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Loss of TACI Causes Fatal Lymphoproliferation and Autoimmunity, Establishing TACI as an Inhibitory BLyS Receptor

Dhaya Seshasayee,[1] Patricia Valdez,[1] Minhong Yan,[2] Vishva M. Dixit,[2] Daniel Tumas,[1] and Iqbal S. Grewal[1,*]

[1]Department of Immunology

[2]Department of Molecular Oncology

Genentech, Inc.

1 DNA Way

South San Francisco, California 94080

Summary

BLyS, a key cytokine that sustains B cell maturation and tolerance, binds three receptors: BR3, BCMA, and TACI. Results from knockout mice implicate a major functional role for BR3 and a redundant one for BCMA in B cell function. TACI's role is controversial based on defects in TI antibody responses accompanied by B cell hyperplasia in knockout mice. We have presently characterized a precise role for TACI in vivo. TACI$^{-/-}$ mice develop fatal autoimmune glomerulonephritis, proteinurea, and elevated levels of circulating autoantibodies. Treatment of B cells with TACI agonistic antibodies inhibits proliferation in vitro and activation of a chimeric receptor containing the TACI intracellular domain induces apoptosis. These results demonstrate the critical requirement for TACI in regulating B cell homeostasis.

Introduction

BLyS (B lymphocyte stimulator, also called BAFF, TALL-1, and THANK) is a member of the TNF ligand superfamily and is known to be a critical molecule for B cell survival, maturation, and tolerance (Groom et al., 2002; Gross et al., 2001; Khare et al., 2000; Moore et al., 1999). BLyS is expressed primarily on dendritic cells and monocytes/macrophages and can be cleaved into a soluble form by furin-type proteases like other members of the TNF superfamily (Moore et al., 1999; Schneider et al., 1999). BLyS is most closely related in sequence to APRIL, another member of the TNF superfamily. BlyS$^{-/-}$ mice do not have mature B cells, resulting in low circulating Ig levels and decreased antibody-mediated immune responses (Gross et al., 2001; Schiemann et al., 2001). In contrast, BLyS transgenic mice have increased numbers of B cells and develop autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS), indicating an important role for BLyS-mediated signals in the development and long-term survival of autoreactive B cells (Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999).

BLyS has been shown to bind to three receptors, BCMA (B cell maturation antigen), BR3 (BLyS receptor 3, also called BAFF-R), and TACI (transmembrane activator and calcium modulator and cyclophilin ligand [CAML] interactor), all of which are expressed on B cells (Gross et al., 2000; Marsters et al., 2000; Shu and Johnson, 2000; Thompson et al., 2000; Wu et al., 2000; Yan et al., 2000a; Yu et al., 2000). BR3 and TACI are also expressed on resting and activated CD4^{+} T cells, respectively (Yan et al., 2001a). BR3 binds BLyS exclusively, while BCMA and TACI also bind to APRIL (Marsters et al., 2000; Yu et al., 2000). All three receptors for BLyS lack an intracellular death domain, suggesting a role for BLyS primarily in cell survival and/or differentiation. While an essential function for BLyS in B cell development has been established, distinct roles for each of the three receptors in BLyS signaling have not been clearly defined to date. BCMA$^{-/-}$ mice have no discernable phenotype indicating a redundant, or as yet undiscovered, role for BCMA (Xu and Lam, 2001). On the other hand, a natural strain of mice, A/WySnJ, expressing a mutant nonfunctional BR3 (Yan et al., 2001a) exhibit a block in B cell development with a severe decrease in the number of peripheral B cells. In addition, B cells with mutated BR3 receptor from A/WySnJ mice are unable to respond to soluble BLyS demonstrating a critical role for BR3 in B cell activity (Thompson et al., 2001; Yan et al., 2001a). Initial reports on TACI$^{-/-}$ mice have revealed conflicting roles for TACI in BLyS signaling. von Bulow et al. (2001) observed normal splenic architecture, B cell maturation, and T-dependent humoral responses but a greatly decreased T-independent response (von Bulow et al., 2001). These results suggested a positive role for TACI in T-independent antibody production. In contrast, Yan et al. (2001b) observed increased B cell accumulation in vivo. In addition, TACI$^{-/-}$ B cells hyperproliferated and produced increased immunoglobulins in vitro. These conflicting results have complicated the interpretation of TACI's role in BLyS signaling.

The finding that cytoplasmic tails of BR3 and TACI are neither conserved nor possess a recognizable signaling motif such as a death domain or a consensus TRAF binding sequence (Yan et al., 2001a), and that BLyS binds to both of these receptors with equivalent affinity, adds additional complexity to this system. The similarity in phenotype of mice lacking BLyS and functional BR3, coupled to the finding that B cells with mutated BR3 receptor do not respond to soluble BLyS, suggests that BLyS stimulates B cells primarily through BR3. Claudio et al. (2002) and our studies by Kayagaki et al. (2002) have shown that binding of BLyS to BR3 promotes processing of NF-κB2/p100 resulting in a survival signal that is critical for maintenance of peripheral B cells. Thus, while the physiological roles of BR3 as a crucial receptor for BLyS in B cell survival are clear, the relative role of TACI is not fully understood at present.

To determine the in vivo role for TACI, we have fully characterized the phenotype of TACI$^{-/-}$ mice in the present study. These mice develop lymphoproliferation and massive multi-organ infiltration of lymphocytes in the liver and kidney. Loss of TACI also results in development of fatal lupus-like autoimmune disease as evidenced by proteinurea and circulating α-dsDNA and α-nuclear autoantibodies, suggesting a dominant inhibi-

*Correspondence: iqbal@gene.com

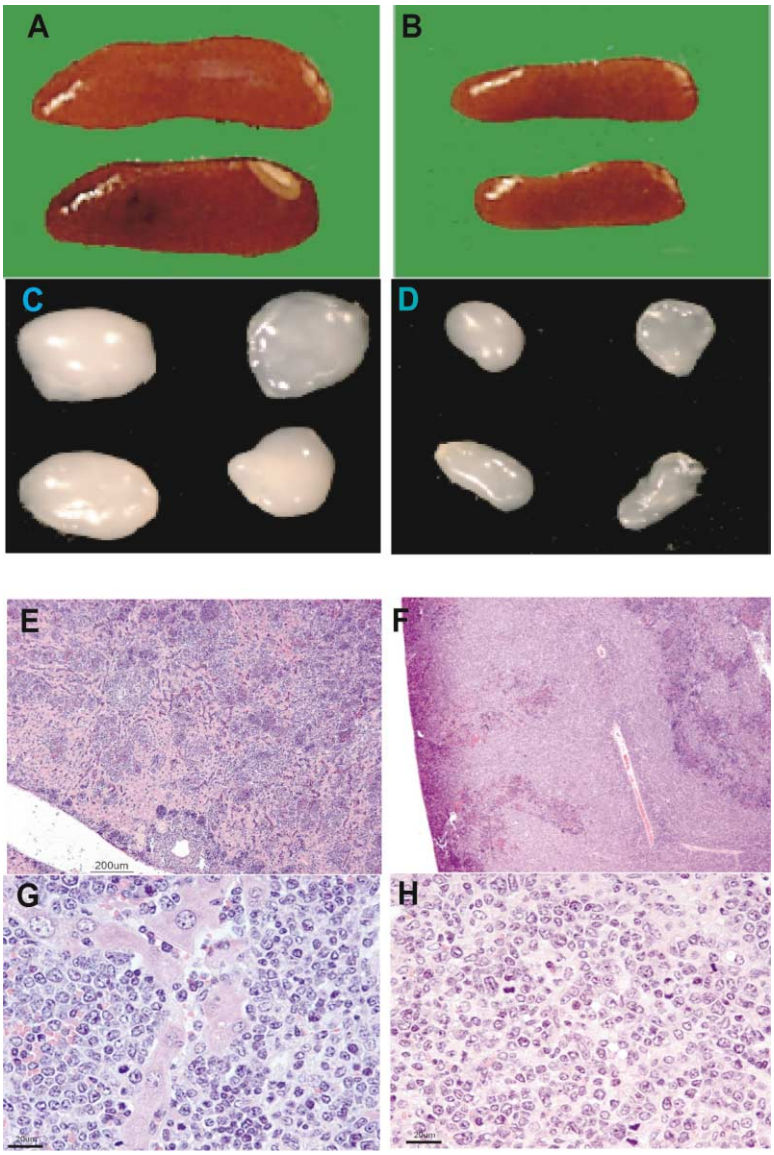

Figure 1. Lymphadenopathy, Splenomegaly, and Development of Lymphoma in TACI$^{-/-}$ Mice

Enlarged spleens and lymph nodes from TACI$^{-/-}$ mice (A and C) and wild-type control littermates (B and D) are shown. Histology of lymphoma in the spleen of TACI$^{-/-}$ mice is shown in (E) and (G). Metastasis of this lymphoma to the liver is shown in (F) and (H). (E) and (F) are under low magnification and (G) and (H) are under high magnification. 15% of TACI$^{-/-}$ mice developed lymphoma at 7 months of age. 20 mice from age-matched groups of wild-type and knockouts were analyzed ($p = 0.0465$).

tory role for TACI in maintaining B cell homeostasis. This was confirmed in vitro using both TACI agonistic antibodies and a chimeric receptor comprising the intracellular domain of TACI. Addition of antibody and specific activation of the chimeric receptor through soluble EDA.A1 ligand inhibited B cell proliferation in a dose-dependent manner. This inhibition of B cell proliferation may be due to the induction of apoptosis by the TACI intracellular domain. Thus, the dramatic phenotype of TACI$^{-/-}$ mice coupled with the in vitro studies of TACI activation demonstrates the in vivo importance of negative regulation of B lymphocyte activity by TACI.

Results

Lymphoproliferation and Multi-Organ Infiltration in TACI$^{-/-}$ Mice

To determine the role of TACI in BlyS-mediated effects in vivo, we studied mice lacking TACI. TACI$^{-/-}$ mice have enlarged spleen and lymph nodes, as shown in Figures 1A–1D. This increase in size is accompanied by an average 2- to 3-fold increase in the numbers of B cells in the spleen and lymph nodes (data not shown). Histology of enlarged spleens from TACI$^{-/-}$ mice demonstrated expansion of the red pulp areas, and that of enlarged lymph nodes demonstrated normal follicular architecture with expansion in all areas of the cortical follicles and interfollicular T cell regions (data not shown). This histology is characteristic of a lymphoproliferative disorder. TACI$^{-/-}$ mice also exhibited lymphocyte infiltration in various organs including liver and kidney (data not shown). Interestingly, TACI$^{-/-}$ mice developed low frequency lymphoma/leukemia in the spleen that metastasized to the liver (Figures 1E–1H). 15% of TACI$^{-/-}$ mice developed the lymphomas at 7–8 months of age as compared to none of the control wild-type or heterozygotes, observed up to 18 months of age. 20 mice from age-matched groups of wild-type and knockouts were analyzed. In panels (E) and (G), the white pulp in the spleen was diffusely infiltrated and ablated by a dense sheet of neoplastic round cells with pale basophilic cytosol, indistinct cell borders, and oval,

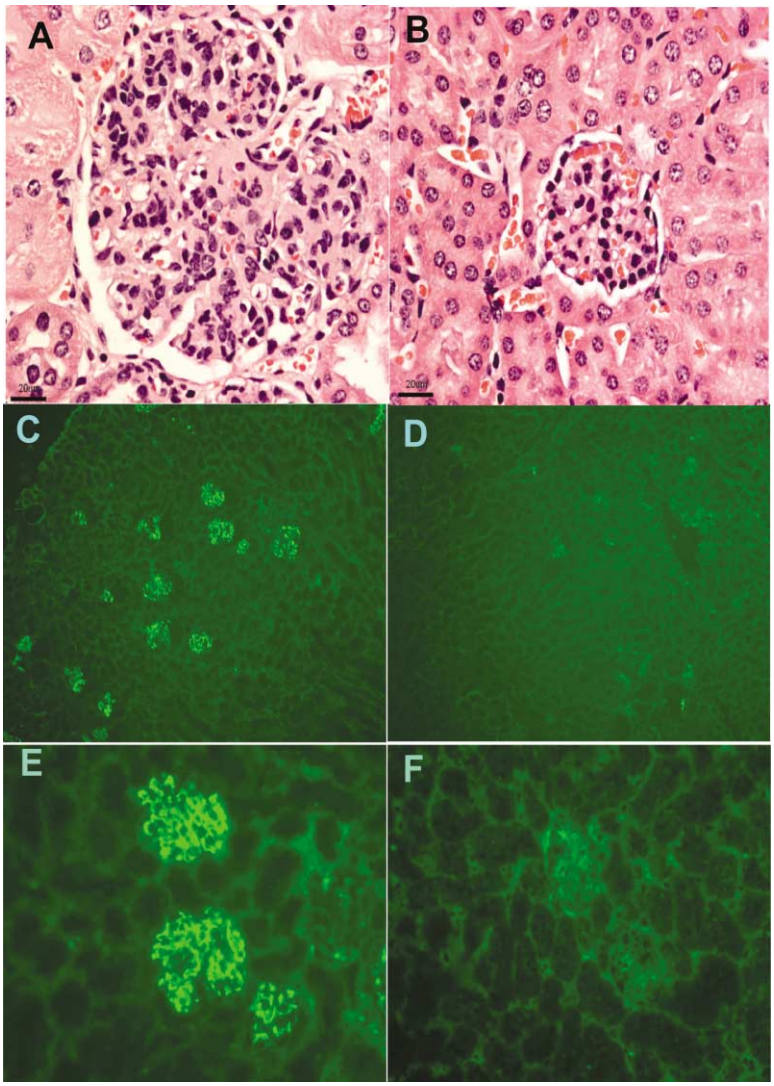

Figure 2. TACI$^{-/-}$ Mice Develop SLE-like Autoimmune Disease

Kidney sections from 7-month-old TACI$^{-/-}$ and wild-type littermates stained with haematoxylin and eosin show amyloid deposition and thickened mesangium of the glomeruli in the TACI$^{-/-}$ mice (A) and absent in the wild-type controls (B). Deposition of IgG in renal glomeruli was detected by direct immunofluorescence using frozen kidney sections from 7-month-old TACI$^{-/-}$ and wild-type littermates (C–F). IgG deposition in the kidney is shown in TACI$^{-/-}$ mice (C and E) and no such deposition was seen in wild-type controls (D and F). (C) and (D) are under low magnification and (E) and (F) are under high magnification. Ten TACI$^{-/-}$ and ten wild-type animals were evaluated.

open-faced nuclei and a high mitotic index (10–15 per high-power field). The liver was similarly infiltrated with massive numbers of malignant lymphocytes. The hepatic sinusoids contained a similar population of neoplastic round cells that formed coalescing masses that impinged upon and disrupted hepatic cords. Intravascular neoplastic round cells (lymphoma) were also present in most tissues evaluated. Immunohistochemistry with α-B220 antibody identified numerous strong staining B220^{+} cells in the liver. These lymphomas were most likely B cell lymphomas due to their staining with B220. These data suggest that in the absence of TACI, lymphocytes undergo deregulated proliferation leading to multiorgan tissue damage and lymphoma development.

TACI$^{-/-}$ Mice Show Histological Signs of Glomerulonephritis

To determine if lymphoproliferation shown in Figure 1 resulted in autoimmune disease, we analyzed TACI$^{-/-}$ mice for symptoms of systemic lupus erythematosus (SLE). TACI$^{-/-}$ mice succumbed to diffuse membranoproliferative glomerulonephritis characterized by mesangial proliferation and inflammatory cell infiltrates (Figures 2A–2B). Fibrinoid necrosis and sclerosis were prominent in affected glomeruli accompanied by occasional crescent formation. Immunofluorescence staining of the kidney revealed IgG deposits (Figures 2C–2F). The TACI$^{-/-}$ mice had significant deposition of IgG in renal glomeruli as determined by direct immunofluorescence. Nine out of ten animals evaluated had high levels of diffuse staining of glomeruli. TACI$^{-/-}$ mice had focal to diffuse global moderate membranoproliferative glomerulonephritis characterized by mesangial proliferation, inflammatory cell infiltration, and subendothelial deposits. These changes and immunoglobulin deposits were absent in age-matched wild-type control animals., suggesting that loss of TACI in vivo results in development of glomerulonephritis.

TACI$^{-/-}$ Mice Develop Autoimmune Disease with SLE-like Symptoms

The histological identification of autoimmune glomerulonephritis in TACI$^{-/-}$ mice suggested that renal function might be impaired in these mice. Renal damage was

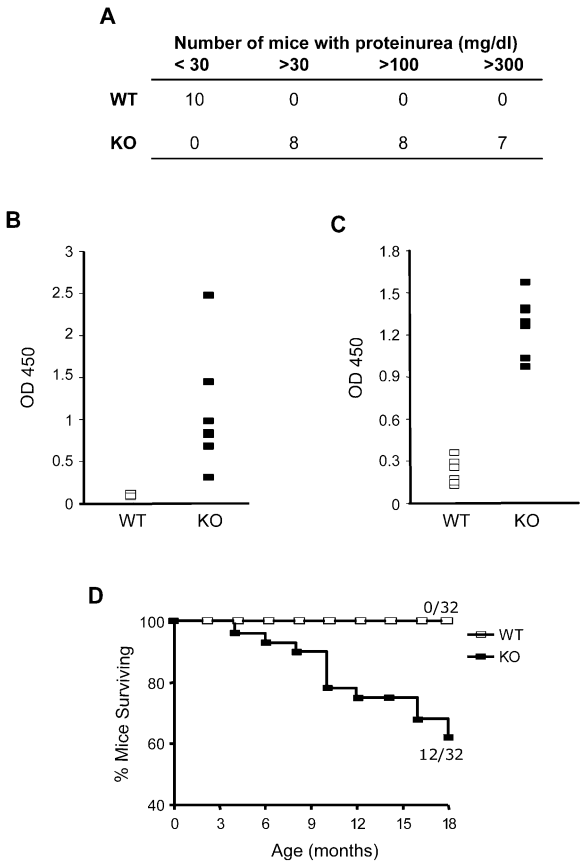

Figure 3. TACI$^{-/-}$ Mice Develop Fatal Lupus-Associated Proteinurea and Autoantibodies

(A) 7-month-old TACI$^{-/-}$ mice and wild-type littermate controls were tested for proteinurea and values are shown in (A). All 10 wild-type mice had proteinurea levels under 30 mg/dl, while seven out of eight TACI$^{-/-}$ mice displayed levels over 300 mg/dl.

(B) Levels of circulating α-dsDNA autoantibodies in the serum of 7-month-old TACI$^{-/-}$ mice and wild-type littermate controls were assayed by ELISA. OD 450 values are shown in the figure.

(C) Titers of α-nuclear antibodies in TACI$^{-/-}$ and wild-type littermates were analyzed using an ANA kit and OD 450 values are shown.

(D) Mortality statistics for TACI wild-type and knockout mice are graphed in (D) ($p = 0.0052$).

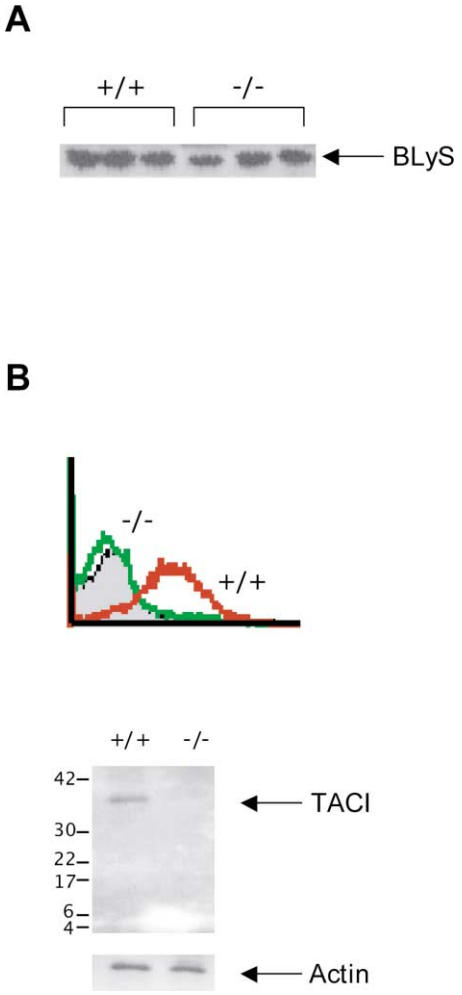

Figure 4. TACI$^{-/-}$ Mice Display Normal Levels of Circulating BLyS and Do Not Express a Truncated TACI Protein

(A) Serum levels of BLyS were analyzed in n = 3 TACI wild-type and knockout mice by Western blotting and were not elevated in TACI$^{-/-}$ mice.

(B) Levels of TACI protein were assayed by FACS (upper panel) and Western blotting (lower panel) using an antibody that recognizes the N-terminal extracellular domain of TACI. Splenocytes from n = 5 TACI wild-type and knockout littermates were gated for B220 and analyzed for TACI expression (isotype control in gray, wild-type in red, and knockout in green). Protein lysates from purified CD19^{+} splenic B cells were also analyzed by Western blotting. Expression of TACI was detected in wild-type but not knockout mice. Equal loading was confirmed by probing for β-actin.

confirmed by the presence of significant proteinurea in TACI$^{-/-}$ mice (Figure 3A). Levels of proteinurea gradually increased in TACI$^{-/-}$ mice, and by 7 months of age, while none of the wild-type littermates displayed levels of proteinurea greater than 30 mg/dl, 100% of TACI$^{-/-}$ had levels greater than 100 mg/dl and ~90% had levels over 300 mg/dl. Proteinurea was observed in both male and female knockout mice with equal frequency. Other hallmarks of lupus, such as circulating dsDNA and antinuclear autoantibodies, were also detected at high levels in TACI$^{-/-}$ mice while being absent in age-matched wild-type control littermates (Figures 3B–3C). Lymphoproliferation and autoimmune disease resulted in decreased survival of TACI knockouts when compared to wild-type littermates (Figure 3D). 12 out of 32 knockout mice (~40%) died by 18 months of age, while 100% of wild-types and heterozygotes survived. These results indicate the important requirement for TACI-mediated signals to control autoimmune diseases such as lupus mediated primarily by B cells.

Since BLyS transgenic mice also develop lupus (Gross et al., 2000; Khare et al., 2000; Mackay et al., 1999), it is possible that the observed phenotype in TACI mice is due to an indirect effect of increased circulating levels of BLyS resulting from lack of TACI. To rule out this possibility, we assayed levels of BLyS in the serum of wild-type and TACI$^{-/-}$ mice by Western blotting (Figure 4A) and ELISA (data not shown). Levels of BLyS were comparable in both wild-type and knockout mice, confirming that the observed phenotype of autoimmunity

results from a loss of TACI signaling and is not an indirect effect of increased available BLyS. The targeting strategy for generating TACI knockout mice involved the deletion of its *trans*-membrane and intracellular domains. This brings up the possibility that autoimmune disease may result from a dominant-negative effect on the other BLyS receptors, BR3 and BCMA, by a truncated protein comprising only the extracellular domain of TACI. Toward this end, levels of TACI were assayed by both FACS and Western blotting using an antibody that recognizes the N-terminal extracellular domain of TACI (Figure 4B). Splenocytes from five wild-type and TACI knockout littermates were gated for B220 and analyzed for TACI expression (upper panel). FACS analyses revealed the absence of any detectable truncated protein in TACI$^{-/-}$ B cells. Purified CD19^{+} B cells were also isolated from spleens and analyzed by Western blotting (lower panel). Expression of TACI was detected in wild-type but not knockout mice. Equal loading was confirmed by probing for β-actin. The complete absence of a truncated TACI protein in TACI$^{-/-}$ mice may be due to improper folding of the mutant protein, resulting in its inability to reach the cell surface. The above results, however, indicate that the observed phenotype is not due to a dominant-negative effect of a truncated TACI protein.

Activation of TACI Intracellular Domain Inhibits B Cell Proliferation In Vitro

A 2-fold in vitro approach was also adopted to confirm the inhibitory role for TACI in B cell signaling in vivo. First, agonistic antibodies to TACI were generated and effects of these antibodies on signaling in human primary B cells were studied. Figure 5A demonstrates binding of the three monoclonal antibodies, 6D11, 7B6, and 4C7, used in the study to 293 cells transfected with full-length human TACI. No binding of the TACI antibodies to mock-transfected 293 cells was observed (data not shown). In Figure 5B, the agonistic activity of two of the three antibodies (6D11 and 7B6) is shown, as evidenced by the activation of an NFκB-luciferase reporter in 293 cells (Yan et al., 2000a). 6D11 and 7B6 were able to activate the NF-κB reporter when compared to soluble human BLyS, which was used as a control. The third antibody 4C7 did not stimulate reporter activity and is not an agonistic antibody. The 6D11 antibody blocked binding of BLyS to TACI; however, 7B6 and 4C7 did not (ELISA, data not shown). As shown in Figure 5C, α-CD40 antibody-induced B cell proliferation was inhibited in a dose-dependent manner by the two agonistic monoclonal antibodies to TACI. All three antibodies are the same mouse isotype (IgG1), and 4C7 served as a matched isotype control antibody. The level of background B cell proliferation in the absence of any stimulus was subtracted from each of the indicated values in the graph. The observation that both 6D11 and 7B6 could stimulate NF-κB activity in 293 cells and inhibit B cell proliferation, while the nonagonistic antibody 4C7 could do neither, indicates that the observed effects on proliferation are due to an active inhibitory signal induced by TACI.

Second, we adopted a chimeric receptor approach to study potential inhibitory signals mediated through the TACI intracellular domain. Toward this goal, we molecularly engineered a chimeric receptor composed of the mouse TACI *trans* membrane and intracellular domains fused to an EDAR extracellular domain (Figure 6A). EDAR is the receptor for a TNF superfamily ligand, EDA.A1, and is not expressed in the immune system (Yan et al., 2000b). A mouse B cell line A20 (Shimonkevitz et al., 1983) was stably transfected with this chimeric receptor and expression was confirmed through FACS analyses of FLAG-tagged soluble EDA.A1 ligand binding (Figure 6B). No binding of EDA ligand was detected to control mock-transfected A20 cells (left panel), while specific binding of the α-FLAG antibody was observed to two different clones of A20.EDAR/TACI cells (middle and right panels). Control A20 cells (Figure 6C) and a clone expressing high levels of the chimeric receptor (Figure 6D) were then stimulated with LPS or a combination of α-CD40 antibody/IL4. When the chimeric receptor was activated in the presence of EDA ligand, an average of 2- to 3-fold inhibition was observed in proliferation, indicating that activation of the TACI intracellular domain could repress LPS- or α-CD40 antibody-induced proliferation. Thus, the above results from both in vitro approaches in human and mouse B cells systems clearly reinforce the inhibitory nature of TACI-mediated signals during B cell activation.

TACI Intracellular Domain Can Induce Apoptosis in Mouse A20 Cells

The ability of the chimeric receptor to suppress both LPS- and α-CD40 antibody-induced proliferation may result from its ability to induce apoptosis in these cells. To examine this possibility, survival assays using annexin-FITC were performed using control mock-transfected A20 (Figure 7A) and two separate clones of A20.EDAR/TACI cells (Figures 7B–7C). Control A20 cells in the presence of EDA.A1 ligand and stimulated with LPS or α-CD40 antibody/IL4 did not undergo apoptosis. However, activation of the TACI intracellular domain by EDA.A1 in A20 cells expressing the chimeric receptor induced apoptosis and reduced survival by 30%–40%. This induction was detected at comparable levels both in the presence and absence of activation signals such as LPS or α-CD40 antibody/IL4, suggesting that the TACI intracellular domain is able to actively induce apoptosis in these cells. Mechanisms of inhibition other than apoptosis by TACI in B cells are also being investigated.

Discussion

The in vivo role of TACI in the regulation of B cell activation is presently highly controversial and is further complicated by its ability to bind another TNF superfamily ligand, APRIL (Gross et al., 2000; Marsters et al., 2000). While APRIL$^{-/-}$ mice have not been characterized to date, data from APRIL transgenic mice (Stein et al., 2002) rule out a dominant role for this ligand in B cell activation. These results and the published data (Gross et al., 2000; Khare et al., 2000; Schiemann et al., 2001) strongly implicate BLyS as the sole ligand for TACI in the regulation of B cell function. Defining a precise physiological role for TACI in vivo has been complicated by conflicting reports from several labs. Studies by von Bulow et al.

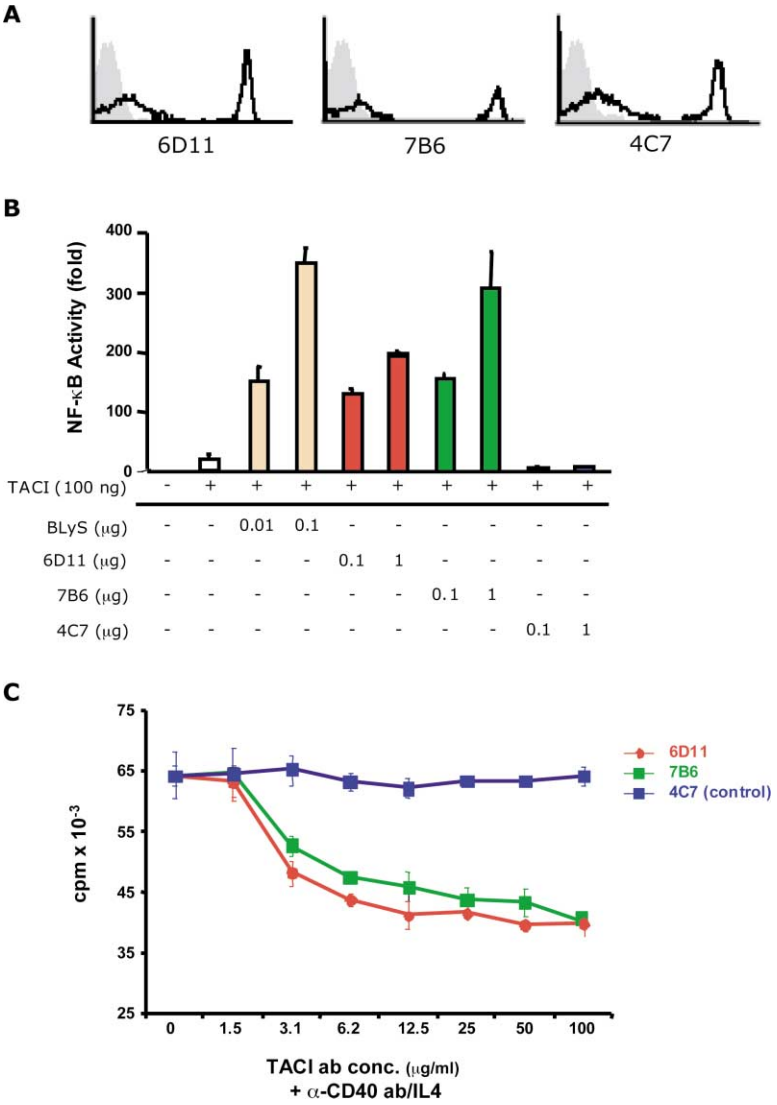

Figure 5. TACI Agonistic Antibodies Inhibit B Cell Proliferation

(A) Three monoclonal antibodies generated in mouse to human TACI (6D11, 7B6, and 4C7) bind to 293 cells transfected with 0.1 μg full-length human TACI for 24 hr and analyzed by FACS using a PE-conjugated α-mouse IgG1 secondary antibody. Isotype control is shown in gray.

(B) Human 293 cells were transfected with 0.1 μg of a full-length human TACI expression plasmid along with 1 μg of ELAM-luciferase reporter plasmid and 0.1 μg control pRL-TK plasmid. After 4 hr, indicated amounts of soluble recombinant human BLyS or TACI antibodies were added for 20 hr and reporter gene activity determined. Two out of three antibodies (6D11 and 7B6) display agonistic activity as evidenced by activation of the NFκB-luciferase reporter. Variations in transfection efficiencies were controlled for by using equal amounts of protein and an internal Renilla reporter control.

(C) 5×10^5 human B cells isolated from peripheral blood by positive selection using magnetic beads were stimulated with α-CD40 antibody (10 μg/ml) and IL-4 (100 ng/ml) and increasing concentrations of two different clones of TACI agonistic antibodies for 72 hr. [H^3] counts are plotted as a function of TACI agonistic antibody concentration. All three antibodies are the same mouse isotype (IgG1) and 4C7 served as a matched isotype control antibody. The level of background B cell proliferation in the absence of any stimulus has been subtracted from each of the indicated values in the graph. The two TACI agonistic antibodies 6D11 and 7B6 significantly inhibit B cell proliferation induced by α-CD40 antibody/IL4, while the nonagonistic antibody 4C7 does not.

(2001) using $TACI^{-/-}$ mice suggested an important positive role for TACI in thymus-independent and a redundant role in thymus-dependent B cell responses. Interestingly, they also observed increased splenic accumulation of B cells, but these cells matured normally. Yan et al. (2001b)'s reports using $TACI^{-/-}$ mice have added more complexity to this issue. In this report, B cells lacking TACI were observed to be hyperresponsive in vitro. Thus, delineating the exact nature of TACI's contribution to B and T cell function has not been performed to date. Previous studies have established an essential role for BLyS in B cell stimulation mediated primarily through BR3 (Kayagaki et al., 2002; Yan et al., 2001a). Our studies presented here represent conclusive evidence that unlike BR3, TACI is an inhibitory receptor, which regulates B cell functions in vivo. Our results reveal that loss of TACI results in lymphoproliferation, lymphoma development, and severe autoimmune nephritis with autoantibody production, leading to death of the mice. Furthermore, our in vitro studies using agonistic anti-TACI antibodies and molecular approaches utilizing a chimeric TACI receptor confirm the inhibitory properties of TACI observed in vivo, thus supporting its physiological roles.

The mechanisms by which TACI may mediate BLyS-induced inhibitory signals are presently unclear. Results from BLyS transgenic and knockout mice strongly suggest that the crucial requirement for BLyS in B cell development may be due to its ability to inhibit apoptosis and promote survival. Furthermore, our recently reported studies by Kayagaki et al. (2002) indicate that BR3 may be the sole BLyS receptor mediating these antiapoptotic effects through its ability to activate the NF-κB2 survival pathway. One possible method by which TACI is mediating its inhibitory function may be due to its ability to sequester BLyS and prevent its binding to BR3, thus inhibiting B cell survival. In this context, it is of interest to discuss interactions between BR3 and TACI signaling when B cells expressing both receptors encounter BLyS in vivo. Resting B cells express higher levels of BR3 transcripts than BCMA or TACI (D.S. and I.S.G., unpublished data) and this may serve to deliver a constant survival signal. Interestingly, upon activation with BLyS/α-IgM, TACI transcripts are selectively upreg-

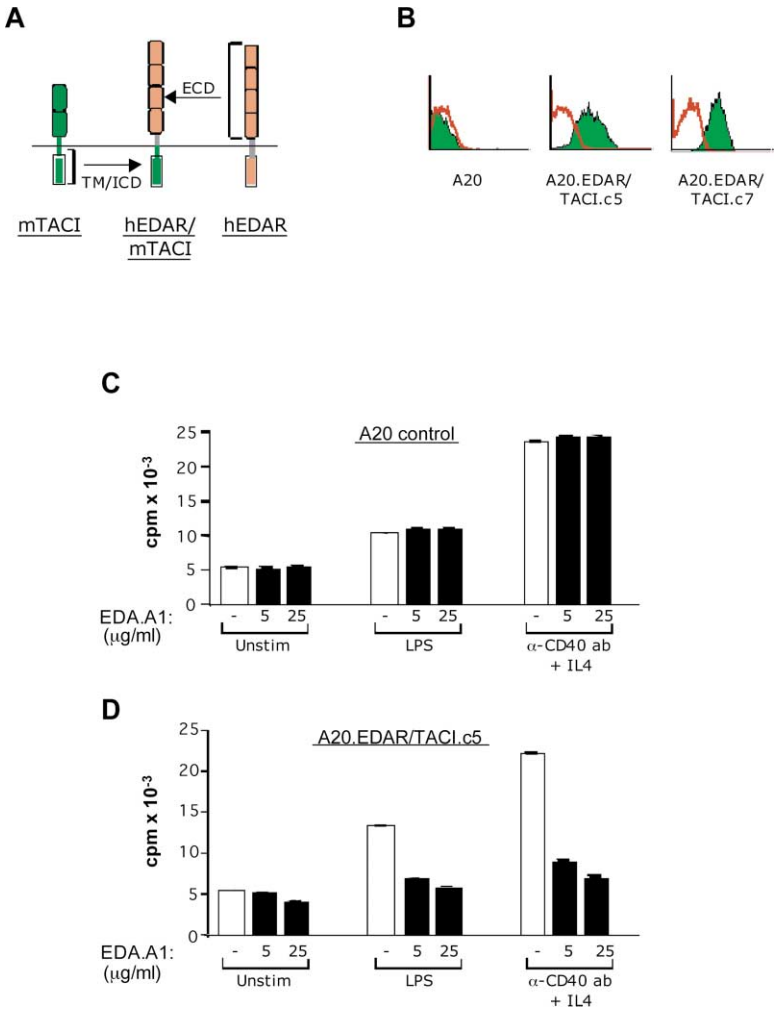

Figure 6. EDAR/TACI Chimeric Receptor Signals Inhibit Proliferation of Mouse A20 Cells

(A) Design of the EDAR/TACI chimeric receptor is shown. The fusion protein was constructed by fusing the extracellular domain of human EDAR (residues 1–183) and the transmembrane and intracellular domains of murine TACI (residues 130–249).

(B) Two different clones of murine A20 cells electroporated with pCIneo-EDAR/TACI were analyzed for FLAG-tagged EDA.A1 ligand binding using a FITC-conjugated α-FLAG antibody. Binding of ligand to control A20 cells and two different clones of A20.EDAR/TACI cells is shown. Isotype control is shown in red and binding of α-FLAG antibody in green.

(C and D) 5×10^5 control mock-transfected A20 (C) and A20.EDAR/TACI.c5 cells (D) were stimulated with LPS (0.5 mg/ml) or α-mouse CD40 antibody (5 μg/ml) and IL-4 (100 ng/ml) in the presence or absence of indicated amounts of soluble recombinant human EDA.A1 protein. Addition of soluble EDA ligand significantly inhibited LPS- and α-CD40 antibody-induced proliferation in A20 cells expressing the chimeric receptor, while having no effect on control A20 cells.

ulated to levels higher than BR3, suggesting that BLyS may selectively be able to signal through TACI upon B cell activation due to TACI receptor upregulation. TACI may thus play an inhibitory role and serve to terminate responses in the later stages of B cell activation. This is reminiscent of roles for CD28 and CTLA-4 in regulating T cell responses (Gross et al., 1992; Linsley et al., 1996) and will be further studied upon the availability of antibodies to track cell surface expression of BR3, TACI, and BCMA. Hence, TACI may function as an inhibitory receptor by out-competing BR3 for binding to BLyS or by directly antagonizing BR3-mediated signaling. These mechanisms may not be mutually exclusive and need to be investigated in future studies. At this stage, however, the former possibility cannot fully account for its observed inhibitory function in vivo based on the comparable levels of serum BLyS in wild-type and $TACI^{-/-}$ mice and results from our studies using TACI agonistic antibodies and an EDAR-TACI chimeric receptor, which indicate that activation of the intracellular domain of TACI can inhibit B cell activation.

The observed phenotype of lymphoproliferation and autoimmunity in $TACI^{-/-}$ mice suggests that TACI may be able to promote apoptosis in activated B cells, and this was observed in our studies with the TACI chimeric receptor in mouse A20 cells. Activation of the TACI intracellular domain induced apoptosis and reduced survival by 40%–50% as assayed by annexin-FITC staining. Studies of potential mechanisms of action of TACI-induced apoptosis are complicated by the absence of a canonical death domain in the TACI intracellular domain (Marsters et al., 2000; Yan et al., 2000a). However, TNF receptors such as the LT-β receptor and TNF-RII have been shown to activate apoptosis upon ligand binding despite the absence of a death domain (Browning et al., 1996; Force et al., 2000; Li et al., 2002). These mechanisms involve association with ubiquitin ligases such as c-IAP1, leading to ubiquitination and proteasomal degradation of survival molecules (Li et al., 2002), the activation of TRAF molecules such as TRAF 1, 2, 3, and 5 (Nakano et al., 1996; Rothe et al., 1995, 1994), and prolonged activation of the JNK pathway (Wallach et al., 1999). Intriguingly, TACI activation by BLyS in a B cell line has been shown to result in JNK activation (Xia et al., 2000), and in this context it is of interest to mention that TACI has been shown to associate with TRAFs 2, 5, and 6 (Xia et al., 2000), and this TRAF association may lead to the observed activation of NF-κB by TACI (Marsters et al., 2000). This paradoxical ability of TACI to induce both survival and apoptotic pathways is similar

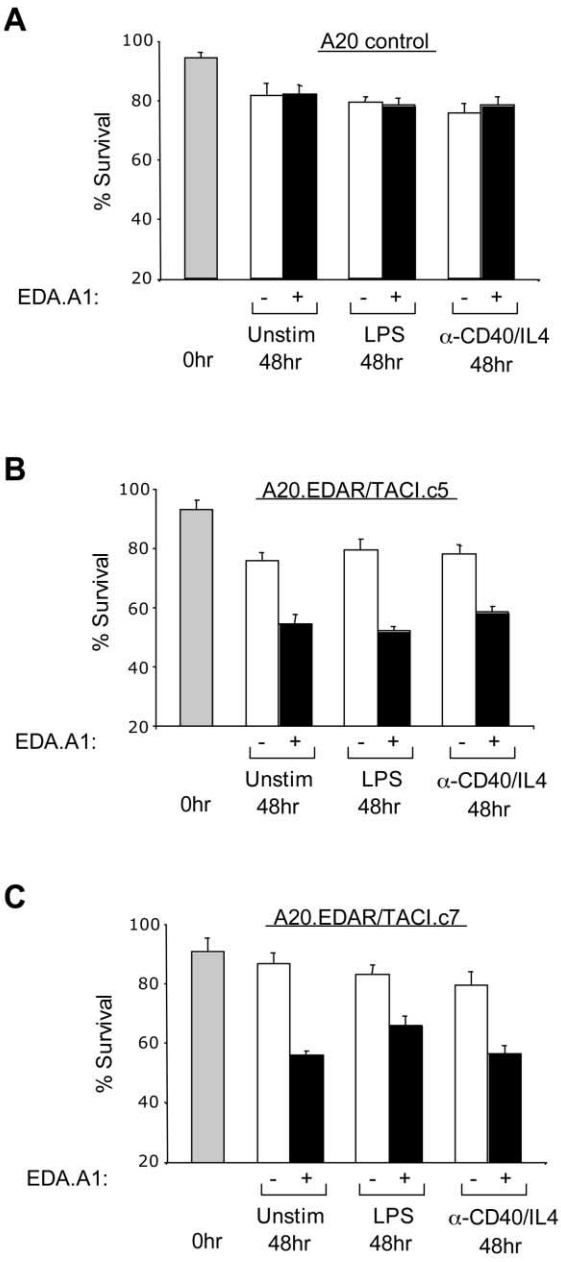

Figure 7. Activation of the TACI Intracellular Domain Induces Apoptosis in A20 Cells Expressing a Chimeric Receptor

2×10^5 control A20 (A), A20.EDAR/TACI.c5 (B), and A20.EDAR/TACI.c7 (C) were stimulated with LPS (0.5 mg/ml) or α-mouse CD40 antibody (5 μg /ml) and IL-4 (100 ng/ml) in the presence or absence of soluble recombinant human EDA.A1 protein (5 μg /ml). Cells were stained with annexin-FITC/PI at 0 and 48 hr postactivation to assay survival. Activation of the chimeric receptor by addition of soluble EDA ligand induced apoptosis in A20 cells expressing the chimeric receptor, while having no effect on control A20 cells.

to that observed for TNF-RII (Pimentel-Muinos and Seed, 1999). The activation of these dual pathways may be context dependent and also provide a possible explanation for the diminished TI responses in $TACI^{-/-}$ mice. Interestingly, B cells from A/WySnJ mice expressing a mutant nonfunctional BR3 protein have been reported to accumulate elevated levels of the proapoptotic gene *blk* (*bik*-like killer), which may be one potential reason for the decreased survival of these B cells (Amanna et al., 2001). It is not yet known if this deregulated expression of *blk* is due to a lack of BR3 signaling or deregulated TACI signaling by BLyS. Further studies are needed to identify downstream adaptor molecules that can induce apoptosis in B cells through TACI signaling. It is also possible that TACI is inhibiting B cell activation through mechanisms other than apoptosis and these are currently being investigated.

The mechanisms by which a multiple ligand-receptor system (BLyS-BR3/TACI/BCMA) can maintain homeostasis and regulate B cell function in vivo have hitherto been a puzzle. In this respect, our data provide the following strong evidence for a mechanism by which the same ligand is able to induce both positive and negative signals by binding to alternate receptors. First, our studies demonstrate the ability of a single ligand, BLyS, to act as both an activator and inhibitor of B cells, which represent a system in which this has not been shown before. An analogous system exists for T cells, where CD80/CD86-CD28/CTLA-4 ligand-receptor interactions operate to regulate function (Sharpe and Freeman, 2002). As in the T cell system, where CD28 functions as a costimulatory receptor while CTLA-4 provides an inhibitory signal upon engagement by their ligands, activation of the two receptors (BR3 and TACI) by BLyS could also result in opposing effects on B cell function. Second, considering the dominant role played by BR3 in B cell responses (Schneider et al., 2001; Thompson et al., 2001; Yan et al., 2001a), no definitive role for TACI in B cell activity has been demonstrated previously. In this respect, our studies clearly establish an essential role for BLyS-TACI interactions in regulation of B cell function in vivo. Third, our studies also demonstrate a crucial role for TACI in keeping the immune system in check by regulating lymphoproliferation and preventing spontaneous autoimmunity. The severe phenotype of $TACI^{-/-}$ mice accompanied by results from our in vitro studies demonstrates the critical requirement for TACI-mediated negative regulation of lymphocyte function and strongly supports a model for the dual regulation of B cells by BLyS, in which signals through TACI receptor inhibit B cell activity and signals through BR3 lead to stimulation of B cell survival.

While our studies clearly demonstrate a negative role for TACI in B cell homeostasis, its role in T cell function is still unclear. TACI has been shown to be expressed on T cells and studies by Huard et al. (2001) indicate a positive role for BLyS signaling in T cells. They demonstrated the ability of BLyS to induce T cell proliferation and cytokine secretion when combined with α-TCR stimulation. This result raises an intriguing question about the identity of the receptor mediating these effects. BCMA expression has not been detected on T cells to date, suggesting that it may not contribute to the observed BLyS effects on T cells. In a previous report from our lab, analyses of BR3 and TACI transcript levels revealed a differential pattern of expression in T cells (Yan et al., 2001a). BR3 was predominantly expressed on resting cells while TACI was upregulated upon activation. The ability of T cells from $TACI^{-/-}$ mice to respond to BLyS (D.S. and I.S.G., unpublished data) indicates

that BR3 may be the major positive receptor. Interestingly, when compared to wild-type cells, TACI$^{-/-}$ T cells were observed to hyperproliferate when stimulated with α-CD3 antibody and BLyS. The results strongly suggest an inhibitory role for TACI in T cell function. However, the possibility that TACI may be playing a positive role in T cell function cannot be ruled out and studies are currently underway to resolve this issue.

Experimental Procedures

Cell Culture and Transfections

The EDAR/TACI chimeric protein was constructed by fusing together PCR fragments of the extracellular domain of human EDAR (residues 1–183) and the transmembrane and intracellular domains of murine TACI (residues 130–249). The construct was confirmed by sequencing in a pGEMT vector (Promega Corporation) and cloned into pCIneo expression vector (Promega Corporation). Murine A20 cells maintained in RPMI 1640, 10% FBS, and L-glutamine were electroporated with 20 μg of pCIneo or pCIneo-EDAR/TACI plasmid. Cells were selected in 1 mg/ml G418 for 2 weeks and clones isolated by limiting dilution.

Antibody ELISAs and Proteinurea

Levels of circulating dsDNA antibodies in serum were analyzed by ELISA using plates coated with poly dA-dT/polyL-Lysine (Sigma-Aldrich) and HRP-conjugated anti-mouse IgG1 antibody (Caltag Laboratories). Titers of α-nuclear antibodies were detected by using ANA screen kit (Sigma-Aldrich, St. Louis, MO) and HRP-conjugated α-mouse IgG antibody (Caltag Laboratories). The presence of proteins in mouse urine was measured using Uristix (Ames).

Histopathology and Immunostaining

Spleens, lymph nodes, livers, and kidneys were collected and embedded in OCT and snap frozen in isopentane for cryotomy or were fixed in 10% neutral buffered formalin and embedded in paraffin. Three micron-thick sections stained with hematoxylin and eosin were examined by light microscopy. Localization of immunoglobulin in renal glomeruli was done by direct immunoflourescence on 5 μ thick frozen sections of kidneys. Sections were fixed in acetone, briefly rehydrated, blocked in 3% BSA/PBS, and incubated for 1 hr at room temperature with 5 μg/ml of Alexafluor 488-conjugated donkey anti-mouse IgG (Molecular Probes). Sections were washed with PBS, mounted with Vectashield, and observed with a fluorescent microscope. Ten TACI$^{-/-}$ and ten wild-type animals were evaluated.

Western Blotting and FACS Analyses

B cells were purified from the spleen using CD19 MACS beads according to manufacturer's instructions (Miltenyi Biotec). 1×10^7 cells were lysed in buffer (20 mM Hepes [pH 7.4], 2 mM EGTA, 50 mM -glycerophosphate, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, and 1 tablet complete protease inhibitor mixture [Roche Molecular Biochemicals]). The lysates were centrifuged at 10,000 $\times$ g for 15 min at 4°C. The supernatant was collected and used as whole cell lysate. Lysates (30 μg) or serum harvested from TACI$^{-/-}$ and wild-type mice were separated via SDS-polyacrylamide gel electrophoresis using 4%–20% Tris-glycine gels (Novex Electrophoresis) in SDS Running buffer (25 mM Tris, 0.2 M glycine, and 3.5 mM SDS), and transferred onto PVDF membrane (Invitrogen Corporation) in transfer buffer (48 mM Tris-Base, 39 mM Glycine, 0.0375%(w/v) SDS, and 20% Methanol). The membrane was incubated in blocking buffer composed of 5% skim milk in TBST (20 mM Tris [pH 7.4], 137 mM NaCl, and 0.5% Tween 20) followed by primary antibodies for BLyS (Active Motif), TACI (R&D Systems), or β-actin (Sigma). Antibody-antigen complexes were detected using a horseradish peroxidase-conjugated secondary antibody and ECL system (Amersham Pharmacia Biotech). For FACS analyses, splenocytes were incubated with biotinylated-α-TACI antibody (R&D Systems) for 15 min at 4°C followed by PE-streptavidin and FITC-conjugated amB220 (BD Pharmingen). Following another wash step, cells were analyzed by FACS using CELLQUEST software (BD BioSciences). Clones of A20 cells expressing the EDAR/TACI chimeric receptor were analyzed for FLAG-tagged EDA.A1 binding using a FITC-conjugated α-FLAG M2 antibody (Sigma-Aldrich).

Proliferation and Survival Assays

Human B cells were isolated from blood from donors using Lymphocyte Separation Medium (ICN Pharmaceuticals) followed by purification using CD19$^+$ MACS beads (Miltenyi Biotech). 5×10^5 cells were induced to proliferate using a combination of α-human CD40 antibody (10 μg/ml) (BD Pharmingen) and IL-4 (100 ng/ml) (R&D Systems) for 72 hr. Mouse A20 cells were stimulated with LPS (0.5 mg/ml) (Sigma-Aldrich) or α-mouse CD40 antibody (5 μg/ml) and IL-4 (100 ng/ml) (R&D Systems), in the presence or absence of soluble recombinant human EDA.A1 protein for 72 hr. Cells were pulsed with 1 μCi [H^3]-dT for the last 6 hr of culture and harvested. For survival assays, 2×10^5 cells were incubated with LPS (0.5 mg/ml) or α-mouse CD40 antibody (5 μg/ml) and IL-4 (100 ng/ml), in the presence or absence of soluble recombinant human EDA.A1 protein (5 μg/ml). At the indicated time points, cells in the respective cultures were stained with Annexin V-FITC/PI (Clontech Labs) and analyzed by FACS.

NF-κB Reporter Assays

Human 293 cells were transfected with 0.1 μg of a full-length human TACI expression plasmid along with 1 μg of ELAM-luciferase reporter plasmid and 0.1 μg control pRL-TK plasmid (Promega Corporation). After 4 hr, indicated amounts of soluble recombinant human BLyS or TACI antibodies were added for 20 hr and reporter gene activity was determined using a dual-luciferase reporter assay system (Promega Corporation).

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