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Article

TRAF2 and TRAF3 Signal Adapters Act Cooperatively to Control the Maturation and Survival Signals Delivered to B Cells by the BAFF Receptor

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SUMMARY

Tumor necrosis factor receptor-associated factors 2 and 3 (TRAF2 and TRAF3) were shown to function in a cooperative and nonredundant manner to suppress nuclear factor-κB2 (NF-κB2) activation, gene expression, and survival in mature B cells. In the absence of this suppressive activity, B cells developed independently of the obligatory B cell survival factor, BAFF (B cell-activating factor of the tumor necrosis factor family). However, deletion of either TRAF2 or TRAF3 from the T cell lineage did not promote T cell survival, despite causing extensive NF-κB2 activation. This constitutive, lineage-specific suppression of B cell survival by TRAF2 and TRAF3 determines the requirement for BAFF to sustain B cell development in vivo. Binding of BAFF to BAFF receptor reversed TRAF2-TRAF3-mediated suppression of B cell survival by triggering the depletion of TRAF3 protein. This process was TRAF2 dependent, revealing dual roles for TRAF2 in regulating B cell homeostasis.

INTRODUCTION

Adaptive immunity depends on the production and maintenance of a pool of mature peripheral B and T lymphocytes throughout life. The expression of a functional antigen receptor is a prerequisite for the generation and subsequent survival of both of these populations (Lam et al., 1997; Polic et al., 2001). However, mature lymphocytes also require the delivery of antigen receptor-independent signals from the extracellular milieu in order to survive. The nature of these signals differs fundamentally between T and B cells. Thus, mature naive T cells require signals delivered via the interleukin-7 receptor (Schluns et al., 2000; Tan et al., 2001) whereas mature naive B cells depend on signals delivered by B cell-activating factor of the tumor necrosis factor family (BAFF = TNFSF13B). Mice that lack BAFF expression (*Tnfsf13b*$^{-/-}$) or that have undergone treatments designed to block the action of BAFF fail to produce or maintain a mature B cell pool (Schiemann et al., 2001; Schneider et al., 2001; Thompson et al., 2000). Three different members of the TNF receptor (TNFR) superfamily act as receptors for BAFF, namely BAFF-R (TNFRSF13C), TACI (TNFRSF13B), and BCMA (TNFRSF17) (Mackay et al., 2007). Mature B cells express both BAFF-R and TACI but require only BAFF-R to survive in vivo (Sasaki et al., 2004; Shulga-Morskaya et al., 2004; von Bulow et al., 2001; Yan et al., 2001).

Despite the essential role of the BAFF-BAFF-R signaling axis in establishing and maintaining the peripheral B cell pool, the intracellular events that link BAFF to its biological effects remain only partially characterized. Unlike most members of the TNFR superfamily, BAFF-R is a relatively weak inducer of acute responses such as the canonical nuclear factor-κB (NF-κB) and the c-*jun* N-terminal kinase (JNK) pathways (Grech et al., 2004; Morrison et al., 2005). In contrast, BAFF-R is a potent activator of the kinetically slower alternative NF-κB pathway and is entirely responsible for signaling the processing of the p100 NF-κB2 protein to its active p52 component in primary B cells (Claudio et al., 2002; Kayagaki et al., 2002). Activation of the alternative NF-κB pathway by BAFF is a major component of its biological activity, because B cells lacking NF-κB2 or other critical upstream activators of NF-κB2 processing, such as NF-κB-inducing kinase (NIK) or I-κB kinase α (IKKα), display greatly impaired survival and maturation in vivo (Kaisho et al., 2001; Miosge et al., 2002; Senftleben et al., 2001; Yamada et al., 2000).

Members of the TNFR superfamily that lack cytoplasmic death domains typically deliver intracellular signals by recruiting members of the TNFR-associated factor (TRAF) family of signaling proteins (Grech et al., 2000). Most non-death domain members of the TNFR superfamily recruit multiple TRAFs to their cytoplasmic domains. BAFF-R, however, has only a single TRAF-binding site that is specific for TRAF3 (Xu and Shu, 2002). Unlike TRAFs 2, 5, and 6, TRAF3 does not activate the canonical NF-κB or JNK pathways when it is overexpressed or recruited to members of the TNFR superfamily (Morrison et al., 2005; Rothe et al., 1995; Song et al., 1997). Instead, TRAF3 appears to play a negative regulatory role, particularly within the alternative NF-κB pathway (Hauer et al., 2005; He et al., 2006). In vitro studies using a transformed B cell line have shown that TRAF3 suppresses the alternative NF-κB pathway by binding NIK and targeting it for proteosomal degradation (Liao et al., 2004). Upon BAFF

treatment of this cell line, extensive proteolysis of TRAF3 occurs with the result that the amount of NIK gradually increases, thereby promoting NF-κB2 processing (Liao et al., 2004).

Mice lacking B cell expression of TRAF3 have recently been generated and display increased NF-κB2 activation as well as increased B cell survival (Xie et al., 2007), suggesting that TRAF3 may act as a negative regulator of BAFF signaling in vivo. However, the mechanisms whereby TRAF3 mediates NIK degradation or is itself targeted for degradation are unclear because the "really interesting new gene" (RING) finger domain of TRAF3 does not display detectable ubiquitin ligase activity (Liao et al., 2004). Although it is not directly recruited to BAFF-R, a role for TRAF2 as well as TRAF3 in BAFF signaling has been suggested by the increased NF-κB2 activation and survival exhibited by primary B cells lacking expression of TRAF2 (Grech et al., 2004).

To clarify the roles of TRAF2 and TRAF3 in regulating the survival of primary lymphocytes, we produced mice that lack B cell or T cell expression of TRAF2 and/or TRAF3. According to our results, TRAF2 and TRAF3 act cooperatively and nonredundantly to suppress B cell but not T cell survival. BAFF-BAFF-R signaling was shown to reverse TRAF2-TRAF3-mediated signal repression during normal B cell development by inducing TRAF2-mediated depletion of TRAF3. TRAF2 therefore plays dual roles in determining BAFF-dependent nature of B cell development, thereby underpinning the independent regulation of B and T lymphocyte homeostasis.

RESULTS

Production of *Traf3*$^{lox/lox}$ Mice

To achieve lineage-specific inactivation of TRAF2 expression, we previously produced mice carrying *loxP* sites homologously recombined either side of the second coding exon of *Traf2* (Grech et al., 2004). A similar strategy was employed to produce mice carrying a "floxed" version of *Traf3* (see Figure S1 available online). Gene targeting was performed with a C57BL/6 embryonic stem (ES) cell line, and mice carrying the targeted *Traf3* allele were maintained on a C57BL/6 inbred background. *Traf3*$^{lox/lox}$ mice, like *Traf2*$^{lox/lox}$ mice (Grech et al., 2004), were phenotypically normal and showed wild-type protein expression from the targeted TRAF alleles in B cells and other cell types (Figure S2B).

TRAF2- and TRAF3-Deficient B Cells Exhibit Identical Phenotypes

Mice with TRAF2-deficient B cells were generated previously by crossing the type I interferon-inducible *Mx1*-CRE transgene onto the *Traf2*$^{lox/lox}$ background (*Traf2*$^{lox/lox}$*Mx1*-CRE mice) (Grech et al., 2004). Although this transgene does not direct CRE expression specifically to B cells (Kühn et al., 1995), TRAF2-deficient B cells generated at low frequencies in these mice proceed to dominate the peripheral B cell pool because of their extended lifespan (Grech et al., 2004). In the current study, inactivation of TRAF2 or TRAF3 expression was achieved specifically within B cells by producing *Traf2*$^{lox/lox}$ and *Traf3*$^{lox/lox}$ mice that were also heterozygous for the *Cd19*-CRE allele (Rickert et al., 1997). These mice were designated *Traf2ΔB* and *Traf3ΔB*, respectively, and were verified by immunoblot analysis to have extensive B cell-specific elimination of the corresponding TRAF protein (Figure 1A).

As expected, the phenotype of *Traf2ΔB* mice resembled that seen previously in *Traf2*$^{lox/lox}$*Mx1*-CRE mice (Grech et al., 2004). Thus, compared to control animals, *Traf2ΔB* mice contained greatly increased numbers of mature B cells, particularly in the lymph node and splenic marginal zone (MZ) B cell compartments (Figures 1B–1D). Upregulation of cell-surface CD21 was also observed on B cells from *Traf2ΔB* mice (Figure 1B). Analysis of *Traf3ΔB* mice revealed a phenotype that was indistinguishable from that of *Traf2ΔB* animals. Thus, not only did *Traf3ΔB* mice exhibit increased numbers of lymph node and splenic MZ B cells as well as increased B cell expression of CD21, but the magnitude of these changes compared to control animals reproduced those observed in *Traf2ΔB* mice (Figures 1B–1D).

TRAF2- and TRAF3-Deficient B Cells Survive In Vitro without Continued Exposure to BAFF

The increased B cell numbers and expansion of the MZ B cell compartment in *Traf2ΔB* and *Traf3ΔB* mice resembled the phenotypic changes observed previously in BAFF-transgenic (BAFF-tg) mice that express supraphysiological amounts of BAFF (Khare et al., 2000; Mackay et al., 1999). The convergence of these phenotypes suggested that both TRAF2 and TRAF3 might act as negative regulators of BAFF-BAFF-R survival signals in B cells. To test this, the survival of *Traf2ΔB* and *Traf3ΔB* B cells during ex vivo culture in unsupplemented medium was measured. In the absence of the survival signals normally provided by BAFF in vivo, wild-type (control) B cells underwent rapid cell death in vitro (Figure 2A). Similarly, BAFF-tg B cells, despite their extended lifespan in vivo, remained dependent on BAFF and also died rapidly in culture (Figure 2A). By contrast, approximately half of the B cells from *Traf2ΔB* and *Traf3ΔB* mice remained viable for up to 10 days when cultured in the absence of BAFF (Figure 2A). Collectively, these data indicate that TRAF2 and TRAF3 do inhibit the survival pathways normally activated by BAFF. Indeed, the absence of either TRAF2 or TRAF3 from mature B cells allowed these survival pathways to remain active without the requirement for ongoing exposure to BAFF.

B Cells Lacking either TRAF2 or TRAF3 Show Increased Activation of the Alternative NF-κB Pathway

The signaling event triggered by BAFF-BAFF-R that is most strongly associated with B cell survival is activation of the alternative NF-κB pathway. As was previously observed in *Traf2*$^{lox/lox}$*Mx1*-CRE mice (Grech et al., 2004), *Traf2ΔB* B cells displayed increased processing of the p100 NF-κB2 protein to p52 (Figure 2B). Comparable increases in cytoplasmic p100 processing was observed in *Traf3ΔB* B cells, and both TRAF2- and TRAF3-deficient B cells exhibited increased nuclear translocation of p52 as well as its binding partner RelB (Figure 2B). The absence of either TRAF2 or TRAF3 in mature B cells, therefore, resulted in hyperactivation of the alternative NF-κB pathway, similar to that observed in BAFF-tg B cells (Figure 2B).

B Cells Lacking either TRAF2 or TRAF3 Share a Common Gene-Expression Profile

The similar phenotypes exhibited by *Traf2ΔB* and *Traf3ΔB* mice strongly suggested that TRAF2 and TRAF3 function cooperatively to regulate signaling and survival in mature B cells.

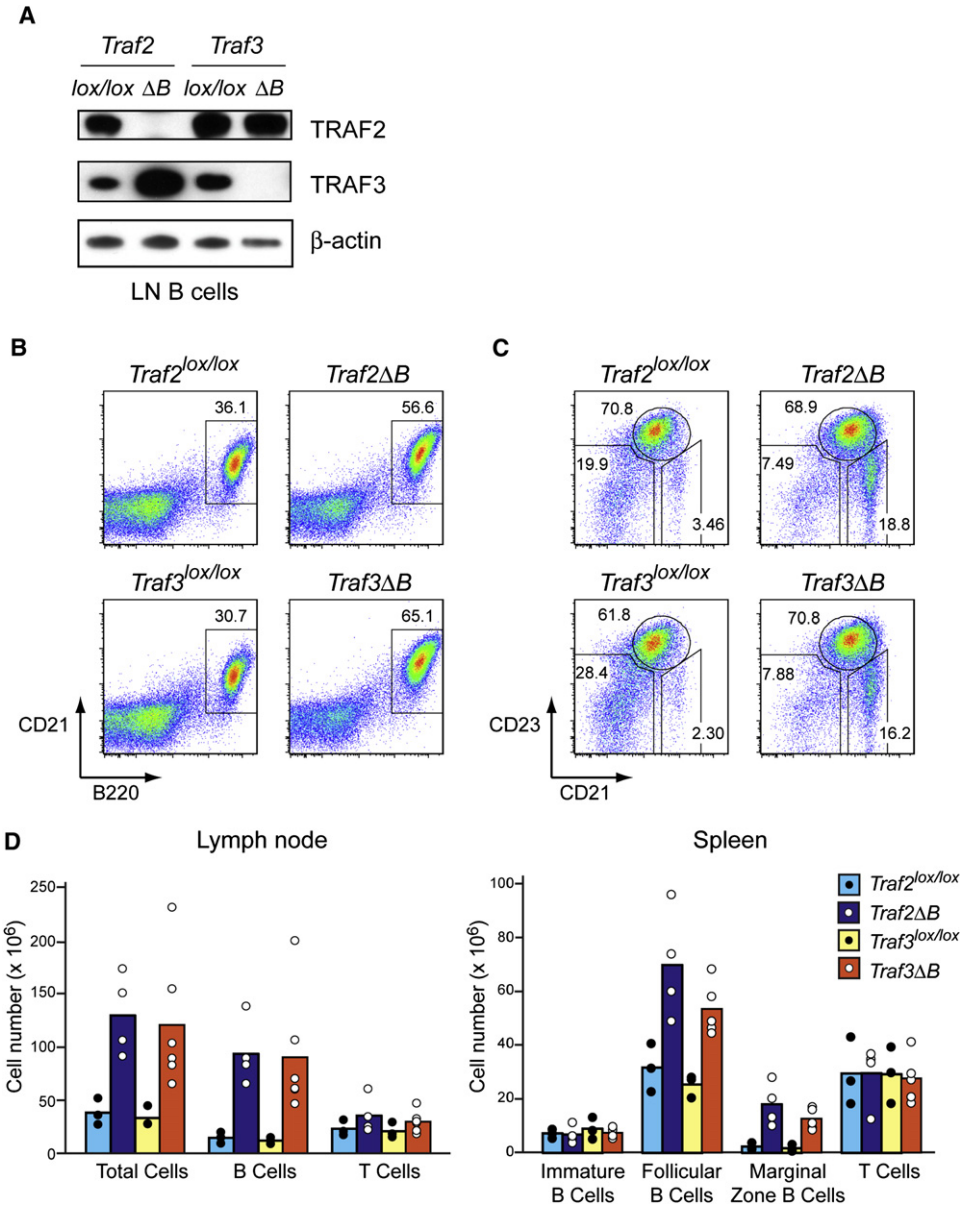

Figure 1. Efficient TRAF Inactivation and Increased B Cell Numbers in *Traf2ΔB* and *Traf3ΔB* Mice

(A) Lymph node B cells were purified from the indicated mouse lines and cytoplasmic lysates subjected to immunoblot analysis with antisera directed against the indicated proteins.

(B) Flow cytometric analysis of lymph node cells from the indicated mouse lines. Data represent cells exhibiting a lymphocyte scatter profile and the window indicates mature B cells.

(C) Flow cytometric analysis of spleen cells. Data are presented for total B cells ($B220^+$ gate). Windows on the data show, from left to right: immature ($CD21^{lo}$, $CD23^{lo}$), follicular ($CD21^{int}$, $CD23^{hi}$), and marginal zone ($CD21^{hi}$, $CD23^{lo}$) B cell populations.

In both (B) and (C), the numbers indicate the proportion of displayed events falling within the associated windows.

(D) Enumeration of lymphocytes from pooled lymph nodes and spleens of various mouse lines exhibiting the indicated lymphocyte phenotypes as determined by flow cytometry. Points represent data from individual mice and bars indicate the arithmetic means.

According to this scenario, the removal of either TRAF2 or TRAF3 from B cells would be expected to result in similar changes to the overall gene-expression profile when compared to that of wild-type B cells. To test this, gene microarray analysis was performed on B cells purified from the lymph nodes of two *Traf2ΔB*, two *Traf3ΔB*, and four control mice. This analysis identified 34 genes for which the mRNA expression in B cells from either *Traf2ΔB* or *Traf3ΔB* mice was more than 2-fold higher (27 genes) or 2-fold lower (7 genes) compared to the amount expressed in control B cells. Strikingly, every mRNA identified to be upregulated or downregulated in TRAF2-deficient B cells was similarly altered in TRAF3-deficient B cells, and vice versa (Figure 2C). In other words, global analysis of mRNAs derived from more than 39,000 different genes revealed that the absence of either

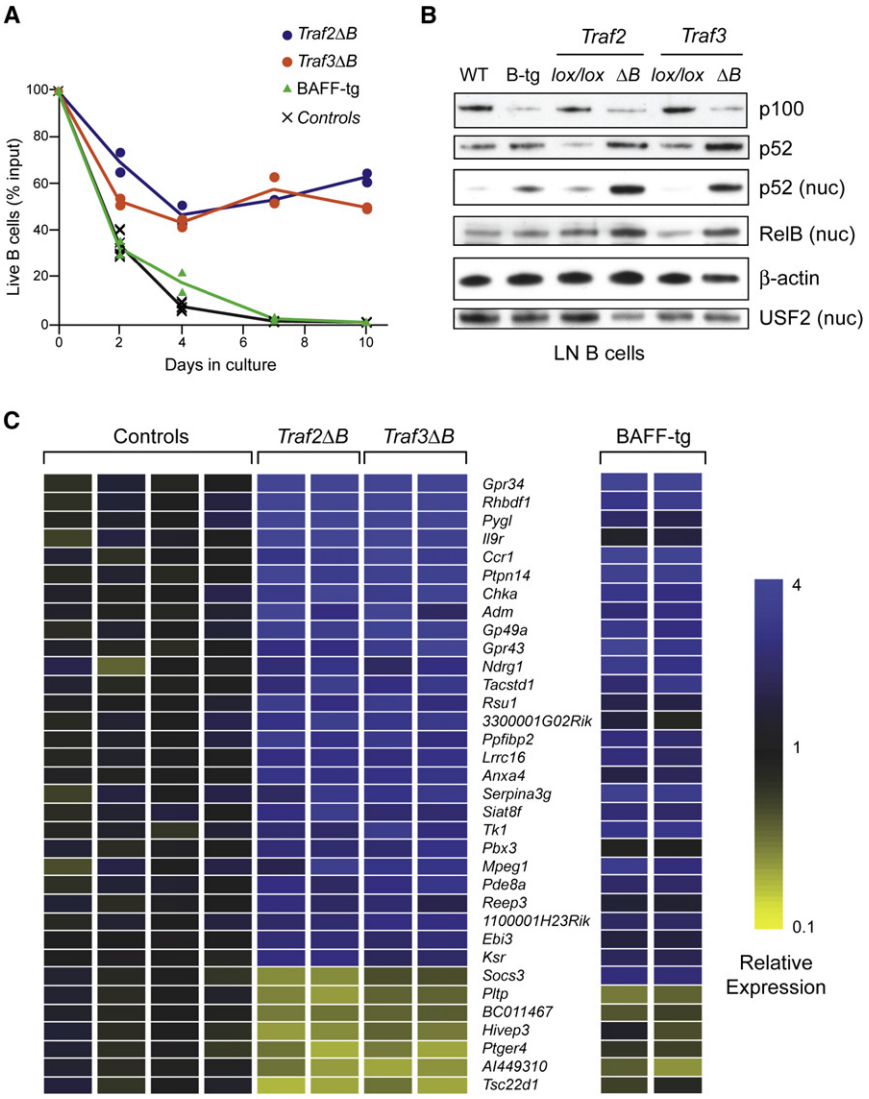

Figure 2. Extended In Vitro Survival, Hyperactivity of the Alternative NF-κB Pathway, and Coincident Regulation of BAFF Target Genes in B Cells from *Traf2ΔB* and *Traf3ΔB* Mice

(A) Purified lymph node B cells from the indicated mouse lines were incubated in unsupplemented culture medium, and the number of live cells present at the indicated time points was assayed by flow cytometry. Points represent data from individual mice and lines connect the means. Overlapping data were obtained from the four control genotypes (wild-type, *Cd19*-CRE^{+}, *Traf2*$^{lox/lox}$, and *Traf3*$^{lox/lox}$), and a single line was applied to these data.

(B) Cytoplasmic and nuclear extracts from purified lymph node B cells were subjected to immunoblot analysis with antisera directed against the indicated proteins. B-tg, BAFF-tg.

(C) Gene-expression analysis was performed on sort-purified lymph node B cells prepared from two *Traf2ΔB* mice, two *Traf3ΔB* mice, and four control mice with an Affymetrix gene chip. Genotypes of the control mice (from left to right): *Traf2*$^{lox/lox}$, *Cd19*-CRE^{+}, *Traf2*$^{lox/lox}$, and *Traf3*$^{lox/lox}$. Gene-expression data were normalized on a per gene basis relative to the mean value obtained from the control samples. Data are shown for mRNAs whose expression in *Traf2ΔB* or *Traf3ΔB* B cells was either more than 2-fold higher or less than half of the mean value obtained from the controls. Gene expression in lymph node B cells from BAFF-tg compared to wild-type mice was analyzed and normalized separately.

TRAF2 or TRAF3 from mature B cells had virtually identical effects on the overall gene-expression profile. Furthermore, the majority of the mRNAs differentially expressed in both *Traf2ΔB* and *Traf3ΔB* compared to control B cells were similarly altered in lymph node B cells from BAFF-tg mice (Figure 2C). Many of these genes therefore constitute normal targets of BAFF signaling in B cells, consistent with the proposition that TRAF2 and TRAF3 act cooperatively and nonredundantly as negative regulators of signaling pathways normally activated by BAFF.

Inactivation of Both TRAF2 and TRAF3 Produces No Further Alterations in B Cell Phenotype

Further support for the codependent roles of TRAF2 and TRAF3 in regulating B cell signaling and survival was obtained through analysis of mice lacking expression of both TRAF2 and TRAF3 in their B cells (*Traf2Traf3ΔB* mice) (Figure S2C). Analysis of these mice revealed that the overall expansion of B cells in lymph node and spleen, the specific expansion of splenic marginal zone B cells, and upregulation of cell-surface CD21 on B cells were all similar in *Traf2ΔB*, *Traf3ΔB*, and *Traf2Traf3ΔB* B cells (Figure 3A). In addition, removal of both TRAF2 and TRAF3 did not result in any further activation of the alternative NF-κB pathway, because the extent of p100 processing to p52 and nuclear translocation of p52 and RelB were comparable in *Traf2ΔB*, *Traf3ΔB*, and *Traf2Traf3ΔB* B cells (Figure 3B). Likewise, B cells from all three types of mice showed similar BAFF-independent survival in vitro (Figure 2A and data not shown). When taken in conjunction with the gene-expression analysis (Figure 2C), these results confirm that TRAF2 and TRAF3 each play complementary and nonredundant roles in regulating specific pathways normally activated by BAFF signaling in mature B cells.

TRAF2- and TRAF3-Deficient T Cells Do Not Show Extended Survival

The fundamental roles of TRAF2 and TRAF3 in regulating the survival of primary B cells led us to investigate whether these molecules might play a similar role in T cells. Two additional lines of mice were produced in which TRAF2 or TRAF3 expression was selectively eliminated from the T cell lineage. To do this, an *Lck*-CRE transgene, which drives predominantly T cell-restricted expression of CRE (Orban et al., 1992), was crossed onto homozygous *Traf2*$^{lox/lox}$ and *Traf3*$^{lox/lox}$ backgrounds to

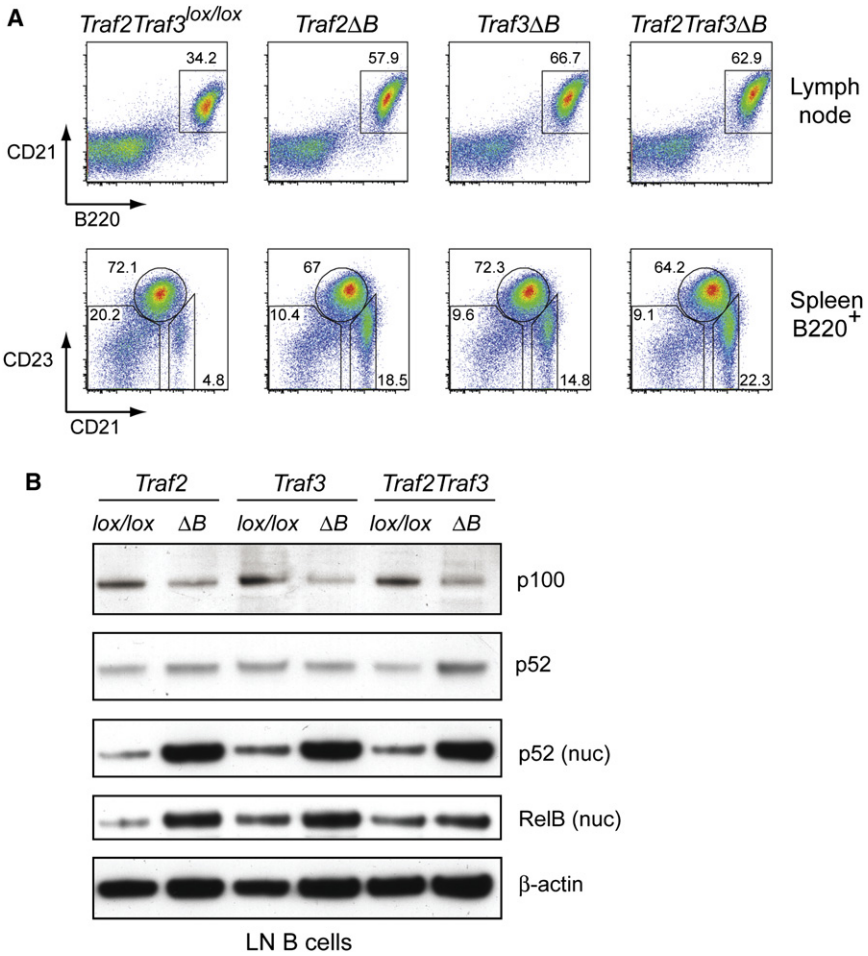

Figure 3. *Traf2Traf3ΔB* Mice, Deficient in B Cell Expression of TRAF2 and TRAF3, Exhibit Expansion of Mature B Cells In Vivo and Increased B Cell NF-κB2 Signaling Similar to that in *Traf2ΔB* and *Traf3ΔB* Mice

(A) FACS analysis of B cell populations from the lymph node (top) and spleen (bottom) of mice of the indicated genotypes were analyzed as for Figure 1B.

(B) Nuclear and cytoplasmic lysates of B cells purified from the lymph nodes of mice of the indicated genotypes were subjected to immunoblot analysis as for Figure 2C.

produce *Traf2ΔT* and *Traf3ΔT* mice, respectively (Figure S2B). In contrast to the dramatic increase in mature B cell numbers observed in *Traf2ΔB* and *Traf3ΔB* mice, *Traf2ΔT* and *Traf3ΔT* mice contained normal or even reduced numbers of peripheral $CD4^+$ and $CD8^+$ T cells (Figure 4A). In addition, T cells purified ex vivo and cultured in unsupplemented medium in vitro were found to die rapidly and with similar kinetics regardless of their expression of TRAF2 or TRAF3 (Figure 4B). Importantly, T cells from *Traf2ΔT* and *Traf3ΔT* mice did show extensive activation of the alternative NF-κB pathway (Figure 4C). These results demonstrate that although TRAF2 and TRAF3 suppress the alternative NF-κB pathway in both T and B cells, their suppression of cellular survival pathways is specific for the B cell lineage.

BAFF Sustains B Cell Development by Reversing the Constitutive Suppression of Survival Signals Enforced by TRAF2 and TRAF3

The results documented thus far clearly demonstrated that TRAF2 and TRAF3 cooperatively inhibit BAFF signaling pathways in mature B cells. However, the precise nature of this inhibitory function still remained to be determined. On the one hand, TRAF2 and TRAF3 could be acting downstream of BAFF signaling by dampening signals initiated by BAFF. Alternatively, their modus operandi might be akin to that of the I-κB proteins (Baldwin, 1996), with TRAF2 and TRAF3 acting as constitutive repressors whose inhibitory activity must be overcome by intracellular events triggered by BAFF signaling. To distinguish between these possibilities, we bred the *Traf2ΔB* mice onto a BAFF-deficient (*Tnfsf13b*$^{-/-}$) background. If TRAF2-TRAF3-mediated signal repression acts downstream of BAFF-signaling, then the elimination of TRAF2 expression would be unable to rescue B cell development in *Tnfsf13b*$^{-/-}$ mice because no BAFF-BAFF-R signaling could be initiated. However, if TRAF2 and TRAF3 act as constitutive repressors of the key pathways activated by BAFF signaling, then the absence of TRAF2 should remove the requirement for BAFF-BAFF-R signaling and thus restore B cell development in *Tnfsf13b*$^{-/-}$ animals.

The clear result obtained was that the development and survival of mature B cells in BAFF-deficient mice was completely rescued by eliminating TRAF2 expression from the B cell lineage. Thus, while mature B cells were virtually absent from BAFF-deficient mice with normal TRAF2 expression (*Tnfsf13b*$^{-/-}$*Traf2*$^{lox/lox}$ mice), *Tnfsf13b*$^{-/-}$*Traf2ΔB* mice contained large numbers of mature ($AA4.1^-$) lymph node B cells (Figure 5A) as well as both follicular ($CD21^{int}$, $CD23^{hi}$) and marginal zone ($CD21^{hi}$, $CD1d^{hi}$) phenotype B cells in the spleen (Figure 5B). These results demonstrate that the cooperative action of TRAF2 and TRAF3 constitutively represses key survival and maturation pathways that operate during normal B cell development. The requirement for BAFF-BAFF-R signaling to sustain B cell development is therefore due to its ability to reverse TRAF2-TRAF3-mediated suppression of these pathways.

TRAF2 Mediates Extensive BAFF-BAFF-R-Dependent Depletion of TRAF3 during B Cell Development

We next investigated the mechanism whereby BAFF-BAFF-R signaling reverses the TRAF2-TRAF3-mediated suppression of B cell survival and maturation pathways. Previous studies with transformed B cell lines have indicated that BAFF signaling can trigger proteolytic degradation of TRAF3 (Liao et al., 2004), which would be predicted to reverse TRAF2-TRAF3-mediated signal repression. This led us to examine whether BAFF-BAFF-R

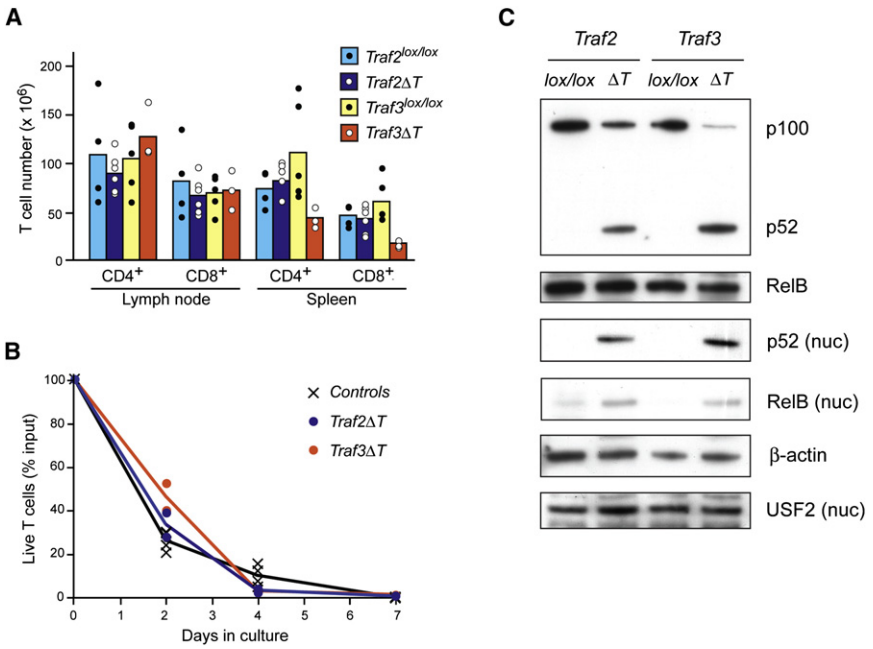

Figure 4. TRAF2- and TRAF3-Deficient T Cells Do Not Accumulate or Survive Preferentially Despite Displaying Spontaneous Activation of the Alternative NF-κB Pathway

(A) Lymph nodes and spleens were obtained from mice of the indicated genotypes and total numbers of $CD4^+$ and $CD8^+$ T cells assessed by flow cytometry. Points represent data from individual mice and bars show the arithmetic means.

(B) Purified lymph node T cells from the indicated mouse lines were incubated in unsupplemented culture medium and the number of live cells present at the indicated time points assayed by flow cytometry. Points represent data from individual mice and lines connect the means. Overlapping data were obtained from the two control genotypes (*Traf2*$^{lox/lox}$ and *Traf3*$^{lox/lox}$) and a single line applied to these data.

(C) Lymph node T cells were purified from the indicated mouse lines and cytoplasmic and nuclear extracts prepared and subjected to immunoblot analysis with antisera directed against the indicated proteins.

signaling triggers TRAF3 degradation during the development of normal primary B cells and, if so, how TRAF2 might be involved in this process.

Two independent observations indicated that the binding of BAFF to BAFF-R during normal B cell development results in the depletion of cellular TRAF3. First, the amount of TRAF3

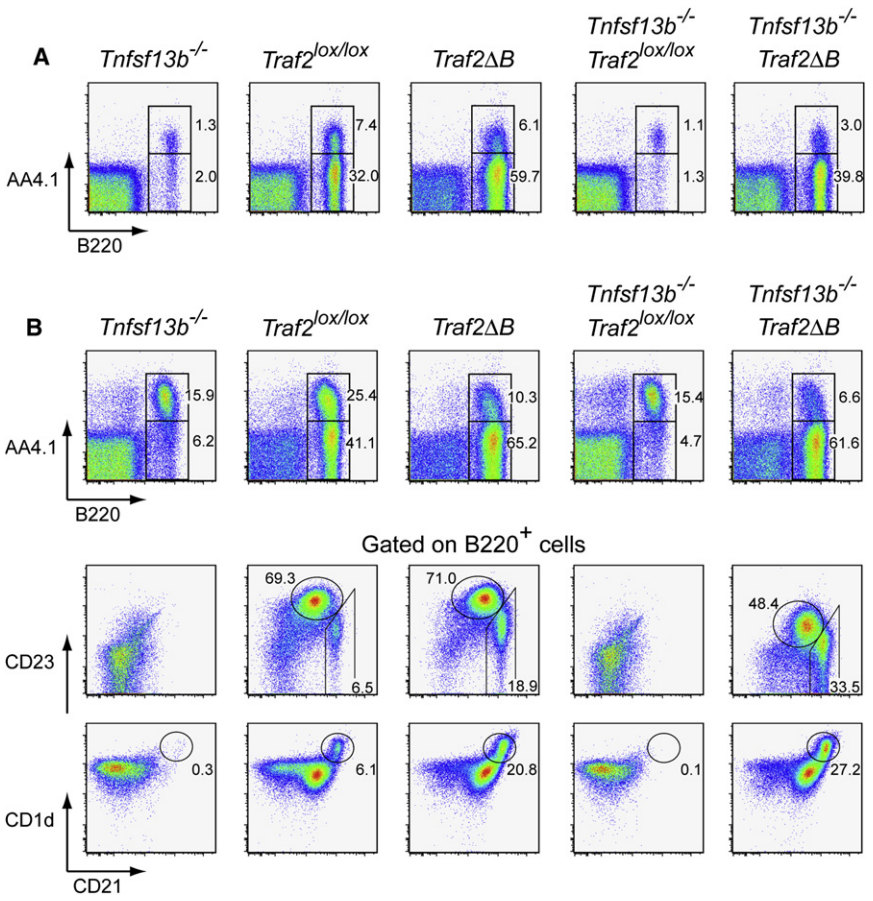

Figure 5. Absence of TRAF2 Expression in the B Cell Lineage Facilitates BAFF-Independent B Cell Development and Survival In Vivo

Traf2$^{lox/lox}$ and *Traf2ΔB* mice were crossed onto a BAFF-deficient (*Tnfsf13b*$^{-/-}$) background. Flow cytometric analysis of lymph node (A) or spleen (B) was carried out as for Figure 1B. Data in B220 versus AA4.1 profiles are from cells falling within the lymphocyte scatter gate. Windows indicate immature ($B220^+$, $AA4.1^+$) and mature ($B220^+$, $AA4.1^-$) phenotype B cells. Data in CD21 versus CD23 and CD21 versus CD1d profiles are from total B cells ($B220^+$ gate) and windows show follicular ($CD21^{int}$, $CD23^{hi}$) and marginal zone ($CD21^{hi}$, $CD23^{lo}$ and $CD21^{hi}$, $CD1d^{hi}$) phenotype B cells. Numbers indicate the proportion of displayed events falling within the associated windows.

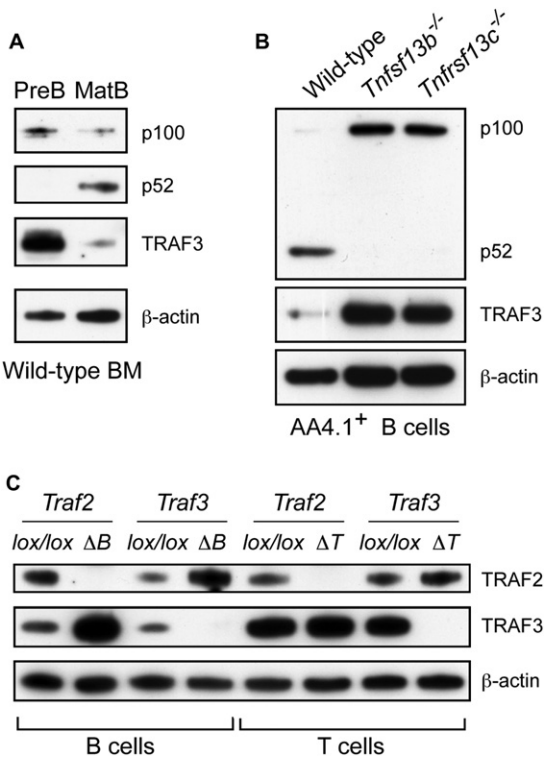

Figure 6. TRAF3 Is Downregulated in a TRAF2-Dependent Manner by BAFF-BAFF-R Signaling during B Cell Development

(A) Precursor B cells (PreB = $B220^+$, IgM^-) and mature B cells (MatB = $B220^+$, $CD23^{hi}$, IgD^{hi}) were sorted from wild-type bone marrow and whole-cell lysates subjected to immunoblot analysis. Note the extensive downregulation of TRAF3 and activation of p100 processing during B cell development.

(B) Immature $B220^+$, $AA4.1^+$ B cells (see Figure 5) were sorted from the spleens of the indicated mice. Whole-cell lysates were obtained and subjected to immunoblot analysis. Note that TRAF3 downregulation and p100 processing require the expression of both BAFF and BAFF-R.

(C) Lymph node B cells (left) or T cells (right) were purified from mice of the indicated genotypes and cytoplasmic lysates subjected to immunoblot analysis. Note that control B cells express low amounts of TRAF3 compared to T cells and TRAF2-deficient B cells. β-actin was used as a loading control in all cases.

protein is drastically reduced as B cells mature from their $B220^+$, IgM^- bone-marrow precursors in vivo (Figure 6A). This observation is consistent with BAFF-R mediating this process because this receptor is not expressed until after B lineage cells commence B cell antigen receptor (BCR) expression (Hsu et al., 2002). Second, immature ($AA4.1^+$) splenic B cells contain large amounts of TRAF3 protein in both BAFF-deficient (*Tnfsf13b*$^{-/-}$) and BAFF-R-deficient (*Tnfrsf13c*$^{-/-}$) compared to wild-type mice (Figure 6B). In each of these cases, the changes in expression of TRAF3 protein did not result from alterations in the amount of *Traf3* mRNA (Figure S3) and correlated with the activation of p100 processing (Figures 6A and 6B). These data indicate that BAFF signaling through BAFF-R triggers extensive post-transcriptional downregulation of TRAF3 protein during B cell development in vivo, most likely by targeting it for proteolytic degradation. B cells from TACI-deficient (*Tnfrsf13b*$^{-/-}$) and wild-type mice were found to contain similar amounts of TRAF3 protein (Figure S4), confirming that BAFF-R is the only BAFF-binding receptor required to trigger TRAF3 depletion during B cell development.

To determine the involvement of TRAF2 in the depletion of TRAF3 in B cells by BAFF-BAFF-R signaling, we examined the amount of TRAF3 protein present in TRAF2-deficient B cells. According to our previous data, TRAF2-deficient B cells from *Traf2*$^{lox/lox}$*Mx1*-CRE mice contain greatly increased amounts of TRAF3 compared to control B cells (Grech et al., 2004). This phenomenon was also observed in the TRAF2-deficient B cells isolated from *Traf2ΔB* mice (Figure 1A) and was not due to upregulation of *Traf3* mRNA (Figure S3). This finding cannot be explained by the modest increase in mature ($AA4.1^-$) phenotype cells among the lymph node B cells from *Traf2ΔB* mice (Figure 5A) because mature and immature peripheral B cells contain similarly low amounts of TRAF3 (Figures 6A and 6B). It appears, therefore, that the extensive BAFF-BAFF-R-mediated depletion of TRAF3 protein that occurs during B cell development (Figures 6A and 6B) requires the presence of TRAF2.

This conclusion was confirmed by additional analysis of the expression of TRAF3 in wild-type and TRAF2-deficient T cells. Normal naive T cells do not undergo BAFF-BAFF-R signaling, as indicated by the absence of p100 processing (Figure 4C). Because T cells and B cells express similar amounts of *Traf3* mRNA (Figure S3), T cells would be expected to contain more TRAF3 protein than wild-type B cells but similar amounts to those in B cells in which post-transcriptional depletion of TRAF3 has been inactivated. The amount of TRAF3 in wild-type T cells were indeed found to be much greater than those in wild-type B cells, whereas they were virtually identical to those present in TRAF2-deficient B cells (Figure 6C). Furthermore, the amount of TRAF3 protein was unchanged in TRAF2-deficient compared to wild-type T cells (Figure 6C), a finding consistent with the dependence of TRAF2-mediated depletion of TRAF3 on upstream signals from BAFF-R. In summary, therefore, BAFF-BAFF-R signaling reverses the TRAF2-TRAF3-mediated suppression of survival pathways during in vivo B cell development by triggering post-transcriptional depletion of TRAF3 protein via a process that is completely dependent on the expression of TRAF2 (Figure S5).

DISCUSSION

The specific effects of TRAF2 and TRAF3 deficiency on B cell phenotype and gene expression documented here indicated that these molecules act by inhibiting signaling pathways normally activated by the obligatory B cell survival factor BAFF. This was further illustrated by the finding that elimination of TRAF2-TRAF3-mediated signal repression completely removed the dependence of B cells on BAFF for their development, maturation, and survival in vivo. This striking result also demonstrated that, rather than simply dampening signals initiated by BAFF, TRAF2 and TRAF3 act cooperatively to constitutively suppress these key survival and maturation pathways. The requirement for BAFF-BAFF-R signaling to sustain B cell development and homeostasis is therefore based on its ability to reverse the TRAF2-TRAF3-mediated suppression of B cell survival.

In contrast to the profound effects observed in B cells, the removal of either TRAF2 or TRAF3 expression from T cell lineage did not increase the survival of mature T cells. TRAF2- and

TRAF3-deficient T cells did nevertheless exhibit extensive NF-κB2 activation. The constitutive suppression of the alternative NF-κB pathway by TRAF2 and TRAF3 therefore appears to be universal, whereas their ability to suppress lymphocyte survival is restricted to the B cell lineage. The precise reasons for this dichotomy are unclear but presumably reflect the distinct intracellular milieu present in B and T lymphocytes. In the context of the mature B cell, therefore, pathways repressed by TRAF2 and TRAF3 (and activated by BAFF) complement BCR-mediated signals to sustain mature B cell survival. On the other hand, these pathways are either not activated or do not complement TCR-mediated survival signals in mature T cells. Instead, signals delivered via interleukin-7 receptor provide critical survival stimuli in the context of the mature T cell (Schluns et al., 2000; Tan et al., 2001). The differential activity of TRAF2 and TRAF3 in regulating B versus T cell survival therefore underpins the independent regulation of homeostasis in these two lymphocyte populations.

A key observation in this study was that the amount of TRAF3 protein in TRAF2-deficient B cells was greatly increased compared to control B cells. This result seems paradoxical: B cells with either no TRAF3 (*Traf3ΔB*) or with large amounts of TRAF3 (*Traf2ΔB*) exhibit the same phenotype. What it emphasizes, however, is that the repression of signaling enforced by TRAF2 and TRAF3 occurs cooperatively and is absolutely reliant on the presence of both proteins. The accumulation of TRAF3 in TRAF2-deficient B cells was in fact found to be due to prevention of the normal depletion of TRAF3 that occurs during the development of wild-type B cells. The fact that this depletion of TRAF3 requires BAFF signaling through BAFF-R as well as the presence of TRAF2 establishes a dual role for TRAF2 in BAFF signaling. Thus, TRAF2 not only mediates the suppression of B cell survival signals in conjunction with TRAF3 but is also required for the depletion of TRAF3 that allows BAFF-BAFF-R signaling to sustain B cell development.

Based on these data, we propose the following model for the regulation of mature B cell development by BAFF. In the absence of BAFF, TRAF2 and TRAF3 act cooperatively to repress the survival of mature B cells by suppressing NF-κB2 activation and possibly other prosurvival pathways. In wild-type mice, B cells express BAFF-R and bind BAFF soon after initial expression of antigen receptor. TRAF3 is subsequently recruited to BAFF-R and then depleted from the cell by a TRAF2-dependent process, possibly involving targeting to the proteosome by the TRAF2-associated ubiquitin ligase c-IAP1 (Li et al., 2002). Depletion of TRAF3 lifts repression of NF-κB2 and/or other prosurvival pathways, thereby facilitating B cell survival. This depletion of TRAF3 by BAFF-R is analogous to the degradation of I-κB repressor proteins induced by other receptors that leads to activation of the canonical NF-κB pathway (Baldwin, 1996). Key survival pathways are constitutively active in the absence of TRAF3, with the result that B cells genetically deficient in TRAF3 no longer require BAFF in order to survive. TRAF2-deficient B cells lack the capacity to deplete TRAF3 but also survive independently of BAFF because of the requirement for TRAF2 as well as TRAF3 to suppress B cell survival. Naive T cells do not receive signals through BAFF-R nor any other receptor that promotes TRAF2-dependent TRAF3 degradation, with the result that the NF-κB2 pathway is inactive in these cells. Thus, in contrast to B cells, naive T cells survive irrespective of TRAF2 or TRAF3 expression because of signals delivered via the interleukin-7 receptor.

Several questions remain to be answered before it can be precisely determined how TRAF2 and TRAF3 control B cell survival. First, how does TRAF2 target TRAF3 for BAFF-mediated degradation when it is not directly recruited to BAFF-R? NIK binds TRAF2 and TRAF3 (Liao et al., 2004; Malinin et al., 1997), raising the possibility that it facilitates TRAF2-mediated depletion of TRAF3 after BAFF-R stimulation. This possibility is supported by the recent observation that TRAF3 protein is present in increased amounts in primary B cells overexpressing a mutant NIK protein that lacks TRAF3-binding activity and which would therefore fail to colocalize TRAF2 and TRAF3 (M. Schmidt-Supprian, personal communication). These studies also confirm that increased expression of NIK in primary B cells, especially the non-TRAF3 binding mutant, results in expansion of the B cell compartment and extended B cell survival similar to *Traf2ΔB* and *Traf3ΔB* mice (M. Schmidt-Supprian, personal communication). It is likely, therefore, that the suppression of signaling mediated by TRAF2 and TRAF3 occurs via their common interaction with NIK. Because TRAF3 acts by targeting NIK for proteolytic degradation (Liao et al., 2004), the requirement for TRAF2 and TRAF3 to suppress NF-κB2 activation may reflect a role for TRAF2 in the degradation of NIK as well as TRAF3. It would therefore be predicted that both TRAF2- and TRAF3-deficient B cells should contain increased amounts of NIK protein. However, we (data not shown) and others (Xie et al., 2007; M. Schmidt-Supprian, personal communication) have been unable to quantify NIK in primary B cells because of the low amounts of NIK protein in this cell type.

Recently published studies support the scenario described above and indicate that the c-IAP proteins are likely to play a key role. Thus, TRAF2-deficient fibroblasts not only have extensive NF-κB2 activation but also have greatly increased amounts of NIK (Vince et al., 2007). c-IAP1-deficient fibroblasts showed a very similar phenotype (Vince et al., 2007), suggesting that TRAF2 may mediate NIK degradation by recruiting the ubiquitin ligase activity of c-IAP1 to NIK. Consistent with this, c-IAP1-mediated ubiquitination and degradation of NIK requires the TRAF2-binding site of c-IAP1 (Varfolomeev et al., 2007). It is not clear why the binding of TRAF3 to NIK is also required for this process. TRAF2- and TRAF3-deficient B cells showed normal expression of c-IAP1 (data not shown), indicating that the constitutive activation of the NF-κB2 pathway in these cells is not due to increased expression of c-IAP1 but presumably to modulation of its ability to mediate NIK ubiquitination.

Given the prominent activation of the alternative NF-κB pathway in TRAF2- and TRAF3-deficient B cells, it was surprising that the genes that were upregulated greater than 2-fold in these cells did not include obvious NF-κB targets. However, target genes of the alternative NF-κB pathway are not well characterized and may in fact include some of these genes. The enhanced BAFF-independent survival of *Traf2ΔB* and *Traf3ΔB* B cells points to a possible role for some of these genes in formation of B cell lineage tumors. Indeed, our results posit that *Traf2* and *Traf3* may act as tumor-suppressor genes in the B cell lineage. In support of this, recent analyses have revealed that mutation of *TRAF3* is a common genetic lesion in human multiple

myeloma (Annunziata et al., 2007; Keats et al., 2007). Moreover, mutations in the genes encoding TRAF2 and TRAF3 may also potentiate autoantibody production and autoimmunity in view of the established role of excessive BAFF signaling in compromising B cell self-tolerance (Lesley et al., 2004; Thien et al., 2004). *Traf2ΔB* and *Traf3ΔB* mice may therefore provide new models relevant to human lymphoma, myeloma, and autoimmune disease.

EXPERIMENTAL PROCEDURES

Mice

Conditionally TRAF2-deficient (*Traf2*$^{lox/lox}$) mice (Grech et al., 2004) as well as *Cd19*-CRE transgenic (Rickert et al., 1997), *Lck*-CRE transgenic (Orban et al., 1992), BAFF-transgenic (Mackay et al., 1999), *Tnfsf13b*$^{-/-}$ (Schiemann et al., 2001), and *Tnfrsf13c*$^{-/-}$ (Sasaki et al., 2004) mice have all been previously described. The production of *Traf3*$^{lox/lox}$ mice is described in detail in Supplemental Data. Mice were screened by PCR analysis of blood or tail-tip DNA. All mice were maintained on an inbred C57BL/6 genetic background at the animal facilities of the Centenary and Garvan Institutes and analyzed between 2 and 6 months of age. All animal experiments were carried under protocols approved by the University of Sydney and Garvan Institute/St. Vincent's Hospital Animal Ethics Committees.

Flow Cytometry

Flow cytometric analysis was performed on a FacsCanto flow cytometer (BD Biosciences) with FacsDiva software (BD Biosciences) and analysis performed with FlowJo (TreeStar). Single-cell suspensions were prepared from spleens and lymph nodes (pooled cervical, inguinal, axillary, brachial, and mesenteric nodes) and stained and analyzed as previously described (Grech et al., 2004). The following antibodies and conjugates were purchased from BD Biosciences: anti-CD16 (2.4G2), anti-B220-FITC, -PE, -PerCP, -APC (RA3-6B2), anti-CD21-FITC (7G6), anti-CD23-PE (B3B4), anti-CD1d-biotin (1B1), anti-CD4-FITC (L3T4), anti-CD8-PE (53-6.7), and streptavidin-PerCP. The following antibody was purchased from eBiosciences: anti-C1qRp-APC (AA4.1). Light scatter gating was performed on all samples to include lymphocytes but exclude doublets, dead cells, and debris.

Lysate Preparation

B and T cells were purified from lymph nodes with Mouse B Cell Isolation Kit and Mouse T Cell Isolation Kit (Miltenyi Biotec), respectively, as per manufacturer's instructions. Labeled cells were separated with AutoMacs Separator (Miltenyi Biotec) and negative fraction collected, counted, and washed in PBS. Nuclear and cytoplasmic lysates were prepared with NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce) as per manufacturer's instructions, with the addition of 100 μg/ml AEBSF (Roche), 10 μg/ml leupeptin, 1 mM sodium pervanadate, and 10 mM sodium fluoride (all Sigma Aldrich). Precursor B cells (B220^{+}, IgM^{-}) and mature B cells (B220^{+}, CD23^{hi}, IgD^{hi}) from bone marrow, as well as immature splenic B cells (B220^{+}, AA4.1^{+}) were isolated by cell sorting on FacsVantage or FacsAria cell sorters (BD Biosciences). Whole-cell lysates were prepared with RIPA buffer (25 mM HEPES, 250 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 100 μg/ml AEBSF, 10 μg/ml leupeptin, 1 mM sodium pervanadate, and 10 mM sodium fluoride).

Immunoblotting

All lysates were subjected to polyacrylamide gel electrophoresis on 4%–12% gradient NuPAGE Bis-Tris gels (Invitrogen) in XCell SureLock Mini-Cell apparatus (Invitrogen) and transferred onto Immobilon-P PVDF membranes (Millipore) with XCell II^{TM} Blot module (Invitrogen). Membranes were blocked and antibodies diluted in 5% BSA/0.2% Tween20/Tris-buffered saline. The following antibodies were purchased from Santa Cruz: anti-TRAF3 (H122), anti-TRAF2 (C20), anti-RelB (C19), anti-USF2 (C20), anti-rabbit IgG-HRP (sc-2030), anti-mouse IgG-HRP (sc2031). Anti-NFκB2 (4882) was purchased from Cell Signaling Technologies and anti-β-actin (AC15) from Sigma Aldrich.

Microarray Experiments

RNA was extracted from cell-sort-purified follicular B cells from the lymph nodes of mice with RNeasy Mini Kit (QIAGEN) as per manufacturer's instructions. RNA was quantitated on a RNA 6000 Nano Chip (Agilent) with 2100 Bioanalyser (Agilent). RNA was then amplified with the GeneChip Two-cycle Target Labeling and Control Kit (Affymetrix) as per manufacturer's instructions. Resulting cRNA was hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix), washed, and stained on GeneChip Fluidics Station 400 (Affymetrix) and scanned with a GeneChip Scanner (Affymetrix). Analysis of data was performed with GeneSpring software (Agilent). All samples were normalized on a per gene basis to the median of the control samples run at the same time. *Traf2ΔB* and *Traf3ΔB* samples were individually compared to the median of their respective controls. Genes were considered 2-fold upregulated only if duplicate samples agreed and raw signals of the TRAF-deficient samples were greater than 50. Genes were considered 2-fold downregulated only if duplicate samples agreed and raw signals of the controls were greater than 50. Genes were excluded if the normalized signal for all controls did not fall between 0.5 and 1.5. For genes identified as differentially regulated by multiple independent probes (*Chka, Adm, Gpr43, Siat8f, Socs3, AI449310, Tsc22d1*), results shown are for the probe with the highest signal intensity. Gene labels used are those prescribed by the Mouse Genomic Nomenclature Committee (MGNC).

In Vitro Survival Assays

B and T cells were purified from lymph nodes with Mouse B Cell Isolation Kit and Mouse T Cell Isolation Kit (Miltenyi Biotec), respectively, as per manufacturer's instructions. Labeled cells were separated with AutoMacs Separator (Miltenyi Biotec) and negative fraction collected, counted, and resuspended at 10^6 cells/mL in RPMI supplemented with 10% FCS, 55 μM β-mercaptoethanol, 1 mM sodium pyruvate, 0.1 M nonessential amino acids, 10 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin. Cells were cultured at 10^6 cells per well in 24-well plates (BD Biosciences). 2×10^5 Calibrite beads (BD Biosciences) were added to each well prior to harvesting and staining for flow cytometry. A known number of beads were collected and the corresponding number of events falling within a live lymphocyte gate, using forward and side light scatter, was used to calculate the total number of live cells in each well. Cell numbers were then expressed as a percentage of the live cells present in the corresponding wells on Day 0.

ACCESSION NUMBERS

The complete data set has been submitted to the GEO database under accession #GSE10422.

SUPPLEMENTAL DATA

Five figures and Experimental Procedures are available at http://www.immunity.com/cgi/content/full/28/3/391/DC1/.

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