Immunity, Vol. 21, 575–587, October, 2004, Copyright ©2004 by Cell Press

Act1, a Negative Regulator in CD40- and BAFF-Mediated B Cell Survival

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Summary

TNF receptor (TNFR) superfamily members, CD40, and BAFFR play critical roles in B cell survival and differentiation. Genetic deficiency in a novel adaptor molecule, Act1, for CD40 and BAFF results in a dramatic increase in peripheral B cells, which culminates in lymphadenopathy and splenomegaly, hypergammaglobulinemia, and autoantibodies. While the B cell-specific Act1 knockout mice displayed a similar phenotype with less severity, the pathology of the Act1-deficient mice was mostly blocked in CD40-Act1 and BAFF-Act1 double knockout mice. CD40- and BAFF-mediated survival is significantly increased in Act1-deficent B cells, with stronger IκB phosphorylation, processing of NF-κB2 (p100/p52), and activation of JNK, ERK, and p38 pathways, indicating that Act1 negatively regulates CD40- and BAFF-mediated signaling events. These findings demonstrate that Act1 plays an important role in the homeostasis of B cells by attenuating CD40 and BAFFR signaling.

Introduction

The CD40 (CD40L/CD154)-CD40 ligand and BAFF-BAFF receptor (BAFFR) cytokine systems, members of the tumor necrosis factor (TNF) superfamily, play critical roles in the homeostatic regulation of B cell functions (Schonbeck and Libby, 2002; van Kooten and Banchereau, 1997; Bishop and Hostager, 2001; Lei et al., 1998; Thompson et al., 2001; Mackay and Browning, 2002; Mackay et al., 2003). The CD40-mediated pathway has been shown to play important roles in T cell-mediated B lymphocyte activation. Ligation of B cell CD40 by CD40L (CD154) expressed on activated T cells stimulates B cell survival, proliferation, differentiation, isotype switching, upregulation of surface molecules contributing to antigen presentation, development of the germinal center (GC), and the memory B cell response. Mice deficient in expression of CD40L or CD40 were unable to mount a primary or a secondary antibody response to a T cell-dependent antigen, did not form GCs, and did not generate antigen-specific memory B cells (Kawabe et al., 1994; Xu et al., 1994).

BAFF (also known as BlyS, TALL-1, zTNF4, THANK, and TNFSF 13B), a recently defined member of the TNF family, has emerged as an important regulator of B cell homeostasis. In BAFF-deficient mice, peripheral B cell survival is severely perturbed, resulting in a complete loss of follicular and marginal zone B lymphocytes (Mackay and Browning, 2002; Thompson et al., 2001; Schiemann et al., 2001; Shu et al., 1999; Khare et al., 2000). Conversely, mice overexpressing BAFF (Blys/TALL-1) display mature B cell hyperplasia and symptoms of systemic lupus erythaematosus (SLE) (Gross et al., 2000). BAFF (Blys/TALL-1) exerts its effect by binding three receptors: transmembrane activator of and CAML interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFFR/BR3) (Thompson et al., 2001; Schiemann et al., 2001; Mackay and Browning, 2002; Mackay et al., 2003). In BCMA-deficient mice, no gross effect on B cell development or antigen-specific immune responses has been observed. In contrast, mature B cells accumulate in TACI-deficient mice, suggesting that TACI may negatively regulate B cell survival and development (Yan et al., 2001; Seshasayee et al., 2003). Lastly, mice expressing a naturally mutated form of BAFFR (the A/WySnJ strain) exhibit peripheral B cell abnormalities comparable to those found in BAFF (Blys/TALL-1)-deficient mice, indicating that BAFF-BAFFR interaction is primarily responsible for peripheral B cell survival and development (Thompson et al., 2001).

CD40 and BAFFR ligation activate the NFκB family of transcription factors, which are critical for the regulation of B cell survival and development. NFκB transcription factors are homo- or heterodimers of a group of structurally related proteins, including Rel (c-Rel), RelA (p65), RelB, NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100) (Ghosh and Karin, 2002). In resting cells, most NFκB/Rel dimers are bound to IκBs and retained in the cytoplasm. Upon stimulation with CD40L and BAFF, the IκB proteins are phosphorylated by IκB kinase (IKK) (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). IKK is composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ.

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It has been shown that IKKβ, but not IKKα, is mainly responsible for the IκB phosphorylation, which is followed by rapid ubiquitination and degradation of the IκB proteins (Karin and Ben Neriah, 2000; Ghosh et al., 1998), releasing NFκB (p65/p50) to activate transcription in the nucleus (the canonical NFκB activation pathway). Conditional deletion of *IKK*β results in the rapid loss of B cells (Li et al., 2003; Pasparakis et al., 2002), indicating that the canonical NFκB activation pathway mediated by IKKβ is probably required for the general differentiation and homeostasis of B cells. CD40 and BAFFR are also able to induce a noncanonical NFκB2 processing pathway; that is, IKKα leads to the phosphorylation and processing of p100, resulting in the formation of an active RelB/p52 heteodimer (Coope et al., 2002; Kayagaki et al., 2002). NFκB inducing kinase (NIK) was shown to play a role in activating IKKα, leading to p100 processing (Ninomiya-Tsuji et al., 1999; Irie et al., 2000; Takaesu et al., 2000; Takaesu et al., 2001; Qian et al., 2001; Deng et al., 2000; Wang et al., 2001; Yin et al., 2001; Garceau et al., 2000). The fact that mature B cell numbers are reduced in mice lacking NIK or p52 and in irradiated mice reconstituted with IKKα-deficient lymphocytes suggests the important role of this noncanonical NFκB activation pathway in CD40 and BAFFR-mediated B cell survival.

CD40 and BAFFR utilize TRAF (TNF receptor-associated factor) molecules as receptor-proximal adapters to mediate the activation of downstream kinases including IKKs and MAP kinases (Xu and Shu, 2002; Hostager and Bishop, 1999). We recently identified a novel adaptor molecule called Act1 that may function as an important regulator in signaling pathways mediated by CD40 and BAFF. Endogenous Act1 is recruited to CD40 in B cells upon stimulation with CD40 ligand (CD40L). Act1 does not have any enzymatic domains; instead it contains a helix-loop-helix at the N terminus and a coiled-coil at the C terminus (Qian et al., 2002; Li et al., 2000; Leonardi et al., 2000). In addition, Act1 contains two putative TRAF binding sites, EEESE (residues 38–42), and EERPA (residues 333–337) (Qian et al., 2002). To elucidate the physiological functions of Act1, we generated Act1-deficient mice. Act1-deficient mice revealed major lymphoid system defects, which is marked with lymphadenopathy, hypergammaglobulinemia, and production of autoantibodies. A general increase in the numbers of peripheral B cells, and CD40- and BAFFR-mediated B cell survival is significantly increased in Act1-deficient mice, indicating that Act1 is an important modulator in humoral immune responses by regulating CD40 and BAFFR signaling in B cells.

Results

Lymphoid System Defects in Act1-Deficient Mice

To generate Act1 null mice, the Act1 targeting construct bearing a disrupted exon 2 (encoding residues 1–268) was transfected into ES cells derived from the 129/sv mouse strain, followed by the transfection of Cre to remove the Neo drug marker and the exon 2 of *Act1* (Figure 1A). Targeted ES clones containing the complete Act1 null allele were injected into blastocysts derived from BALB/c mice (Act1 null allele, Figures 1A and 1B). Germline- transmitted chimeras were obtained and bred to BALB/c. Heterozygous mice (F1) were obtained and bred to homozygotes (Figure 1C).

Act1 expression is induced in mouse tissues upon intraperitoneal injection of lipopolysaccride (LPS) (Zhao et al., 2003). Act1 expression is also induced in mouse splenic B cells upon stimulation with BAFF, CD40L, and LPS, implicating its important role in B cell function (Figure 1D). The LPS-induced expression of Act1 in mouse splenic B cells was no longer detectable in Act1-deficient mice (Figure 1D). The expression patterns are consistent with a negative modulating role for Act1 during B cell activation.

The Act1-deficient mice displayed major lymphoid system abnormalities as early as 3 weeks of age. The lymph nodes (cervical, axillary, and brachial) were massively enlarged (Figure 2A). Lymphadenopathy is due to lymphoid hyperplasia, increased germinal centers, and the accumulation of large numbers of immunoglobulin-producing plasma cells (Syndecan-1-positive cells) in the medulla of the lymph nodes (Figure 2B). Act1-deficient mice also developed enlarged spleens (Figure 2A). The microscopic structure of the spleen was largely unaltered, although increased density of B cell zones was observed in some of the Act1-deficient spleens as compared to wild-type littermate controls (KO2 compared to WT2 in Figure 2C). Furthermore, the Act1-deficient mice developed inflammation in multiple tissues, including upper respiratory airway and skin (Figure 2D and Supplemental Figure S2 available online at http://www.immunity.com/cgi/content/full/21/4/575/DC1/).

Hypergammaglobulinemia in Act1-Deficient Mice

In the CD40-deficient mice, the constitutive levels of immunoglobulins including IgG1, IgG2a, IgG2b, and IgE are much lower as compared to wild-type control mice (Figure 3A and Supplemental Figures S3A and S3B). In contrast, IgG subclasses and IgE were substantially increased, more than 10-fold, in the Act1-deficient mice as compared to those in the wild-type control mice. However, the levels of IgM, IgG3, and IgA were not changed in either CD40- or Act1-deficient mice (Figure 3A and Supplemental Figure S3A). The hypergammaglobulinemia in Act1-deficient mice is consistent with the increased numbers of plasma cells in the secondary lymphoid system of the Act1-deficient mice. Interestingly, mice that overexpress BAFF display a very similar phenotype to the Act1-deficient mice, including lymphadenopathy and hypergammaglobulinemia (Gross et al., 2000; Mackay et al., 1999). Taken together, Act1 probably functions as an important modulator in humoral immune responses, possibly through its involvement in CD40- and/or BAFF-mediated pathways.

To determine whether Act1 indeed exerts its regulatory role in humoral immune responses through the CD40 and BAFF pathways, we generated CD40-Act1 and BAFF-Act1 double knockout mice. Both the CD40-Act1 and BAFF-Act1 knockout mice had much reduced enlargement of lymph nodes and spleen as compared to Act1-deficient mice (data not shown). The hypergammaglobulinemia observed in Act1-deficient mice was abolished in CD40-Act1 and BAFF-Act1 double knockout mice (Figure 3A and Supplemental Figure S3A). These results strongly suggest that Act1 modulates the humoral immune responses through its interaction with the CD40- and BAFF-mediated pathways.

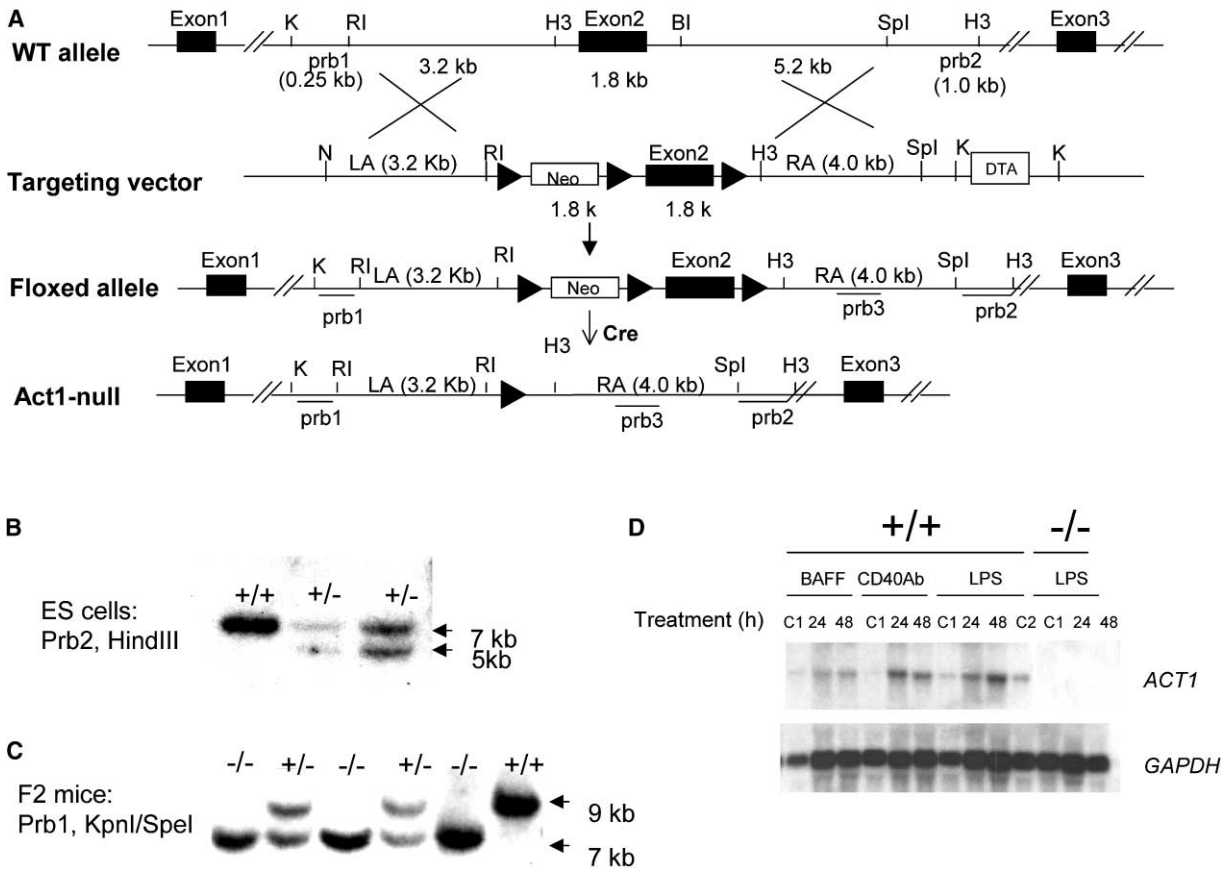

Figure 1. Generation of Act1-Deficient Mice

(A) Construction of the Act1 gene-targeting vector. See the Experimental Procedures for details. The gene encoding Act1 (wt allele); the targeting vector (targeting vector); loxP sequences (filled triangles), left arm (LA); right arm (RA), Diphtoxin A gene (DTA); the targeted allele with the targeting vector (floxed allele); Cre-mediated complete deletion of the Act1 null allele (Act1 null).

(B and C) Southern blot analyses of DNA from Act1-deficient mice and ES cells. Genomic DNA was extracted from mouse tail tissue or ES cells, digested with HindIII or KpnI/SpeI, and analyzed by Southern blot by using the two probes (prb1 and prb2) shown in (A). Southern analysis with prb1 (digested with Kpn1/Spe1) generates a 9.0 kb for the wild-type allele and a 7.0 kb for the Act1 null allele. Southern blot with prb2 (digested with HindIII) generates a 7.0 kb for the wild-type allele and a 5.0 kb band presents the Act1 null allele.

(D) RNA blot analysis. Total RNA was extracted from wild-type (+/+) or Act1-deficient (−/−) splenic B cells, untreated (C1 and C2, incubated without ligand for 24 or 48 hr, respectively), or treated with BAFF, CD40 antibody, or LPS for 24 or 48 hr. The probes are full-length mouse cDNAs (right margins: *ACT1*, Act1; *GAPDH*, glyceraldehydes phosphodehydrogenase).

In Vivo Responses to Antigen Challenge

To examine the role of Act1 in T cell-dependent (TD) antigen-specific responses, the Act1-deficient and littermate control mice were challenged with nitro-phenol-conjugated chicken γ-globulin (NP_{28}-CGG), and specific antibody responses were measured (Figure 3C). Upon in vivo challenge with NP-CGG, Act1-deficient mice developed much higher titers of both total (NP30-BSA, Figure 3C) and high-affinity (NP4-BSA, Figure 3C) NP-specific IgG2b antibodies. Although the total NP-specific IgG1 level was slightly increased (NP30-BSA, Figure 3C), the Act1-deficient mice developed significantly increased high-affinity IgG1 (NP4-BSA, Figure 3C). However, NP-specific IgM antibody production was unchanged in Act1-deficient mice. The Act1-deficient mice and littermate control mice were then immunized with the T cell-independent (TI) antigen NP-Ficoll, and specific antibody responses were measured. Upon in vivo challenge with NP-Ficoll, Act1-deficient mice developed much higher titers of total NP-specific IgG2a and IgG2b antibodies (Figure 3D). However, NP-specific IgM and IgG1 production was unchanged in Act1-deficient mice (Figure 3D). The hyper TD and TI antigen-specific responses observed in Act1-deficient mice suggests that Act1 probably plays a negative regulatory role in both T cell-dependent and -independent humoral immune responses, probably through its interaction with the CD40- and/or BAFFR-mediated pathways (Kawabe et al., 1994; Xu et al., 1994).

Production of Autoantibodies

Dysregulation of the humoral immune response often leads to autoimmune disease. Both anti-ssDNA and anti-dsDNA IgG antibodies were detected in most of the Act1-deficient mice, but not in the littermate control mice (Figure 3E). Furthermore, anti-histone IgG antibod-

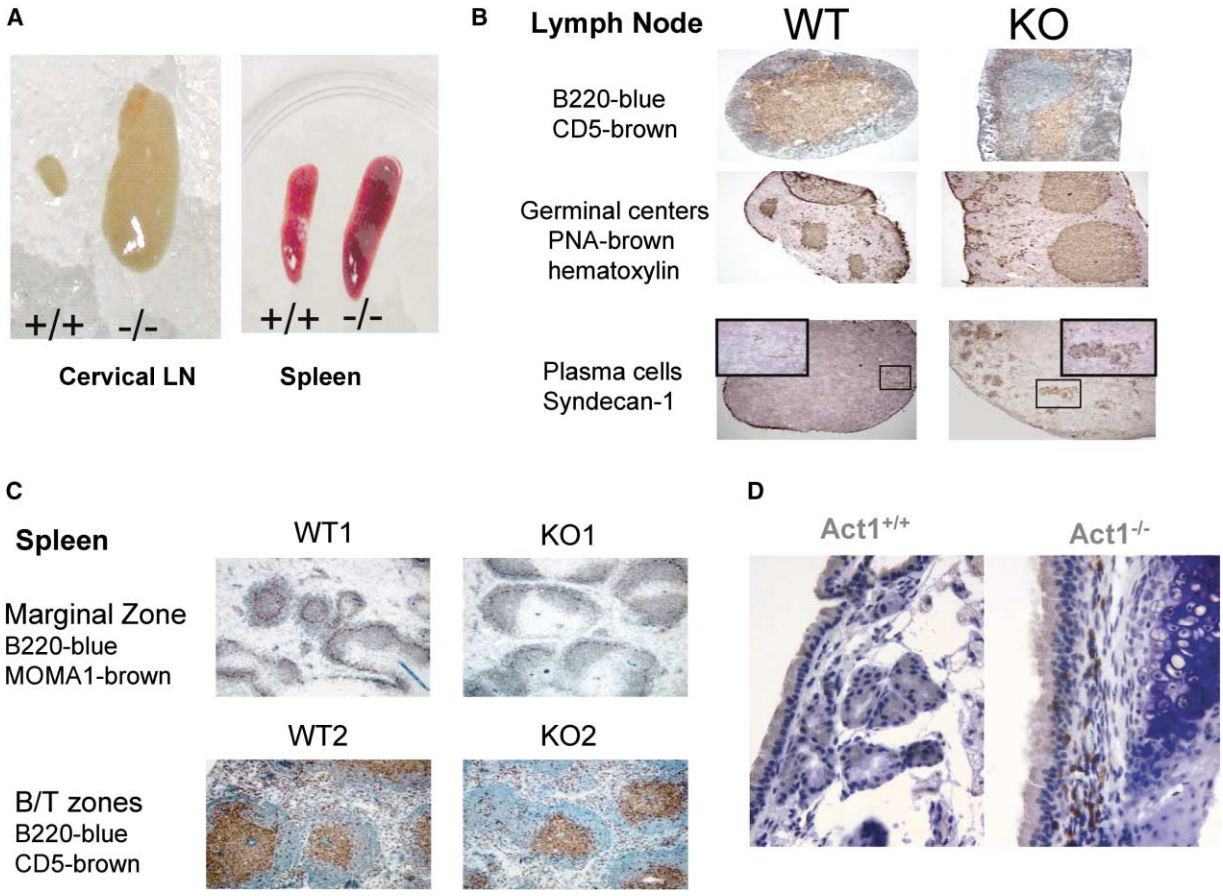

Figure 2. Lymphoid System Abnormality in Act1-Deficient Mice

(A) Enlarged cervical lymph node and spleen in Act1-deficient mice. Cervical lymph nodes and spleens dissected from wild-type (+/+) and Act1-deficient (−/−) mice were compared.

(B) Histology of lymph node. Frozen sections from wild-type (+/+) and Act1-deficient (−/−) cervical lymph node were immunostained for T/B cell zones with anti-B220 (blue) and anti-CD5 (brown); for germinal centers with biotinylated-PNA (brown) and for plasma cells with anti-Syndecan-1 (brown).

(C) Histology of spleen. Frozen sections from wild-type (+/+) and Act1-deficient (−/−) spleens were simultaneously immunostained for metallophilic macrophages and B cells with anti-MOMA1 (brown) and anti-B220 (blue). They were also immunostained for T/B cell zones with anti-B220 (blue) and anti-CD5 (brown).

(D) Immunostaining of trachea. Tracheas from wild-type or Act1-deficient mice were immunostained with anti-CD3 (brown). The brown-stained CD3+ T cells are clearly detected below the epithelium in the Act1-deficient trachea.

ies were also detected in the Act1-deficient mice, but not in the littermate control mice. Consistent with the above conclusion that Act1 is involved in BAFF-BAFFR pathway, transgene-mediated overexpression of BAFF causes breakdown of B cell tolerance and leads to production of autoantibodies and a SLE-like condition in mice (Gross et al., 2000; Mackay et al., 1999).

Increased Peripheral B Cells

We next analyzed the T and B cell compartments of thymus and spleen of Act1-deficient and littermate control mice by flow cytometry. The total T cells (Thy1+) and the proportion of T cells in the subsets defined by CD4 and CD8 cell-surface markers were unaltered (Table 1 and data not shown). However, the total splenic B cell (B220+) population was significantly increased in Act1-deficient spleen, as compared to that of littermate control mice that were gender matched (Table 1 and Figure 4A). The decreased percentage of T cells in the spleen (Thy.1+, Figure 4A) was probably due to increased B cells, since the total numbers of T cells per spleen were unaltered (Table 1). In addition to the increased B cell population, the dendritic cell population ($I\text{-}A^+CD11c^+$) was also increased in Act1-deficient spleen as compared to the wild-type control (Table 1).

Analysis of $B220^+$ cells in the bone marrow of Act1-deficient mice indicated no changes in pro-B cells ($B220^+CD43^+IgM^-$), pre-B cells ($B220^+CD43^-IgM^+$), and immature B lymphocytes ($B220^+IgM^+IgD^-$) compartments (Table 1). Thus, lack of Act1 does not affect the generation or differentiation of bone marrow lymphoid progenitor cells that give rise to fully committed B lymphocytes. As indicated above, $B220^+$ B cells in secondary lymphoid organs were significantly increased (Table

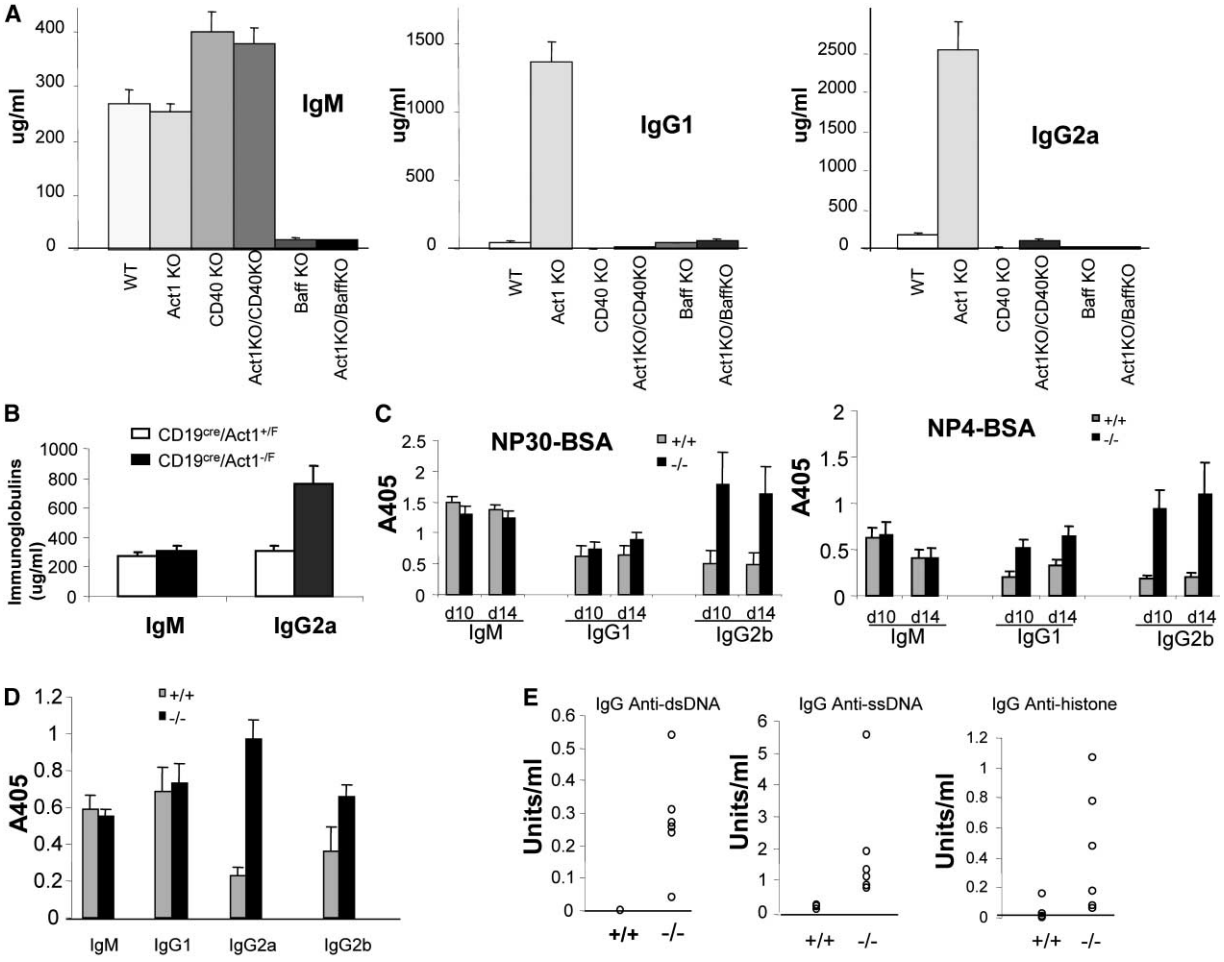

Figure 3. Antibody Production

(A and B) Serum immunoglobulin levels. (A) Sera from 7-week-old Act1-deficient mice ($Act1^{-/-}$) (n = 5), wild-type littermates ($Act1^{+/+}$) (n = 4), CD40-deficient mice ($CD40^{-/-}$) (n = 6), double knockout mice of Act1 and CD40 ($Act1^{-/-}CD40^{-/-}$) (n = 5), BAFF-deficient mice ($Baff^{-/-}$) (n = 8), and double knockout mice of Act1 and BAFF (Act1−/−Baff−/−) (n=12) were analyzed by ELISA for different immunoglobulin levels, including IgM, IgG1, and IgG2a. (B) Sera from 8-week-old control mice ($CD19^{cre}Act1^{+/F}$) (n = 4) and B cell-specific Act1 knockout mice ($CD19^{cre}Act1^{-/F}$) (n = 6) were analyzed by ELISA for IgM and IgG2a levels.

(C) T cell-dependent immune responses. Four pairs of 7-week-old Act1-deficient and wild-type littermate (+/+) mice were immunized with 20 μg of NP_{28}-CGG in PBS. Blood was taken at day 10 and day 14 after immunization. The sera were analyzed by ELISA for total NP-specific antibodies with NP_{30}-BSA or NP-specific high-affinity antibodies with NP_4-BSA. Each sample was measured by using multiple dilutions, and the data presented are an average of 50% of the maximum binding to NP4-BSA or NP30-BSA from four mice.

(D) T cell-independent immune responses. Four pairs of 7-week-old mice were immunized with 20 μg/ml NP-Ficoll in PBS. Blood was taken at day 0, day 8, and day 15 and analyzed for TI antibody isotypes with capture antigen NP_{30}-BSA by ELISA. Data are shown here from the blood at day 8 after immunization. Each sample was measured by using multiple dilutions, and the data presented are an average of 50% of the maximum binding to NP30-BSA from four mice.

(E) Autoantibodies in Act1-deficient mice. Levels of circulating anti-dsDNA, anti-ssDNA, and anti-histone IgG autoantibodies in the serum of 7-month-old Act1-deficient (−/−) and wild-type littermate controls (+/+) were assayed by ELISA. The units of autoantibodies are an arbitrary unit scale defined relative to a standard linear curve by using dilutions of monoclonal antibodies to the appropriate nuclear antigen. BWD-1(1D12) was used to generate the anti-ds-DNA and anti-ssDNA standard curves, whereas BWH-1 (2B1) was used for the standard antihistone curve (Kotzin et al., 1984).

1 and Figure 4A). Analysis of splenic B cells showed elevated mature B cells ($B220^{+}IgM^{lo}IgD^{+}$; Table 1), follicular B cells ($B220^{+}CD21^{int}CD23^{hi}$; Figure 4B and Table 1), marginal zone B cells ($B220^{+}CD21^{hi}CD23^{lo}$, Figure 4B and Table) and T1 ($B220^{+}IgM^{hi}CD21^{-}$) and T2 ($B220^{+}IgM^{hi}CD21^{+}$) transitional B cells (Figure 4C and Table 1). These data show that the absence of Act1 led to uniform increase in cellular components of the B cell compartment in secondary lymphoid organ. Taken together, these results indicate that Act1 probably plays a critical regulatory role in the survival of peripheral B cells.

Specific Deletion of the Act1 Gene in B Cells

To determine the role of Act1 in B cells, we decided to specifically delete the Act1 gene in B cells. The Act1-deficient ($Act1^{-/-}$) mice were first bred onto CD19cre

Table 1. B Cell, T Cell, and Dendritic Cell Populations

Bone Marrow ($\times 10^6$)	Act1$^{+/+}$	Act1$^{-/-}$	Fold			
Pro-B cells (B220$^+$CD43$^+$IgM$^-$)	1.6 ± 0.3	1.6 ± 0.3	1.0			
Pre-B cells (B220$^+$CD43$^-$IgM$^+$)	4.5 ± 0.7	4.1 ± 0.6	0.9			
Immature B cells (B220$^+$IgM$^+$IgD$^-$)	3.3 ± 0.6	2.9 ± 0.6	0.9			
Recirculating mature B cells (B220$^+$IgM^{lo}IgD$^+$)	0.43 ± 0.1	0.77 ± 0.1	1.8			
Spleen ($\times 10^6$)	**Act1$^{+/+}$**	**Act1$^{-/-}$**	**Fold**	**CD19cre/Act1$^{+/F}$**	**CD19cre/Act1−/F**	**Fold**
Total B cells	120.9 ± 8.4	269.9 ± 5.1	2.2	105.2 ± 7.4	136.3 ± 8.9	1.3
Mature B cells	37.5 ± 1.8	102.3 ± 2.0	2.7	34.9 ± 3.3	52.1 ± 4.8	1.5
Marginal zone B Cells	5.6 ± 0.4	18.4 ± 0.8	3.3	6.2 ± 0.6	12.8 ± 1.3	2.1
Follicular B cells	31.1 ± 1.7	77.6 ± 2.1	2.5	28.9 ± 2.4	40.9 ± 4.1	1.4
T1 transitional B cells	8.2 ± 0.3	16.0 ± 1.0	1.9	6.8 ± 0.7	9.6 ± 0.9	1.4
T2 transitional B cells	4.1 ± 0.3	10.2 ± 0.5	2.5	3.6 ± 0.4	5.5 ± 0.5	1.5
Dendritic cells	6.1 ± 1.0	15.8 ± 2.2	2.6	4.2 ± 0.4	5.8 ± 0.7	1.4
Total T cells	37.7 ± 3.0	34.2 ± 2.1	0.9	43.5 ± 4.1	43.9 ± 3.7	1.0
CD4$^+$ cells	27.1 ± 2.3	24.4 ± 3.1	0.9	31.1 ± 2.8	31.6 ± 3.1	1.0
CD8$^+$ cells	10.6 ± 1.0	9.5 ± 0.5	0.9	12.4 ± 1.1	12.1 ± 1.2	1.0

Total B cells (B220$^+$), mature B cells (B220$^+$IgM^{lo}IgD$^+$), marginal zone B cells (B220$^+$CD21^{hi}CD23^{lo}), follicular B cells (B220$^+$CD21^{int}CD23^{hi}), T1 transitional B cells (B220$^+$IgM^{hi}Cd21$^-$), T2 transitional B cells (B220$^+$IgM^{hi}CD21$^+$), dendritic cells (I-A$^+$CD11c$^+$); total T cells (Thy.1$^+$).
Data are shown as mean ± SEM. from three pairs of mice each group. Fold stands for the ratio of cell number from Act1 knockout mice to cell numbers from Act1 control mice. BM cell counts represent the number of cells isolated from two hindleg femurs. Mice were age (6–8 weeks) and gender matched.

transgenic mice (CD19cre$^{+/+}$ [Li et al., 2003]) to generate CD19cre$^{+/-}$Act1$^{+/-}$ mice. These mice were further bred onto Act1 floxed mice (Act1$^{flox/flox}$) to generate the control mice (CD19cre$^{+/-}$Act1$^{+/flox}$) and B cell-specific knockout mice (CD19cre$^{+/-}$Act1$^{-/flox}$). The deletion efficiency mediated by CD19 promoter driven Cre was about 80% as determined by Southern blot with the prb3 (Figure 1 and Supplemental Figure S1). We found that the B cell-specific Act1 knockout mice (CD19cre$^{+/-}$Act1$^{-/flox}$) had similar phenotypes as the Act1-deficient mice, although with less severity. The CD19cre$^{+/-}$Act1$^{-/flox}$ mice developed enlarged lymph nodes and spleen (data not shown). Hypergammaglobulinemia was also detected in these mice (Figure 3B). While the T cell population was not altered, the total B cell population and all the subsets of B cells were increased in the CD19cre$^{+/-}$Act1$^{-/flox}$ (Table 1). These results strongly indicate that Act1 plays an important role in B cells, which is consistent with the phenotype observed in the Act1-deficient mice. Interestingly, the dendritic cell population was also increased in these B cell-specific Act1 knockout mice, suggesting that the defect in B cells may have an indirect impact on the dendritic cells.

Increased CD40- and BAFFR-Mediated B Cell Survival

While CD40- and CD40L-deficient mice showed loss of T cell-dependent B cell survival, proliferation, and activation, recent studies have also demonstrated the importance of BAFF-BAFFR interaction in B cell survival (Mackay et al., 2003; Mackay and Browning, 2002). The fact that Act1-deficient mice and B cell-specific Act1 knockout mice showed increased peripheral B cells implicates a possible role of Act1 in CD40- and BAFFR-mediated B cell survival. To test this hypothesis, resting mature B cells were purified from spleen and cultured with or without anti-CD40 antibody or BAFF for 4 days. These cultured cells were examined for cell survival by flow cytometry by using forward light scatter (FSC)/side light scatter (SSC) plots (Rolink et al., 2002) (Supplemental Figure S4A). Treatment of wild-type splenocytes with CD40 antibody for 4 days increased the percentage of live cells from 7.0 to 22.2, whereas the same stimulation increased the viability of Act1-deficient splenocytes, from 5.2% to 38.7% (Figure 5A). BAFF treatment also led to greater increase in cell survival in the Act1-deficient splenocytes, as compared to the cells from littermate control mice (Figure 5A). However, IL-4- and IgM-mediated B cell survival was unaltered in Act1-deficient mice (Figure 5A and data not shown). It is important to point out here that CD40- and BAFF-mediated cell survival was also enhanced in splenic B cells isolated from B cell-specific Act1 knockout mice (Figure 5B), indicating an intrinsic defect in Act1-deficient B cells. We then examined the role of Act1 in CD40-mediated B cell proliferation. CD40-mediated proliferation was increased by 40% in Act1-deficient B cells as compared to that in wild-type cells (Supplemental Figure S4B), which probably contributes to the enhanced CD40-mediated B cell survival in Act1-deficient mice described above (Figure 5A).

The prosurvival proteins Bcl-xl and Bcl2 have been implicated in CD40- and BAFFR-mediated B cell survival (Mackay et al., 2003). Western blot analysis showed that Act1-deficient B cells had much induced levels of Bcl-xl in response to stimulation with CD40 antibody (Figure 5C), strongly suggesting that Bcl-xl is probably one of the important effectors responsible for Act1-regulated CD40-mediated B cell survival.

Increased CD40- and BAFFR-Mediated Signaling

We determined whether Act1 had any direct effects on the immediate signaling events mediated by CD40 and BAFF. Act1-deficient B cells showed stronger IκB phos-

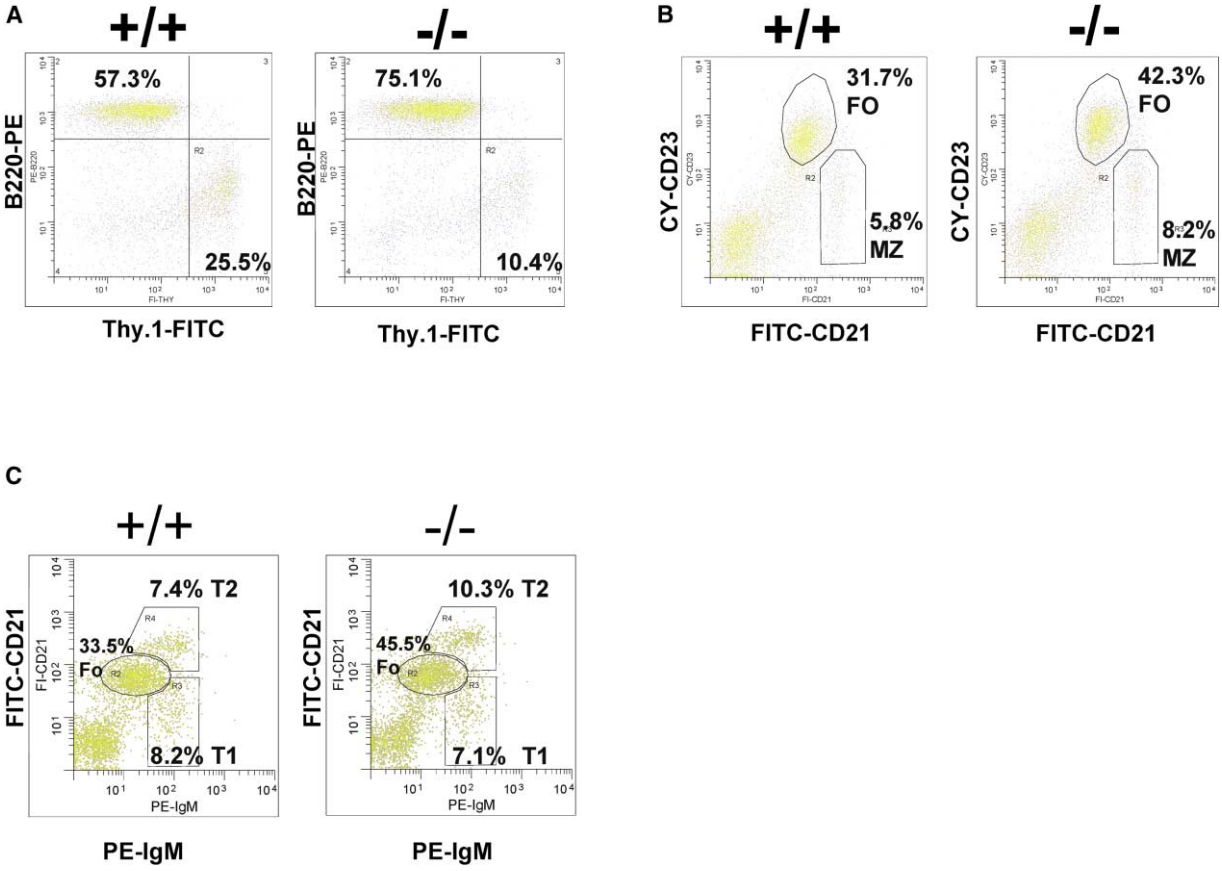

Figure 4. FACS Analysis

Splenocytes from 7-week-old Act1-deficient (−/−) (n = 3) and wild-type littermate control (+/+) (n = 3) mice were stained with the indicated antibodies, and then analyzed after electronic scatter gating.

(A) Increased B220+ cells in Act1-deficient spleen.

(B) Increased follicular ($CD21^{int}CD23^{hi}$) and marginal zone ($CD21^{hi}CD23^{lo}$) B cells in Act1-deficient spleen.

(C) Increased transitional B cells in Act1-deficient spleen. The data are presented as percentages of total splenocytes from one pair of mice. Similar results were obtained from three pairs of mice. Since the Act1-deficient spleens were enlarged, the different B cells populations are presented as actual cell numbers in Table 1.

phorylation and enhanced p100 processing to p52 in response to CD40 antibody and BAFF stimulation, implicating a negative regulatory role of Act1 in both canonical and noncanonical NFκB activation pathway (Figures 6A–6C). Interestingly, the CD40-induced IκB phosphorylation was much stronger than that induced by BAFF treatment in Act1-deficient B cells. As activation of NFκB (through the canonical pathway) induces the expression of p100, Act1-deficient B cells showed higher levels of NFκB2/p100 precursor in response to CD40 antibody stimulation (Figure 6A). Furthermore, Act1-deficient B cells showed enhanced activation of MAP kinases, including JNK, ERK, and p38 in response to CD40 antibody and BAFF (Figures 6B and 6C and data not shown). It is important to point out here that the expression of CD40-CD40L and BAFF-BAFFR was not altered in Act1-deficient mice as compared to wild-type controls (Figure 6B, Supplemental Figure S5 and data not shown). Taken together, these results indicate that Act1 negatively regulates the CD40- and BAFFR-mediated pathways, which probably results in its inhibitory effect on B cell survival. As controls, we showed that the levels of April- (a ligand for TACI, BCMA, but not BAFFR) and BCR-mediated signaling are the same between wild-type and Act1-deficient B cells (Figures 6D and 6E).

In addition to B cells, we also examined the role of Act1 in CD40- and BAFFR-mediated signaling in primary mouse embryonic fibroblasts (MEFs) (Supplemental Figure S6). Act1-deficient MEFs showed increased CD40- and BAFFR-mediated NFκB-dependent E-selectin promoter activity, indicating that Act1 can also negatively regulate the CD40- and BAFFR-mediated signaling in non-B cells.

Interaction of Act1 with Other Signaling Molecules

To elucidate the mechanism of action of Act1, we assessed its interaction with known components of the CD40 and BAFFR pathway. Endogenous Act1 was recruited to CD40 and BAFFR upon CD40L and BAFF stimulation in the B cell line IM9 and as well as in primary human splenocytes (Figures 7A–7C). Since TRAF molecules have been shown to function as important adapt-

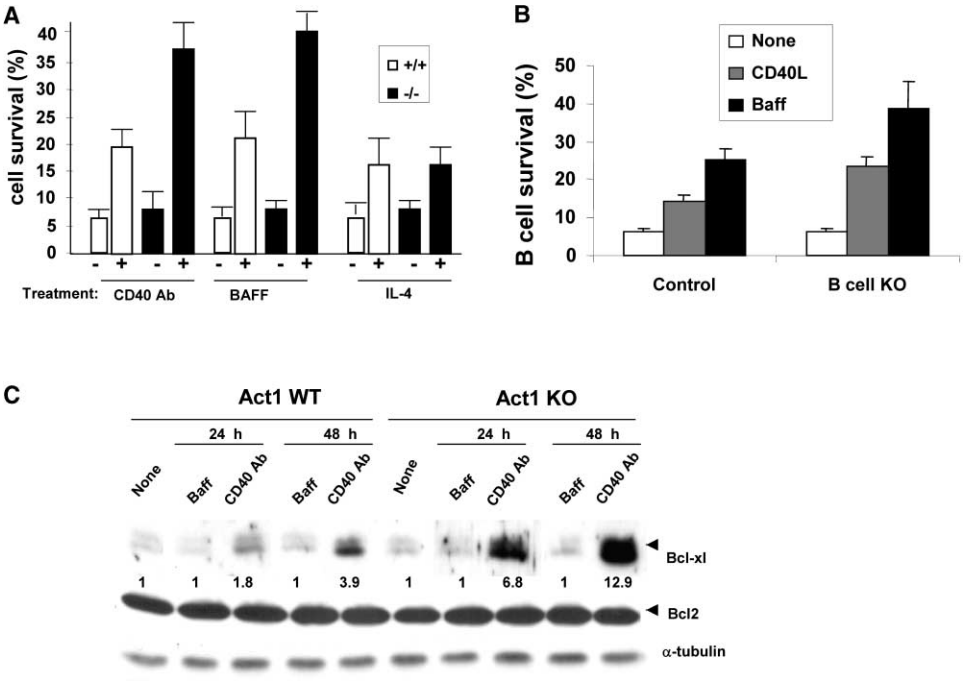

Figure 5. B Cell Survival

(A) Enhanced B cell survival in Act1-deficient mice. Purified splenic B cells from 7-week-old Act1-deficient (−/−) and littermate control (+/+) mice untreated or treated with CD40 antibody for 4 days. These cultured B cells were then analyzed by flow cytometry on FSC/SSC plots (Supplemental Figure 4A). Annexin V staining for apoptotic cells and PI staining for dead cells were used in the FACS analysis. The data presented are the average of percentage of cell survival from three pairs of mice.

(B) Increased percentage of live B cells from B cell-specific Act1 knockout mice upon stimulation with CD40 antibody or BAFF ligand. The data are shown as the average of percentage of B cell survival from B cell specific Act1 knockout mice ($CD19^{cre}Act1^{-/F}$) and control mice ($CD19^{cre}Act^{+/F}$).

(C) Enhanced CD40-mediated induction of Bcl-xl in Act1-deficient splenic B cells. Splenic B cells from 7-week-old Act1-deficient (−/−) and littermate control (+/+) were untreated or treated with CD40 antibody or BAFF for 24 hr or 48 hr. The cell extracts were analyzed by Western blot with the antibodies against Bcl-xl and Bcl-2. α-Tubulin was used as a loading control. The Bcl-xl levels were analyzed by Scion Image 1.62C alias and presented as relative fold of induction of the untreated samples.

ers for CD40 and BAFFR, we examined the interaction between Act1 and different TRAFs. Previously, Act1 was shown to specifically interact with TRAF3, but not with the other TRAFs when they were overexpressed in 293T cells (Qian et al., 2002). Under conditions of normal expression, endogenous Act1 interacted strongly with TRAF3, weakly with TRAF2, but failed to interact with TRAF6 upon CD40L stimulation as revealed by coimmunoprecipitation (Figure 7A). On the other hand, endogenous Act1 interacted with TRAF3, but not with TRAF2 or TRAF6 upon stimulation with BAFF (Figure 7B). Taken together, these preliminary results suggest that Act1 might regulate the CD40- and BAFFR-mediated signaling through its interaction with the TRAF molecules, particularly TRAF3.

Discussion

This manuscript shows that CD40- and BAFF-mediated survival is significantly increased in Act1-deficient B cells, implicating a negative or attenuating regulatory role of Act1 in B cell survival. Consistent with this finding, Act1-deficient mice revealed a general increase in peripheral B cells, culminating in lymphadenopathy, splenomegaly, hypergammaglobulinemia, inflammation in multiple tissues, and the formation of autoantibodies. While the B cell-specific Act1 knockout mice displayed a similar phenotype, the SLE-like pathologies of the Act1-deficient mice were blocked in CD40-Act1 and BAFF-Act1 double knockout mice.

The enhanced CD40- and BAFF-induced p100 processing of p52 in the Act1-deficient B cells is likely to be important for the expanded B cell compartment in the Act1-deficient spleen. Nevertheless, the enhanced canonical NFκB activation pathway and the activation of MAP kinases probably also contribute to the increased survival of Act1-deficient peripheral B cells. The control of cell survival is believed to rely on the regulation of key antiapoptotic and proapoptotic regulators. Previous studies have shown that antiapoptotic proteins Bcl2 and Bcl-xl play important roles in B cell survival. Interestingly, we found that stimulation with anti-CD40 resulted in a much higher levels of Bcl-xl in the Act1-deficient B cells than in the wild-type control cells. These elevated levels of Bcl-xl provide a mechanism for the increased CD40-mediated B cell survival in the Act1-deficient mice. BAFF stimulation did not increase the levels of Bcl-xl, indicating that B cell survival is probably not

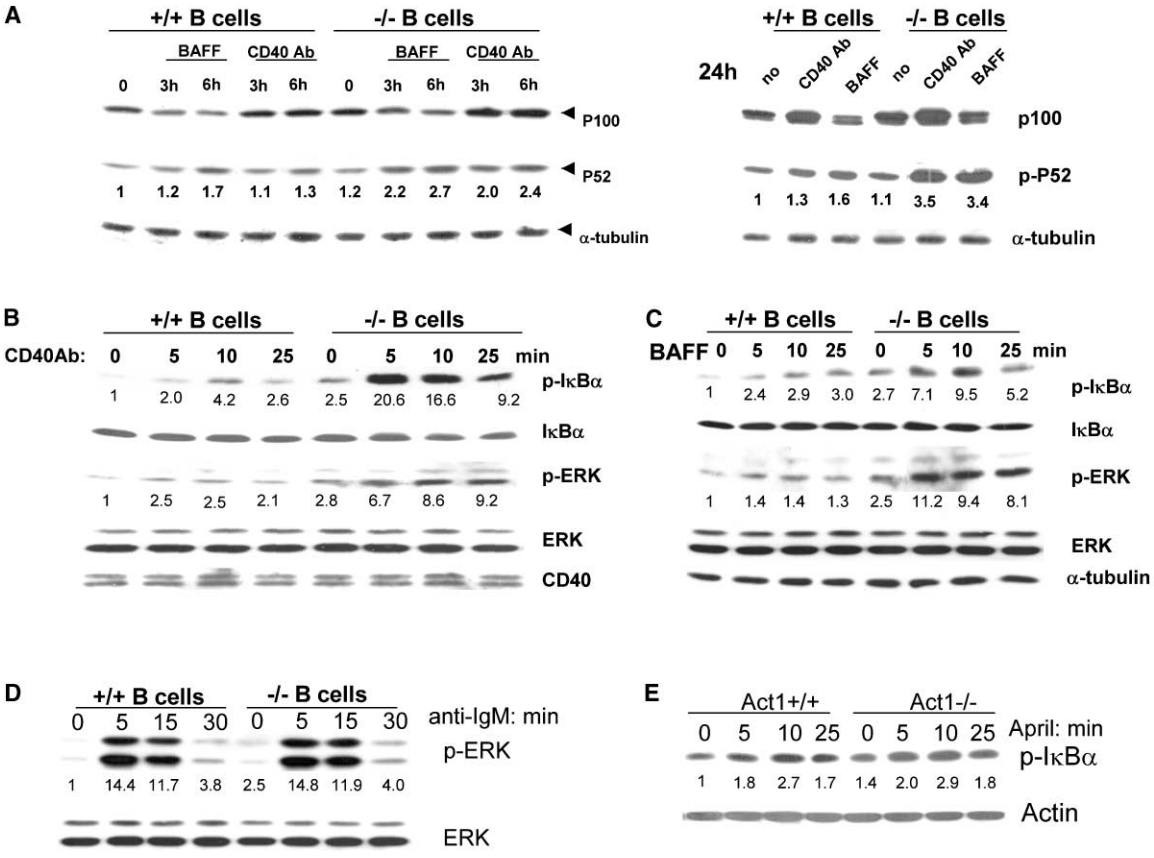

Figure 6. Increased CD40 and BAFF Signaling in Act1-Deficient Splenic B Cells

Splenic B cells from 7-week-old Act1-deficient (−/−) and littermate control (+/+) mice were untreated or treated with CD40 antibody, mBAFF, anti-IgM or mApril for the indicated times. The whole cell extracts were examined by western blot analysis.

(A) Cells were treated with CD40 antibody or BAFF for 3, 6, or 24 hr, and extracts were analyzed with antibodies against P100/P52 and α-tubulin.

(B) Extracts from cells treated with CD40 antibody were analyzed with antibodies against p-IκBα, IκBα, p-ERK, ERK, and CD40.

(C) Extracts from cells treated with BAFF were analyzed with antibodies against p-IκBα, IκBα, p-ERK, ERK, and tubulin.

(D) Extracts from cells treated with anti-IgM were analyzed with antibodies against p-ERK and ERK.

(E) Extracts from B cells treated with mApril for different times were analyzed with antibodies against p-Iκ-Bα and Actin. α-tubulin and Actin were used as a loading control. The levels for p52, p-IκBα, p-ERK, and p-JNK were analyzed by Scion Image 1.62C alias and presented as relative fold of induction of the untreated samples.

dependent on this antiapoptotic factor in Act1-deficient mice. While Act1-deficient B cells showed similar levels of p100 processing in response to CD40 antibody and BAFF, the CD40-induced IκB phosphorylation was much stronger than that induced by BAFF treatment. The differential effects of Act1 on CD40- and BAFF-mediated pathways probably result in altered expression of different target genes in Act1-deficient cells. Future efforts are directed toward identifying the Act1-modulated target genes that are responsible for attenuating CD40- and/or BAFF-mediated B cell survival.

The fact that Act1 specifically interacts with TRAF3 upon stimulation with CD40L and BAFF suggests that TRAF3 may play an important role in Act1-mediated negative regulation. Interestingly, TRAF3 has been shown to play a negative regulatory role in both CD40 and BAFFR signaling (Xu and Shu, 2002; Hostager et al., 2003; Xie et al., 2004; Liao et al., 2004). TRAF3$^{-/-}$ B cells displayed enhanced CD40-mediated JNK activation. Furthermore, in TRAF3$^{-/-}$ B cells, the recruitment of TRAF2 to CD40 was increased upon CD40 engagement, suggesting that TRAF3 may exert its inhibitory effects on CD40 signaling by competing with TRAF2 for association with CD40. Moreover, TRAF3 was shown to interact with NIK, targeting NIK for degradation by the proteasome and inhibiting NIK-mediated p100 processing. It is possible that Act1 exerts its inhibitory role in CD40 and BAFFR signaling through its interaction with TRAF3 by either competing with the positive regulators (such as TRAF2) for interactions with receptors and kinases or targeting the positive regulators for degradation through the proteasome pathway. Interestingly, the expression of both Act1 and TRAF3 are increased in mouse splenic B cells upon stimulation with BAFF, CD40L, and LPS (Grammer and Lipsky, 2000) (unpublished data, Z. Zhao and X.L.), suggesting that these two molecules might work together as a feedback control to dampen signaling for B cell survival and activation, regulating homeostasis of B cell functions.

While we have now clearly shown that Act1 plays a

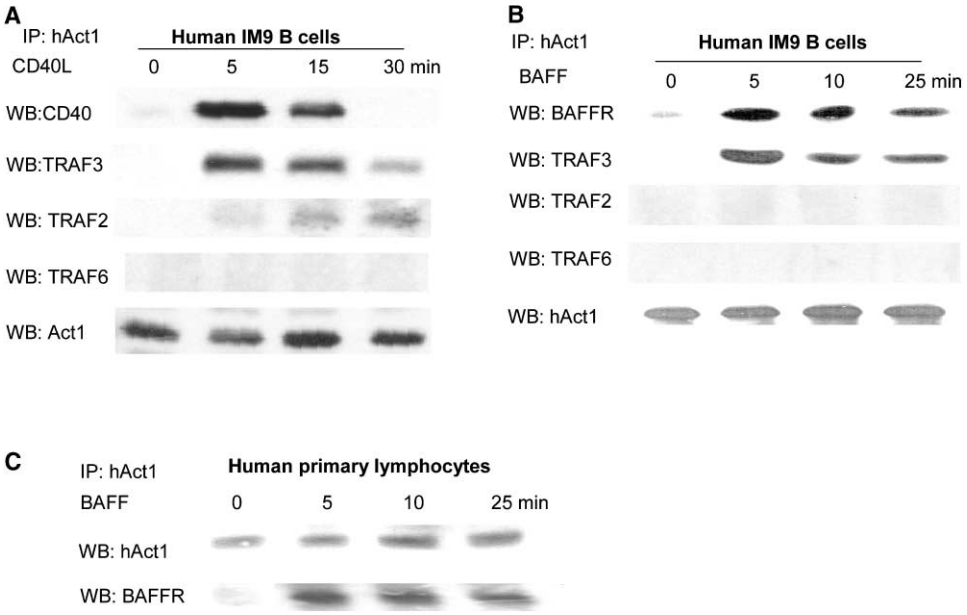

Figure 7. CD40L- and BAFF-Induced Interaction of Act1 with CD40, BAFFR, and TRAF3

Cell extracts from IM9 (A and B) or primary human splenocytes (C) untreated or treated with CD40L (A) or BAFF (B and C) for the indicated times were immunoprecipitated with anti-Act1, followed by Western analyses with antibodies against CD40 (A), BAFFR (B and C) Act1 (A–C), TRAF3 (A and B), TRAF2 (A and B) and TRAF6 (A and B).

negative role in CD40 and BAFF-mediated pathways, our previous studies showed that overexpression of Act1 can lead to constitutive NFκB activation. One possibility is that Act1 may play differential functions in different cell types. However, we found that CD40- and BAFF-mediated signaling is also elevated in Act1-deficient MEFs, suggesting that Act1 is able to function as a negative regulator in cell types other than B cells. Furthermore, siRNA knocking down of Act1 in Hela cells led to enhanced CD40-mediated JNK activation, indicating that Act1 also functions as a negative regulator in cultured epithelial cells (Supplemental Figure S7). Our recent results suggest that the Act1-mediated constitutive NFκB activation is probably due to nonspecific protein-protein interaction carried out by overexpression of Act1. Interestingly, when Act1 and TRAF3 were cotransfected into 293T or Hela cells, TRAF3 specifically and completely inhibited Act1 mediated NFκB activation, whereas TRAF3 had no effect on IRAK-mediated NFκB activation (Supplemental Figure S7). This result indicates that when Act1 is in complex with its natural interaction partner (TRAF3), Act1 does not lead to NFκB activation. Upon Act1 overexpression, Act1 probably interacts nonspecifically with other signaling molecules, resulting in constitutive NFκB activation.

In addition to its role in CD40 and BAFFR signaling in B cells, Act1 may also play a role in other cell types in signaling events mediated by other members of the TNFR superfamily, especially the subset of TRAF3-utilizing TNFRs. The fact that the dendritic cell population is increased in Act1-deficient mice suggests a potential role of Act1 in dendritic cell maturation and survival. Although the T cell population and proliferation is not affected in Act1-deficient mice (Supplemental Figure S4C), we still cannot completely exclude possible functions of Act1 in T cells. Therefore, in addition to our effort to understand the detailed mechanism of CD40-Act1- and BAFFR-Act1-mediated signaling in B cells, future research will also explore the role of Act1 in other TNFR-mediated signaling pathways in different cell types by breeding conditional Act1-deficient mice onto cell- and tissue-specific Cre-transgenic mice.

Experimental Procedures

Generation of Act1-Deficient Mice

The Act1 genomic clone was obtained from screening of a 129/sv BAC library. The HindIII/BamHI genomic fragment containing exon 2 of the Act1 gene (residuals 1–268, containing the first ATG) was subcloned into the Tri-Neo vector (containing three loxP sites). While the neomycin-resistance gene (Neo) was inserted between the first and the second loxP sites, HindIII/BamHI genomic fragment containing exon 2 of the Act1 gene was flanked by the second and the third loxP sites. The resulting DNA fragment including lox-Neo-lox-exon2-lox was then subcloned into the pBS (pBlueScript) vector. For construction of the Act1 gene-targeting vector, the 5′ arm (or left arm [LA] consisting of a 3.2 kb fragment from the first intron of the Act1 gene) was subcloned upstream of lox-Neo-lox-exon2-lox in pBS, while the 3′ arm (or right arm [RA] consisting of a 4.0 kb fragment from the second intron of the Act1 gene) was subcloned downstream of lox-Neo-lox-exon2-lox in pBS. Diphtoxin A gene was inserted downstream of the 3′ arm for negative selection. The resulting Act1 gene-targeting vector was then linearized with NotI, electroporated into 129/sv ES cells, and then followed by selection for G418-resistant ES clones. The G418-resistant ES clones were then transfected with Cre to remove the Neo drug marker and exon 2 of Act1, followed by Southern blots with two probes (prb1 and prb2) located outside of each end of the targeting construct. Targeted ES cells (Act1 null) were injected into mouse blastocysts to generate wild-type, heterozygous, and homozygous mice (BALB/c).

Act1-deficient mice and their age- and gender-matched wild-type littermates from these intercrosses were used for experiments. The Cleveland Clinic Foundation Animal Research Committee approved all of the animal protocols used in this study.

Generation of B Cell-Specific Act1-Deficient Mice

To generate B cell-specific Act1 knockout mice, the Act1-deficient mice were bred onto CD19cre mice ($CD19cre^{+/+}$) to generate $CD19cre^{+/-}Act1^{+/-}$ mice. These mice were further bred onto Act1 floxed mice ($Act1^{flox/flox}$) to generate control mice ($CD19cre^{+/-}Act1^{+/flox}$) and B cell-specific knockout mice ($CD19cre^{+/-}Act1^{-/flox}$). CD19cre-mediated deletion efficiency was determined by Southern blot with the probe (prb3) shown in Figure 1.

Generation of the CD40-Act1 and BAFF-Act1 Double Knockout Mice

To generate the double knockout mice, Act1 complete knockout mice ($Act1^{-/-}$) were first bred onto CD40 knockout mice ($CD40^{-/-}$) (purchased from Jackson Laboratory; Kawabe et al., 1994) or BAFF knockout mice ($BAFF^{-/-}$) to first generate heterozygous mice ($Act1^{+/-}CD40^{+/-}$ or $Act1^{+/-}BAFF^{+/-}$), then CD40 null Act1 heterozygous mice ($CD40^{-/-}Act1^{+/-}$), and BAFF null Act1 heterozygous mice ($BAFF^{-/-}Act1^{+/-}$). These mice were then bred among themselves to generate CD40-Act1 and BAFF-Act1 double knockout mice.

Southern and RNA Blots

Genomic DNA was extracted from ES cells or mouse tail tissue, digested with HindIII or KpnI / SpeI (New England Labs), separated by 1% agarose gel electrophoresis, and analyzed with either a 5′ external (prb1 for DNA digested with KpnI /SpeI) or a 3′ external probe (prb2 for DNA digested with HindIII). For RNA analysis, total RNA was isolated by using Trizol reagent (Invitrogen) according to the manufacturer's instruction.

Western Blot Analysis

Splenic B cells were untreated or treated 2 μg/ml anti-mouse CD40 antibody (BD pharmingen), 2 μg/ml anti-IgM (Jackson ImmunoResearch), 10 ng/ml TNFα (Peprotech), 0.1 μg/ml mouse BAFF ligand (Apotech Biochemicals, Epalinges, Switzerland), or 0.2 μg/ml mouse April ligand (generously provided by Amgen, Inc., California) for different time points. Western analyses were performed with antibodies against phospho-JNK, JNK, phospho-IκBa, IκBa, phospho-ERK, ERK, phospho-P38, P38 (Cell Signaling, Beverly, MA) and CD40, Bcl2, Bcl-x_L, α-tubulin, Actin (Santa Cruz Biotechnology, CA), and P100/P52 (kindly provided by Dr. Green, La Jolla Institute for Allergy and Immunology, San Diego, CA).

B Cell Preparation and B Cell Survival Assay

Total mouse splenic B cells were purified by two rounds of panning with anti-B220. The resting splenic B cells were then purified from the total B cells through 50%, 60%, and 70% Percoll gradient centrifugation.

For the cell survival assay, splenic B cells from naive mice were incubated at concentration of 3×10^6 cells/ml in RPMI medium plus 10% FCS, untreated or treated with CD40 antibody or BAFF ligand for 4 days. The annexin V-FITC apoptosis detection kit was used for detecting apoptotic cells, and propidium iodide was used for detecting dead cells in the flow analysis. The percentage of remaining live cells was determined by flow analysis with a FACS Calibur machine (Becton Dickinson).

Flow Cytometry

Single cell suspension from various tissues was stained with conjugated mAbs (including PE-B220, FITC-CD5, FITC-IgD, PE-IgM, FITC-CD21, Biotin-B220, Biotin-CD23, FITC-GL-7, PE-FAS, FITC-I-A, PE-CD11c, FITC-CD4, PE-CD8, FITC-CD43, and FITC-Thy1 (BD PharMingen). Cell-associated fluorescence was analyzed with a FACScan instrument and associated Winlist 5 software.

Isotype- and Antigen-Specific ELISAs

Serum IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE were determined by isotype-specific ELISA (Southern Biotechnology Associates, Birmingham, Alabama). The data were analyzed by Softmax Pro2.1. For T cell-dependent antigen-specific IgM, IgG1, and IgG2b, antibody titers were determined as described for isotype-specific ELISAs, except that plates were coated with 5 μg/ml capture antigen of either NP_4-BSA or NP_{30}-BSA. Data for the antigen specific antibodies were presented as reading at 405 nm with serum dilution of 1:100. For T cell-independent antigen-specific IgM, IgG1, IgG2a, and IgG2b, plates were coated with 5 μ g/ml capture antigen of NP30-BSA. Autoantibodies to histone, dsDNA, and single-stranded DNA (ssDNA) were measured by ELISA as previously described (Vyse et al., 1996).

Immunizations

7-week-old wild-type or Act1-deficient mice were immunized intraperitoneally with 20 μg of NP_{28}-CGG for TD response or 20 mg of NP-Ficoll for TI response (Biosource Technologies, Vacaville, CA). On days 10 and 14 after immunization for TD or days 8 and 15 for TI, serum was collected from peripheral blood. Circulating antibodies were measured by an isotype-specific, antigen-specific ELISA. NP_{30}-BSA was used to capture all antigen-specific Igs, whereas NP_4-BSA was used to capture only high-affinity Igs. Captured antibodies were detected with enzyme-conjugated rabbit anti-IgM, anti-IgG1, anti-IgG2a, and anti-IgG2b.

Immunohistochemistry

Spleens were embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA) and slowly frozen over liquid nitrogen and stored at −90°C. Cryostat sections were incubated with biotinylated anti-B220, anti-CD5, anti-Syndecan-1 (BD Pharmingen, San Diego, CA), biotinylated-PNA (Vector Labs, Burlingame, CA), or biotinylated anti-MOMA1(Bachem, King of Prussia, PA). Sections were developed by using the avidin/biotin system (Vecastain ABC kit, Vector Labs, Burlingame, CA) per the manufacturer's instructions. Slides were mounted in Crystal Mount (Biomeda, Foster City, CA) and analyzed with a Nikon E800 microscope.

Coimmunoprecipiations

Primary human splenocytes were isolated from discarded human spleen tissue of cadaver transplant donors and cultured in 10% FCS + RPMI. Human B cell line IM9 was cultured in 10% FCS + RPMI. Immunoprecipitations were performed on these cells as previously described (Qian et al., 2002). Antibodies used for immunoprecipitations include CD40 (H120, Santa Cruz Biotechnology), BaffR (Prosci, Inc.), TRAF2 (C20, Santa Cruz Biotechnology), TRAF3 (H122, Santa Cruz Biotechnology), TRAF6 (H274, Santa Cruz Biotechnology), and Act1 (Qian et al., 2002).

Acknowledgments

This work was supported by National Institutes for Health Grant GM 600020 to X.L.

Received: April 1, 2004
Revised: August 30, 2004
Accepted: September 1, 2004
Published: October 19, 2004

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