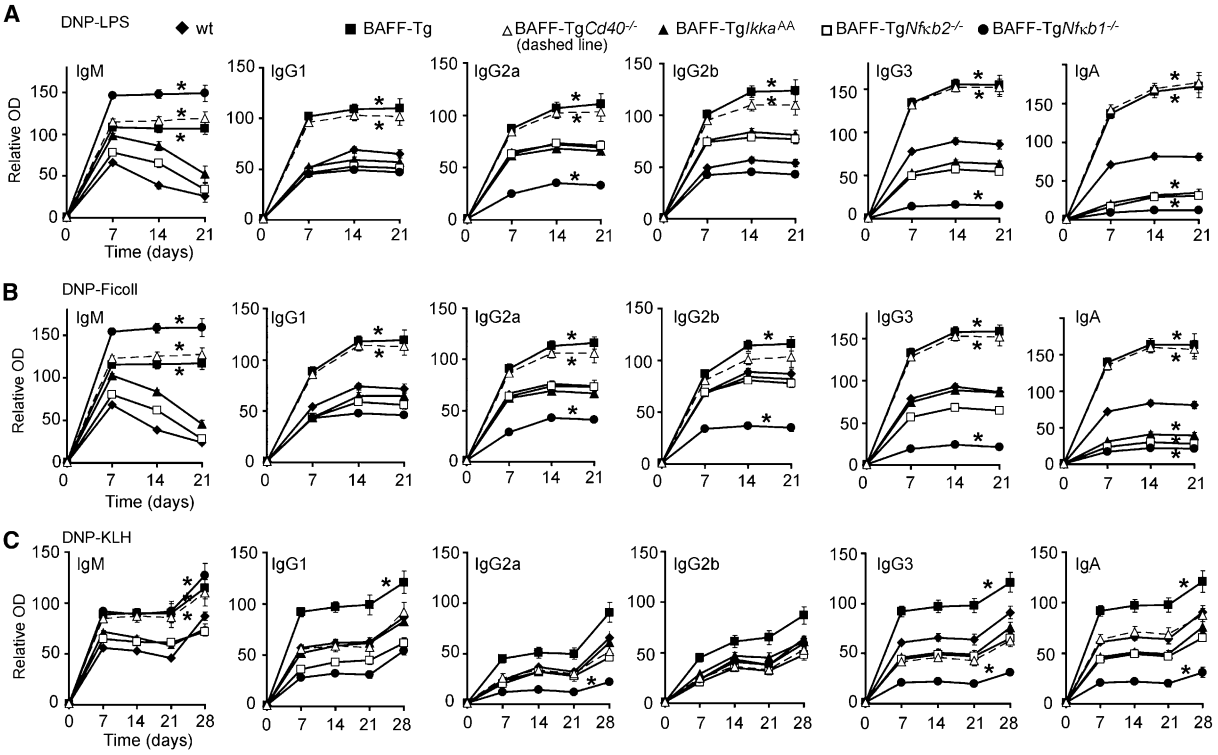

Figure 4. Antibody Responses after Immunizations with T1 and TD Antigens

(A) Antibody responses after DNP-LPS immunizations.

(B) Antibody responses after DNP-Ficoll immunizations.

(C) Antibody responses after DNP-KLH immunizations. These mice were rechallenged at day 21.

From all the mice, serum was taken at days 0, 7, 14, 21, and in the case of DNP-KLH, also at day 28 postimmunization. Values represent mean ± SD. OD_{405} results of antigen-specific ELISAs (n = 5 mice per strain). Asterisks indicate significant differences ($p < 0.01$) from wt controls.

Class switching to IgG1 was markedly impaired in the absence of T cells and could be only weakly enhanced by BAFF. Reduced class switching to IgG2a and IgG3, relative to BAFF-Tg mice, was also observed. Class switching to these isotypes, however, was still higher in BAFF-Tg*Rag2*$^{-/-}$ mice than in *Rag2*$^{-/-}$ mice, indicating that BAFF promotes switching to IgG2a and IgG3 even in absence of T cells. Interestingly, induction of IgG2b and IgA by BAFF was unaffected by the absence of T cells. Similar immune responses were induced under excessive BAFF in *Tcr*$\beta\delta^{-/-}$ mice, which lack mature T cells. Because we did not have these mice available as BAFF-Tg compound mutants, we injected them with 5 μg of recombinant BAFF every other day starting on day of immunization. As seen in B cell-transplanted BAFF-Tg*Rag2*$^{-/-}$ mice, BAFF administration had virtually no effect on the IgG1 response, but substantially enhanced the IgM, IgG2a, IgG2b, IgG3, and IgA responses. Collectively, these results indicate that BAFF can promote class switching to the IgG2a, IgG2b, IgG3, and IgA isotypes without T cells.

The effect of BAFF on Ig class switching in response to TI antigens was not as pronounced when mice were immunized with the thymus-dependent (TD) antigen DNP-KLH (Figure 4C). Not surprisingly, BAFF-Tg*Cd40*$^{-/-}$ mice exhibited reduced class switching relative to BAFF-Tg mice in response to TD antigen, reflecting the missing CD40-based B cell-T cell interactions. Correspondingly, CD40-deficient mice remained defective in GC formation (Oxenius et al., 1996), even upon BAFF overexpression (Figure S4).

BAFF Promotes B Cell Retention in Splenic MZ

BAFF overexpression markedly increased the number of splenic CD21^{hi}CD23^{-} B cells, which represent the dominant B cell population of the MZ compartment (Thien et al., 2004), and expanded the size of the MZ (Figures 5A and 5B). Complete disruption of the alternative NF-κB pathway in BAFF-Tg*Nfκb2*$^{-/-}$ mice caused nearly total loss of CD21^{hi}CD23^{-} B cells and splenic microarchitecture. A similar defect was found upon interference with the classical pathway in BAFF-Tg*Nfκb1*$^{-/-}$ mice. Whereas these mice maintained splenic compartmentalization, they had no MZ B cells. In contrast, loss of CD40 had no effect on the MZ B cell compartment. Incomplete disruption of the alternative NF-κB pathway in BAFF-Tg*Ikka*$^{AA/AA}$ mice led only to a partial reduction in the number of CD21^{hi}CD23^{-} B cells. The spleens of BAFF-Tg*Ikka*$^{AA/AA}$ mice, however, did not show normal compartmentalization and the MZ could not be localized properly (Figure 5B). Nevertheless, the presence of other markers typical of MZ B cells, such as CD1d and CD9 (Roark et al., 1998; Won and Kearney, 2002), confirmed the MZ phenotype of the CD21^{hi}CD23^{-} B cells in these mice (Figure S5). Transplantation of wt BM into lethally irradiated BAFF-Tg*Ikka*$^{AA/AA}$ mice completely restored splenic microarchitecture, indicating that the defect in the BAFF-Tg*Ikka*$^{AA/AA}$ mice is intrinsic to hematopoietic cells. As expected, these radiation chimeras developed dsDNA autoantibodies and glomerulonephritis with similar kinetics and severity as BAFF-Tg mice (data not shown). This observation excludes a pathogenic role for the alternative NF-κB pathway within splenic stromal or kidney parenchymal cells.

Long-term retention of B cells within the MZ was reported to depend on expression of αLβ2 (LFA-1) and α4β1 but not α4β7 integrins (Lu and Cyster, 2002). Integrins αLβ2 and α4β1 are present on MZ B cells and recognize VCAM-1 and ICAM-1, which are expressed by MZ stromal cells and MZ macrophages in response to lymphotoxin-β receptor (LTβ-R) signaling via the classical NF-κB pathway (Dejardin et al., 2002; Lu and Cyster, 2002). Low VCAM-1 and ICAM-1 expression in mice with defective classical NF-κB pathway (not shown) may explain, at least in part, the MZ B cell deficiency in BAFF-Tg*Nfκb1*$^{-/-}$ mice (Figures 5A and 5B).

Because BAFF expanded the MZ B cell compartment, we wondered whether BAFF could induce αL, α4, β1, and β2 integrin subunit mRNAs in cultured wt B cells (Figure S6). This was indeed the case, whereas integrin β7 mRNA expression was not affected. We also examined integrin expression in splenic B cell populations of BAFF-Tg mice with intact or interrupted alternative NF-κB pathway. As found in vitro, BAFF enhanced expression of αL, α4, β1, and β2 mRNAs in CD21^{hi}CD23^{-} MZ and, to a lesser extent, in CD21^{+}CD23^{hi} FO B cells, in a manner dependent on the alternative NF-κB pathway (Figure 5C). Expression of β7 integrin mRNA was downregulated in MZ B cells, but not FO B cells. Partial disruption of the alternative NF-κB pathway, however, led to upregulation of β7 in MZ B cells. Next, we examined the expression of integrins on the surface of CD21^{hi}CD23^{-} MZ and CD21^{+}CD23^{hi} FO B cells (Figure 5D). In concordance with the RNA analysis, αL, α4, and β2 were markedly elevated in MZ B cells compared to FO B cells. BAFF stimulated expression of these integrin subunits in a manner dependent on the alternative NF-κB pathway. Furthermore, α4β7 was downregulated on MZ B cells of BAFF-Tg mice but upregulated when the alternative NF-κB pathway was disrupted. α4 also partners with β1, so downregulation of α4β7 makes more α4 available for dimerization with β1, resulting in more α4β1, which is important for B cell retention in the MZ.

We then examined the functional relevance of BAFF-induced αLβ2 and α4β1 integrin upregulation. As seen in wt mice (Lu and Cyster, 2002), treatment of BAFF-Tg mice with a combination of α4 and αL blocking antibodies rapidly reduced the number of splenic MZ B cells (Figure 5E) and the size of the MZ B cell compartment (data not shown). This treatment also led to appearance of MZ B cells in the blood. Thus, BAFF overexpression may enhance B cell retention in the MZ through upregulation of αLβ2 and α4β1.

Enhanced Integrin-Linked Survival and Anti-dsDNA Autoreactivity of MZ B Cells from BAFF-Tg Mice

Because all mice with an enlarged MZ B cell compartment developed autoimmune disease, but mice with normal or reduced MZ did not, we hypothesized that an enlarged MZ B cell pool is a prerequisite for development of autoimmune disease. Thus, we examined whether the enlarged CD21^{hi}CD23^{-} MZ B cell pool of BAFF-Tg mice serves as a niche for autoreactive B cells by transferring equal amounts of sorted MZ and FO B cells from BAFF-Tg with confirmed autoimmune disease into 4-week-old B cell-deficient BAFF-TgμMT mice. Successful adoptive transfers were verified by

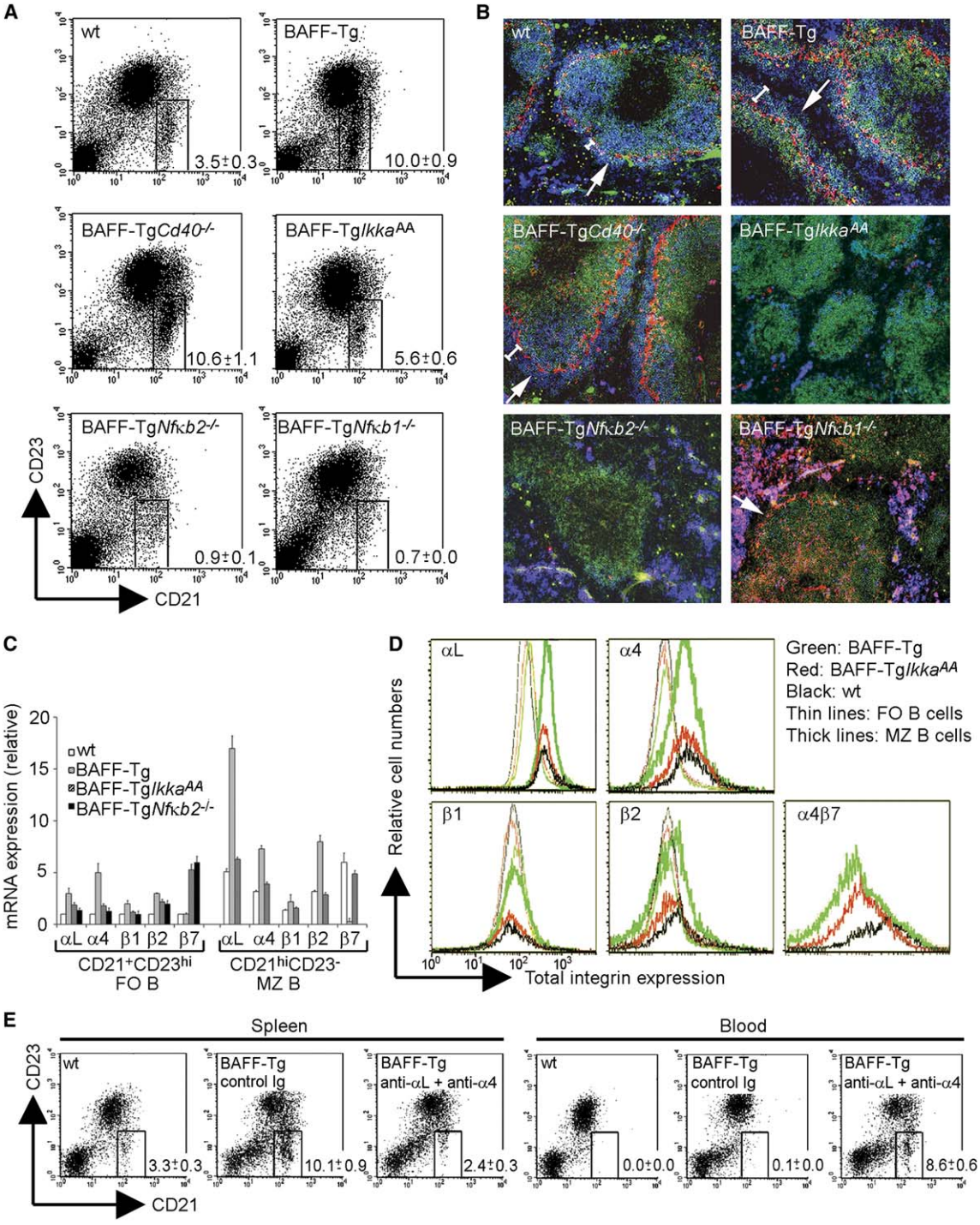

Figure 5. Altered MZ B Cell Compartments in BAFF-Tg Compound Mutant Mice

(A) Splenocytes from wt and different BAFF-Tg mice were analyzed by flow cytometry for expression of CD21 and CD23. B cells with a MZ phenotype are boxed and their percentages mean ± SD, relative to total $CD21^+$ cells, are shown (one representative experiment is shown; n = 3 mice per strain).

(B) Spleen sections from the indicated strains were stained with anti-Moma-1 (red) for sinusoidal macrophages and anti-B220 (green) and anti-IgM (blue) for B cells. The MZ surrounding the sinus is indicated with an arrow and its diameter is marked with a bar, 10× magnification (one representative section out of three analyzed is shown).

(C) Quantification of integrin gene expression by real-time PCR in purified FO and MZ B cells from different mice (mean ± SD; n = 3).

(D) Flow cytometric analysis of surface integrin expression on $B220^+CD21^+CD23^{hi}$-gated FO B cells and $B220^+CD21^{hi}CD23^-$-gated MZ B cells (n = 3). Mean fluorescence intensity values are shown in Table S1.

(E) Inhibition of integrins αLβ2 and α4β1 relocates MZ B cells to the peripheral circulation. Flow cytometric analysis of spleens and blood from mice 3 hr after i.v. injection of 100 μg, each, of αL and α4 antibodies or isotype-matched control Ig (one representative experiment is shown; values are means ± SD for three individual mice). Spleens and blood of untreated wt mice are shown for comparison.

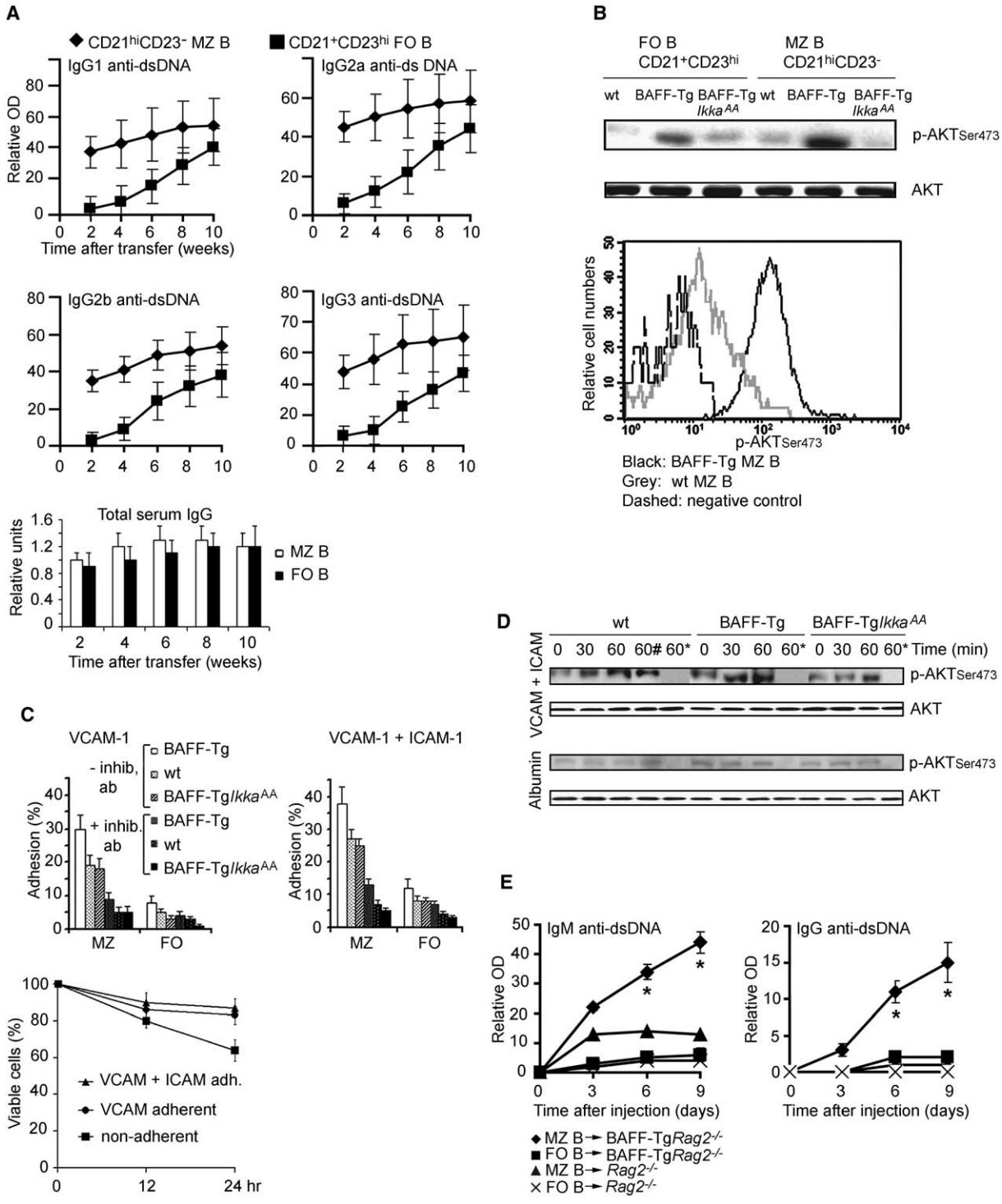

Figure 6. Autoreactive B Cells Accumulate in the MZ

(A) Top: dsDNA autoantibody titers of IgG subclasses from BAFF-TgμMT mice transplanted with either MZ B cells or FO B cells. Values represent mean ± SD relative OD_{405} (n = 3) at indicated times after adoptive transfer. Bottom: Relative total serum IgG amounts in these mice.

(B) Top: Immunoblot analysis of extracts of isolated FO and MZ B cells from different strains with phospho-AKT Ser473 and AKT antibodies. Bottom: Intracellular flow cytometric analysis of $B220^{+}CD21^{hi}CD23^{-}$-gated splenic B cells from wt and BAFF-Tg mice stained with phospho-AKT Ser473 antibody (results from BAFF-Tg*Ikka*$^{AA/AA}$ mice are very similar to those of wt mice, data not shown; n = 3).

(C) Top: Analysis of MZ and FO B cell adhesion to VCAM-1- and VCAM-1 plus ICAM-1-coated plastic dishes. Where indicated, adhesion was blocked by inclusion of α4 antibody for VCAM-1 and α4 plus αL antibodies for VCAM-1 plus ICAM-1-coated plates. Bottom: Survival of adherent or nonadherent MZ B cells from BAFF-Tg mice was determined by trypan blue exclusion. Shown are mean values at different times after plating (mean ± SD; n = 3).

(D) Immunoblot analysis with phospho-AKT Ser473 and AKT antibodies of MZ B cells plated on plastic dishes coated with either VCAM-1 plus ICAM-1 or albumin and, where indicated, incubated with BAFF (#) or LY294002 (*). Cell extracts were prepared at indicated times after plating.

immunohistology (Figure S7). Serum IgG anti-dsDNA subclasses of the transplanted mice were monitored biweekly, starting 2 weeks after transplantation (Figure 6A). Initially, mice receiving MZ B cells from BAFF-Tg donors produced more IgG autoantibodies than mice receiving FO B cells, whereas total serum IgG production was similar in both recipients. With time, however, BAFF-TgμMT mice that received FO B cells also exhibited elevated titers of IgG autoantibodies. Immunohistology of spleens from such mice revealed that some of the FO B cells had migrated into the MZ (Figure S7). In contrast, BAFF-TgμMT mice that were transplanted with MZ or FO B cells from wt donors failed to produce measurable IgG autoantibodies within 4 weeks posttransplantation (data not shown). These results suggest that excessive BAFF induces appearance of autoreactive B cells preferentially within the MZ compartment.

The importance of the MZ as a niche for pathogenic autoreactive B cells was further supported by the absence of marked IgG autoantibody titers in BAFF-TgμMT mice transplanted with αLβ2-deficient BM cells (Figure S8A). While these mice show substantial TI class switching, indicating that their antigen presentation is intact (Figure S8B), they do not form a MZ (not shown).

In the MZ, autoreactive B cells may receive additional prosurvival signals not available elsewhere. Indeed, immunoblot analysis revealed strong AKT (PKB) phosphorylation at Ser473 in MZ B cells from BAFF-Tg mice, which was considerably weaker in FO B cells of the same mice (Figure 6B, top). Little AKT Ser473 phosphorylation was seen in MZ or FO B cells from wt or BAFF-Tg*Ikka*$^{AA/AA}$ mice. Flow cytometry of splenic MZ B cells stained with phospho-AKT Ser473 antibody yielded similar results (Figure 6B, bottom).

BAFF increased integrin expression preferentially on MZ B cells, so we investigated whether integrin binding to VCAM-1 or VCAM-1 plus ICAM-1 affected MZ B cell survival by activating AKT. MZ B cells plated on plastic dishes coated with either VCAM-1 or VCAM-1 plus ICAM-1 exhibited greater adhesion than FO B cells from the same strain (Figure 6C, top). MZ B cells from BAFF-Tg mice developed higher adhesion activity than MZ B cells from wt or BAFF-Tg*Ikka*$^{AA/AA}$ mice. Importantly, adhesion of MZ B cells from any of these strains resulted in increased survival (Figure 6C, bottom) and AKT Ser473 phosphorylation (Figure 6D). AKT Ser473 phosphorylation was not increased by the addition of BAFF to the medium, ruling out a direct contribution of BAFF to AKT Ser473 phosphorylation during this experiment. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY290042 prevented AKT Ser473 phosphorylation, indicating that phosphorylation is PI3K dependent. These findings were also verified by flow cytometry (data not shown).

Our results suggest that some of the BAFF-induced survival signals in MZ B cells may be mediated through integrin ligation resulting in AKT activation. However, given the discrepancy between the strong AKT Ser473 phosphorylation found in MZ B cells of BAFF-Tg mice and the relatively weak AKT phosphorylation in purified B cells after integrin ligation, it appears that other mechanisms also contribute to AKT activation in BAFF-Tg mice.

We next examined whether BAFF causes MZ B cells to lose self-tolerance in a T cell-independent manner. To test this, we injected apoptotic fetal liver cells into BAFF-Tg*Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice that were transplanted 3 days earlier with equal amounts of purified MZ or FO B cells from healthy 4-week-old wt mice. Successful adoptive transfers were verified by immunohistology (data not shown). Within a few days of injection of apoptotic cells, BAFF excess led to a marked increase in production of dsDNA IgM and IgG antibodies in mice that received MZ B cells but not in mice transplanted with FO B cells (Figure 6E). No significant increase in autoantibody titers could be found in MZ B cell-transplanted *Rag2*$^{-/-}$ mice without the *Baff* transgene. These findings indicate that BAFF excess may cause MZ B cells to lose self-tolerance independently of T cells.

Discussion

We demonstrate that the alternative NF-κB pathway, based on IKKα-catalyzed NF-κB2-p100 processing (Bonizzi and Karin, 2004), is indispensable for enhanced survival of peripheral B cells and development of SLE-like autoimmune disease in BAFF-Tg mice. Autoimmunity (i.e., production of autoantibodies) without progression to autoimmune disease (i.e., organ lesions) was observed in BAFF-Tg mice with a defective classical, but intact alternative, NF-κB pathway. Thus, full-blown autoimmune disease in BAFF-Tg mice depends on both NF-κB pathways. However, neither CD40-mediated B cell-T cell interactions nor GC formation have a role in the pathogenesis of the disease in BAFF-Tg mice.

Our results indicate that BAFF activates the classical NF-κB pathway not only via BCMA and TACI, as previously suggested (Claudio et al., 2002), but also through BAFF-R, although activation of the classical pathway by BAFF-R was weak compared to the TACI-generated response. A role of BAFF-R in activation of the classical NF-κB pathway was recently reported (Sasaki et al., 2006). Both NF-κB-signaling pathways contributed to BAFF-enhanced B cell survival in vitro. BAFF-induced survival of BAFF-R-deficient B cells was similar to that of B cells with a defective alternative NF-κB pathway, while survival responses of TACI-deficient B cells resembled those of B cells defective in classical NF-κB signaling. Even though the effect of BAFF on in vitro B cell survival is mediated through both NF-κB pathways, each pathway makes a distinct contribution to expression of antiapoptotic genes. Whereas activation of either pathway enhanced expression of the antiapoptotic proteins A1/Bfl1 and Bcl-X_L, induction of Pim-2 kinase was exclusively dependent on the alternative pathway. Loss of Pim-2 caused a similar defect in BAFF-induced in vitro survival as the one caused by NF-κB2 or BAFF-R deficiency. It should be noted, however, that loss of Pim-2

(E) The indicated mice were transplanted with wt MZ or FO B cells and after 3 days injected with apoptotic fetal liver cells. dsDNA autoantibody titers were monitored at the indicated times after apoptotic cell injections. Values represent mean ± SD relative OD_{405} (n = 3). Asterisks indicate significance relative to autoantibody titers from wt mice ($p < 0.05$).

does not affect B cell development in vivo (Mikkers et al., 2004), suggesting compensation by other survival factors not available in vitro.

Besides its effect on mature B cells, BAFF enhanced survival of transitional B cells, leading to increased T1 and T2 B cell pools, and this process depended mainly on the alternative pathway. Importantly, only BAFF-Tg and BAFF-Tg compound mutants that possessed an enlarged transitional B cell pool showed measurable titers of circulating autoreactive IgM, supporting the hypothesis that the transitional B cell stage may represent a decisive checkpoint for elimination of self-reactivity (Yurasov et al., 2005). Because we found IgG and IgA autoantibodies solely in mice that produced IgM autoantibodies, the former types of autoantibodies are likely to have originated from IgM-producing autoreactive B cells (Rolink et al., 1996).

An association between factors that enhance TI class switching and production of pathogenic autoantibodies was reported (Peng et al., 2002). BAFF strongly promoted TI Ig class switching, and this process mainly depended on an intact classical NF-κB pathway and TACI. In parallel, induction by BAFF of p50:p65 dimers as well as p50 homodimers also required intact classical NF-κB signaling and the presence of TACI. These observations are consistent with p50 homodimers acting as an isotype-specific "switching factor" (Kenter et al., 2004) and suggest that the promoting effect of BAFF on Ig class switching may be mediated by these NF-κB isoforms. This suggestion is supported by our observation that BAFF did not promote in vitro class switching in B cells lacking NF-κB1. While BAFF-TgTACI- (data not shown) and TACI-deficient mice showed some Ig class switching and, consequently, developed a mild autoimmune disease (von Bulow et al., 2001), BAFF-Tg*Nfκb1*$^{-/-}$ mice that had much less class switching and little or no detectable autoreactive IgG developed no autoimmune disease. This underscores the importance of Ig class switching for generation of pathogenic autoantibodies. Adoptive B cell transfers into BAFF-Tg*Rag2*$^{-/-}$, and injection of *Tcrβδ*$^{-/-}$ mice with BAFF, indicated that excessive BAFF can promote class switching after TI antigen immunization independently of T cells. It remains to be seen, however, whether these mice will develop an overt SLE-like autoimmune disease similar to BAFF-Tg mice.

Similar to BAFF-Tg*Nfκb1*$^{-/-}$ mice, BAFF-Tg*Ikka*$^{AA/AA}$ mice failed to produce pathogenic IgG autoantibodies, even though they showed measurable amounts of autoreactive IgM, but, unlike BAFF-Tg*Nfκb1*$^{-/-}$ mice, exhibited some Ig class switching upon stimulation with TI antigens. In search for a possible explanation for these observations, we found that spleens of BAFF-Tg*Ikka*$^{AA/AA}$ mice lacked a proper MZ. The MZ is unique to the spleen and is populated by a special subset of B cells and other leukocytes, including macrophages and dendritic cells (Lopes-Carvalho et al., 2005). It was suggested that MZ B cells, along with peritoneal B1 B cells, play an important role in "innate-like" TI antigen responses and may be involved in autoimmune processes (Lopes-Carvalho and Kearney, 2004; Martin et al., 2001). We excluded a major contribution of peritoneal B1 B cells to BAFF-induced autoimmune disease because these cells were present in splenectomized BAFF-Tg mice (data not shown), which developed no or very mild disease. As we confirmed earlier reports on BAFF-induced enlargement of the MZ B cell pool (Schiemann et al., 2001; Thien et al., 2004), MZ B cells are therefore prime candidates for production of pathogenic autoreactive antibodies. Indeed, we found a strong correlation between an enlarged MZ, IgG autoantibody titers, and development of autoimmune disease. Importantly, adoptive transfer of B cells from different splenic compartments of BAFF-Tg mice with known autoimmune disease confirmed that CD21^{hi}CD23^{-} MZ B cells are the major and most immediate source of autoreactive B cells producing pathogenic IgG autoantibodies.

It seems likely that in BAFF-Tg mice, most autoreactive B cells are retained in the MZ by BAFF-induced upregulation of αLβ2 and α4β1 integrins. Inhibition of integrin interaction with their ligands led to rapid loss of splenic MZ B cells, confirming the requirement of functional αLβ2 and α4β1 for keeping these cells in the MZ. In the MZ, autoreactive B cells may obtain survival signals through binding of their integrins to VCAM-1 and ICAM-1 expressed by MZ stromal cells and macrophages in a LTβ-R-dependent manner (Dejardin et al., 2002; Lu and Cyster, 2002). Although mice with defective LTβ ligand-LTβ-R axis do not have a MZ, they develop signs of autoimmunity due to a defect in central tolerance induction in the thymus, a mechanism different from BAFF-mediated autoimmunity (Boehm et al., 2003). Autoreactive B cells from BAFF-Tg mice without a properly organized MZ, e.g., BAFF-Tg*Ikka*$^{AA/AA}$ mice, fail to obtain survival signals through integrin ligation and may, consequently, die due to reduced competitiveness (Lesley et al., 2004). Our proposal that in BAFF-Tg mice the MZ serves as a critical niche for pathogenic autoreactive B cells is further supported by the absence of marked IgG autoantibody titers in BAFF-TgμMT mice transplanted with αLβ2-deficient BM cells that did not exhibit a MZ.

Besides the possibility of a defective checkpoint at the transitional B cell stage as a source for autoreactive B cells, we obtained more direct evidence that CD21^{hi}CD23^{-} MZ B cells, but not FO B cells, tend to lose self-tolerance in a T cell-independent manner under the influence of excessive BAFF.

We suggest that development of autoimmune disease in BAFF-Tg mice is a gradual process. Two mechanisms may explain development of self-reactivity in BAFF-Tg mice: (1) a defective checkpoint at the transitional B cell stage leading to survival of constantly appearing autoreactive transitional B cells; and (2) a loss of tolerance of MZ B cells exposed to excessive BAFF, which may come in contact with self-antigens presented by adjacent APCs. BAFF then promotes Ig class switching to rapidly increase the levels of pathogenic IgG autoantibodies. Further, BAFF-mediated integrin upregulation retains autoreactive B cells in the MZ and provides them with critical survival signals. Our findings suggest that incomplete inhibition of the alternative NF-κB pathway, as seen in the BAFF-Tg*Ikka*$^{AA/AA}$ mutant, may be a sufficient therapeutic option for patients suffering from autoimmune disease associated with BAFF overproduction. As previously discussed (Bonizzi and Karin, 2004), inhibition of the alternative NF-κB pathway is less likely to cause an immune deficiency, which is

commonly seen after blockade of the classical NF-κB pathway (Senftleben et al., 2001b; Sha et al., 1995). In fact, disruption of the alternative NF-κB pathway enhances innate immunity (Lawrence et al., 2005).

Experimental Procedures

Mice

BAFF-Tg mice were from L. Gorelik (Biogen Idec, Cambridge, MA) (Mackay et al., 1999); BAFF-R-deficient mice from K. Rajewsky (CBR, Harvard University, Boston, MA) (Sasaki et al., 2004); and TACI-deficient mice from R. Bram (Department of Immunology, Mayo Clinic, Rochester, MN) (von Bulow et al., 2001). *Nfκb2*$^{-/-}$ mice were generated by R. Bravo (Bristol Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). *Cd40*$^{-/-}$, *Nfκb1*$^{-/-}$, B cell-deficient μMT, and T cell-deficient *Tcrβδ*$^{-/-}$ mice were from Jackson Laboratories, and *Rag2*$^{-/-}$ mice from Taconic Laboratories. *Ikka*$^{-/-}$ embryos (Hu et al., 1999) and *Ikka*$^{AA/AA}$ mice (Cao et al., 2001) were described. All mouse strains were backcrossed for at least six generations onto the C57Bl/6 background, and homozygous double mutant mice were obtained by heterozygote intercrosses. Genotyping was by PCR. Mice were housed under conventional barrier protection in accordance with UCSD and NIH guidelines, and mouse protocols were approved by the UCSD Institutional Animal Care Committee.

Cell Transplantations

Splenic B cells, BM cells from femurs, or fetal liver cells from 14-day-old mouse embryos were harvested under sterile conditions and injected (5×10^6 cells, or in case of apoptotic fetal liver cells 1×10^4 cells per mouse) into tail veins of lethally (3000 rad) or, in case of B cell-deficient recipients, sublethally (600 rad) irradiated mice. BM cells from αLβ2-deficient mice were from D. Corey (Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX). Prior to transplantation of BAFF-TgμMT mice, spontaneous Ig class switching was excluded by antibody isotype analysis with ELISA after TI antigen immunization.

Splenectomy

Mice were splenectomized under anesthesia as described (Reeves and Reeves, 1991).

Anti-dsDNA ELISA

dsDNA autoantibody titers were analyzed with 96-well MaxiSorp ELISA plates (Nunc) coated with double-stranded calf thymus DNA (Enzler et al., 2003; Mackay et al., 1999). AP-labeled anti-mouse IgM, IgG, IgG subclasses, and IgA (Southern Biotech Associates, Birmingham, AL) were used to detect different antibody isotypes/subclasses. Results were normalized to corresponding Ig isotypes/subclasses (Southern Biotech Associates). Relative ODs were obtained by comparison to antibody titers of 4-week-old wt mice.

Flow Cytometry and Cell Sorting

Splenic single-cell suspensions depleted of erythrocytes were incubated with FcγIII/II blocking antibody (PharMingen, San Diego, CA), following various fluorescent antibodies. FITC-, PE-, APC-, or biotin-labeled antibodies were either from PharMingen or Santa Cruz Biotechnology (Santa Cruz, CA) and were to: CD1d, CD3, CD5, CD9, CD21, CD23, CD24, IgM, IgG subclasses, CD45/B220, AA-4.1, αL, α4, β1, β2, and α4β7. Binding of biotinylated antibodies was detected by Streptavidin-PE (PharMingen). To exclude dead cells, 25 μg/ml of 7-Amino-actinomycin D (7-AAD) (PharMingen) was added, where indicated. Data were collected with a FACScalibur flow cytometer and analyzed with CellQuest software (Beckton Dickinson, San Jose, CA). Cells were also sorted with a Beckton Dickinson Cell Sorter. Intracellular stainings were done according to the saponin method (Perez et al., 2004). AKT and phospho-AKT Ser473 antibodies were from Cell Signaling Technology (Beverly, MA).

B Cell Isolations and Analysis

Splenic or peritoneal B cells were isolated with MACS beads coupled to anti-CD43 and anti-CD5 (Miltenyi Biotec) and, where indicated, depleted of T cells with anti-CD3 beads (Li et al., 2003). B cells were cultured as described (Gu and Rajewsky, 2004). Cultures were stimulated with recombinant human BAFF (0.2 μg/ml) (PeproTech Inc., Rocky Hill, NJ) and incubated with cycloheximide (25 μg/ml) (Sigma), where indicated. Mature *Ikka*$^{-/-}$ B cells were obtained from spleens of *Ikka*$^{-/-}$ (fetal liver cells) → μMT radiation chimeras. *Pim2*$^{-/-}$ B cells were purified from *Pim2*$^{-/-}$ splenocytes provided by C. Thompson (Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA). Cell proliferation was measured with the propidium iodide method (Li et al., 2003). Viable cells were counted by trypan blue exclusion (Enzler et al., 2003). To test B cell survival in presence of selectins, 12-well culture plates (Corning Inc., NY) were coated with VCAM-1 or VCAM-1 plus ICAM-1 (R&D Systems) as described (Lu and Cyster, 2002) and B cells were allowed to adhere in the presence of αL or αL plus α4 antibodies (both at 0.5 μg/ml, Santa Cruz), where indicated. To some cultures, 10 μM of PI3K inhibitor LY294002 (Cayman Chemical) was added.

Apoptotic Fetal Liver Cells

Cells were plated and UV irradiated (0.42 W/cm^2 at 254 nm) for 20 min. Extent of apoptosis was determined and preparations with >80% apoptotic cells were injected.

Molecular Biology

Real-time PCR was as described (Park et al., 2004). Results were normalized to the level of cyclophilin mRNA. Individual primer sequences are available upon request. Cell fractionation and immunoblot analysis was as described (Li et al., 2003). Antibodies to Bcl-X_L, A1/Bfl1, Pim-1, Pim-2, Pim-3, and phospho-Bad Ser112 were from Santa Cruz Biotechnology, and p100/p52 and IκBα antibodies were from Upstate (Charlottesville, VA). EMSA and supershift analysis were as described (Senftleben et al., 2001a). Antibodies to p50, p65, c-Rel, and RelB were from Santa Cruz Biotechnology. p52 antibody was from Upstate. The BAFF antagonist BR3-F_c was kindly provided by Genentech (San Francisco, CA).

Histochemistry and Immunohistology

Spleens were processed as described (Anzelon et al., 2003). Sections were stained with B220-FITC, IgM-APC, CD3-FITC (all PharMingen), Moma-1-biotin antibodies (Bachem Philadelphia Inc., King of Prussia, PA), peanut agglutinin-(PNA)-biotin (Vector Laboratories, Burlingame, CA), and SAv-Cy3 (Zymed, San Francisco, CA). Images were captured with a Zeiss Axioplan. Kidneys were fixed in 10% formalin or frozen. Fixed kidneys were embedded in paraffin and sections stained with PAS. Renal lesions were scored by an observer blinded to the genotypes (Austin et al., 1984). Specific changes evaluated were glomerular cellularity, necrosis, crescent formation, basement membrane thickening, glomerular sclerosis, and interstitial fibrosis with scores of 0, 1, 2, or 3 for normal, mild, moderate, or severe pathology. Frozen kidneys were processed as spleens. Anti-IgG-PE (Jackson Immuno Research) was used to identify glomerular immune complexes.

Immunizations

To induce GCs, mice were immunized with 100 μg of KLH (Calbiochem-Novabiochem Corp., La Jolla, CA) precipitated in alum and analyzed as described (Anzelon et al., 2003). DNP-KLH in alum was used to measure TD, and DNP-LPS (Sigma) or DNP-Ficoll (Solid Phase Sciences) were used to measure TI immune reactions (Haas et al., 2002). Serum was collected, serially diluted, and added to 96-well Maxisorp ELISA plates, pretreated with 50 μl/well of DNP-ovalbumin (5 μg/ml) and analyzed as above.

Urinalysis

Protein in urine was measured with Uristix reagent strips (Bayer Corporation, Elkhart, IN).

Statistical Analysis

To analyze autoantibody titers, Wilcoxon-rank sum tests were used. A significant level of 0.01 was used as Bonferroni correction for comparisons. Antibody titers and B cell survival data were analyzed with Student's t test.

Supplemental Data

Supplemental Data include eight figures and one table and can be found with this article online at http://www.immunity.com/cgi/content/full/25/3/403/DC1/.

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