APRIL modulates B and T cell immunity

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The TNF-like ligands APRIL and BLyS are close relatives and share the capacity to bind the receptors TACI and BCMA. BLyS has been shown to play an important role in B cell homeostasis and autoimmunity, but the biological role of APRIL remains less well defined. Analysis of T cells revealed an activation-dependent increase in APRIL mRNA expression. We therefore generated mice expressing APRIL as a transgene in T cells. These mice appeared normal and showed no signs of B cell hyperplasia. Transgenic T cells revealed a greatly enhanced survival in vitro as well as enhanced survival of staphylococcal enterotoxin B–reactive CD4⁺ T cells in vivo, which both directly correlate with elevated Bcl-2 levels. Analysis of humoral responses to T cell–dependent antigens in the transgenic mice indicated that APRIL affects only IgM but not IgG responses. In contrast, T cell–independent type 2 (TI-2) humoral response was enhanced in APRIL transgenic mice. As TACI was previously reported to be indispensable for TI-2 antibody formation, these results suggest a role for APRIL/TACI interactions in the generation of this response. Taken together, our data indicate that APRIL is involved in the induction and/or maintenance of T and B cell responses.

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Introduction

The TNF family has an important role in inducing various biological responses such as cell proliferation, differentiation, survival, and death (1). Two recently described cytokines, APRIL and BLyS (also called BAFF, THANK, TALL-1, and zTNF4), form a new subfamily of TNF-like ligands (2–7). BLyS and APRIL are synthesized as type II transmembrane proteins and proteolytically cleaved at a multibasic motif (4, 8, 9). APRIL is processed intracellularly by furin convertase prior to its secretion, thus acting solely as a secreted factor, whereas BLyS is released from the cell surface by processing of membrane-bound BLyS (4, 8, 9). Two members of the TNF receptor family, the transmembrane activator and calcium modulator cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA), have been identified as receptors that can bind both APRIL and BLyS with comparable efficacy (7, 10–13). More recently, a third receptor was identified that is unique to BLyS (BAFF-R or BR3); this receptor further enhances the complexity of this subfamily (14, 15). Expression of BCMA, TACI, and BR3 is observed on B cells, whereas TACI is also expressed on activated T cells (16).

A clue to the role of this receptor/ligand subfamily in immunity came from the observation that treatment of mice with a soluble decoy form of TACI or BCMA (TACI-Fc or BCMA-Fc) led to reduced B cell numbers and a block in the humoral response (10, 12, 13, 17, 18). These effects have been attributed to BLyS sequestration, as (a) BLyS acts as a costimulator of B cells in the presence of anti-IgM antibodies (3–5); (b) in vivo administration of a soluble form of BLyS disrupts spleen architecture due to increased B cell numbers (3); (c) mice that express BLyS as a transgene have enlarged spleens and lymph nodes and display autoimmunity due to B cell expansion as a result of increased survival of normally deleted B cells (6, 7, 19); and (d) BLyS-deficient mice have a phenotype comparable to that of TACI-Fc– or BCMA-Fc–treated mice, i.e., almost complete loss of mature B cells and a severely decreased humoral response (20, 21). This last observation in particular suggests that BlyS binding to TACI and/or BCMA is essential for B cell survival and function. Paradoxically, TACI-deficient mice show B cell expansion rather than death, whereas BCMA knockout mice have no overt phenotype (20, 22–24). This points to an unexpected role for TACI in negative regulation of B cell growth and a possibly redundant role for BCMA. Despite the B cell expansion, in at least one report the T cell–dependent humoral response appeared normal in the TACI knockout mice (22). A significant decrease is observed, however, in responses to T cell–independent type 2 (TI-2)

antigens (22, 23). These apparently contradictory findings in BLyS, TACI, and BCMA knockout mice were at least partly clarified with the identification of BR3, the third receptor for BLyS. This receptor appears to mediate most of the B cell survival signal elicited by BLyS and explains to a large extent the phenotype of the BLyS transgenic (Tg) animals.

In addition to a defect in B cell immunity, BLyS Tg mice have increased numbers of activated $CD4^+$ and $CD8^+$ T cells (7, 19). Although these effects have not been characterized to the same extent as have the changes in B cell homeostasis, they do point to a possible role for BLyS in T cell activation. In agreement, BLyS was recently reported to provide costimulation for suboptimally activated T cells (17).

The role of APRIL in immune regulation is less well defined. APRIL was originally described to stimulate growth of tumor cells in vitro and in vivo (2). Recently, reports described the potential of recombinant APRIL to act as a costimulator of primary B and T cells in vitro and to stimulate IgM production by peripheral blood B cells in vitro (18, 25). Similar to BLyS, in vivo application of APRIL results in splenomegaly due to expansion of the B cell population and an increase in the percentage of activated T cells (18), suggesting a role for APRIL in lymphoid homeostasis as well. To better characterize this function, we generated APRIL Tg mice and have determined the role of APRIL in lymphoid development and activation.

Methods

Generation and screening of lck-APRIL Tg mice. The human APRIL cDNA coding region was inserted into a targeting vector containing the lck distal promoter fused to human growth hormone (hGH) intronic and polyadenylation sequences (26). The resulting construct was injected into BALB/c × C57BL/6 embryos, and Tg founders and offspring were screened by PCR of tail DNA using oligonucleotide forward primer H19 5′-ATGGATTACAAAGACGATGACG-3′ and reverse primer H14 5′-TCACAGTTTCACA AACCCCAGG-3′. The APRIL Tg mice were backcrossed on a C57BL/6 background. Mice used in experiments had been backcrossed at least five times onto the C57BL/6 background.

RT-PCR. Naive $CD4^+$ T cells were isolated from the T cell receptor (TCR) Tg DO11.10 mice as described (27). Isolated T cells were restimulated (in the absence of irradiated splenocytes) with 5 mM of the relevant ovalbumin (OVA) peptide and in the presence of IL-12 and anti–IL-4 to generate Th1 cells, or in the presence of IL-4, anti–IL-12, and anti–IFN-γ to generate Th2 cells. Total RNA was isolated from 2×10^6 naive or activated T cells, and cDNA was prepared using a cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). cDNA was screened for expression of murine APRIL (700 bp) employing primers H195 (forward: 5′-AGCCTCATCTCCAGGCCACAT-3′) and H194 (reverse: 5′-AAACCCCAGGAATGTTCCATGCG-3′) and murine BLyS (930 bp) employing primers H198 (forward: 5′-ATGGATGAGTCTGCAAAGACCCTG-3′) and H199 (reverse: 5′-TTACAGCAGTTTTAGG GCACCAAAG-3′). As a control, cDNA was screened for GAPDH as described (28). Samples were amplified for 35 cycles (APRIL and BLyS) or 26 cycles (GAPDH).

Total RNA was isolated from splenocytes and reverse-transcribed to cDNA. Aliquots from cDNA preparations were amplified by PCR, generating the complete cDNA of human APRIL (780 bp) employing primers H14 and H19, the complete cDNA of murine APRIL (700 bp) employing primers H195 and H194, or a 445-bp fragment of actin (forward: 5′-ATCAAGATCCTGACCGAGCG-3′; reverse: 5′-TACTTGCGCTCAGGAGGAGC-3′) (29). The following conditions were used: 1 cycle at 94°C for 5 minutes, followed by 3 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, followed by another 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The products were resolved on a 1% agarose gel. To ensure that the amplified human APRIL signal was not due to contaminating genomic DNA, the entire procedure was performed in the absence of reverse transcriptase. Moreover, cDNA probes were amplified for Thy1 using primers (forward: 5′-CCATCCAGCATGAGTTCAGCC-3′; reverse: 5′-GCATCCAGGATGTGTTCTGA-3′) that amplify a 300-bp fragment of cDNA and a 700-bp fragment of genomic DNA (genomic mouse DNA was used as a positive control).

Western blot analysis. Cell lysates were prepared in NP-40 buffer (1% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF in 50 mM Tris, pH 7.4). Following quantification of protein content to ensure equal loading of the cell samples, lysates were separated electrophoretically by SDS-PAGE, then transferred to nitrocellulose. Equal loading and transfer was verified in each experiment by Ponceau S staining of the membrane (Sigma-Aldrich). For analysis of mouse sera, 1 μl of each sample was analyzed by Western blot analysis. Immunoblots were probed using 2 μg/ml of affinity-purified anti-APRIL antibody (8) and developed using the ECL system (Amersham Pharmacia Biotech).

Flow cytometric analysis. Cells were stained with appropriate antibodies and analyzed on an EPICS XL flow cytometer (Beckman Coulter, Fullerton California, USA). Freshly prepared or cultured lymphocytes were resuspended in FACS buffer (1% BSA, 0.05% sodium azide in PBS). Peripheral blood leukocytes (PBLs) were isolated by density gradient centrifugation of EDTA-treated mouse blood over Ficoll-Paque (Amersham Pharmacia Biotech). Labeled anti-mouse anti-CD4 (L3T4), -CD8 (Ly-2), -CD45R/B220 (RA3-6B2), -IgD (11-26c.2a), -IgM (R6-60.2), -CD69 (H1.2F3), -CD62L (L-selectin), -CD44 (IM7), -CD45RB (16A), -CD23 (IgE Fc receptor), -CD24 (HSA, 30F1), -CD21, -CD5, -Vβ8 (F23.1), and -Vβ6 (RR4-7) antibodies, intracellular cytokine staining kit, and hamster anti–Bcl-2/control hamster Ig kit were purchased from BD PharMingen (Heidelberg, Germany). All antibodies were used according to the manufacturer's specifications. For determination of dead cells, propidium iodide (PI; 2 μg/ml) was added to cell suspensions.

Antigen-specific antibody production in vivo. For analysis of T cell–dependent humoral responses, groups of 6- to 8-week-old APRIL Tg mice and littermates were immunized intraperitoneally with 10^7 plaque-forming unit (pfu) per animal of a recombinant form of Western Reserve strain or a modified version of attenuated Ankara-strain vaccinia virus (rMVA), which expresses the entire envelope (env) gene of HIV-1 strain IIIB (MVAenv) under viral transcriptional control (30). Fourteen days after inoculation (priming), blood was collected from the retro-orbital plexus using a heparinized capillary tube, collected in an Eppendorf tube, and incubated 1–2 hours at room temperature and then overnight at 4°C. The samples were centrifuged, and serum was obtained and stored at –80°C. After serum sample collection, mice received a second intraperitoneal immunization with 10^7 pfu per animal of MVAenv (boost), and another 14 days later, mice were bled again. For analysis of TI-2 humoral responses, mice were immunized intraperitoneally with 100 μl PBS containing 30 μg of 4-hydroxy-nitrophenacetyl–conjugated Ficoll (NP-Ficoll) (Biosearch Technologies). Serum was collected as described above.

Serum Ig and autoantibody analysis. Murine serum Ig's were quantitated by standard ELISA techniques using isotype-specific capture antibodies and specific biotin-conjugated anti-mouse isotype-specific antibodies (BD PharMingen); mouse Ig standards recommended by the manufacturer were used. Dilutions within a linear range of absorbance were chosen for each experiment. To determine antibody titers against vaccinia antigens, β-galactosidase, or 4-hydroxy-nitrophenacetyl (NP), the respective antigen was coated at 1 μg/ml on ELISA plates. Anti-dsDNA autoantibodies were detected by coating ELISA plates with 2.5 μg/ml grade I calf thymus DNA (Sigma-Aldrich). Serum dilutions within the linear range of absorbance were used for quantification. Absorbance was measured at 492 nm on a Labsystems Multiskan Plus plate reader (Thermo Labsystems, Chicago, Illinois, USA).

Cell isolation. T and B cells were purified by negative selection from lymph nodes or spleens using either B220 or Thy1.2-coupled Dynabeads (Dynal Biotech, Oslo, Norway), respectively, according to the manufacturer's instructions. Cell populations isolated by this method were at least 95% positive for B220 (B cells) or CD3 (T cells). In some experiments, $CD62L^+$ cells were sorted using a Beckman Coulter EPICS ALTRA cell sorter.

Proliferation and survival assays. Cells were plated at 5×10^4 cells per well and proliferation quantified by ^{3}H-thymidine uptake (1 μCi/200 μl) during the final 24 hours of culture. T cell proliferation was induced by anti-CD3 (145-2C11; 0.1 μg/ml) alone or in combination with anti-CD28 (37.51; 0.3 μg/ml). Survival of cultured cells was determined by PI uptake. In some experiments, survival was tested in the absence or presence of Mega-APRIL (at indicated concentrations; ALEXIS Biochemicals Corp., Basel, Switzerland). Treatment of Jurkat cells with recombinant APRIL stimulated proliferation in a dose-dependent manner, confirming the activity of the reagent (not shown). For this, cells were stained with the respective markers and analyzed by flow cytometry after incubation with 2 μg/ml of PI. BCMA-Fc and TACI-Fc were obtained from ALEXIS Biochemicals Corp.

In vivo T cell response. Staphylococcal enterotoxin B (SEB) (100 μg in 100 μl PBS; Sigma-Aldrich) was injected intraperitoneally into mice, and the response of SEB-reactive T cells was determined by analyzing the percentage of $V\beta8^+$ $CD4^+$ and $CD8^+$ T cells in blood at different time points.

Results

APRIL Tg mice. The expression pattern of APRIL on lymphoid cells remains ill-defined. Injection of recombinant APRIL nonetheless results in expansion of the B cell population and T cell activation (18), suggesting that it plays a role in the immune system. To analyze whether APRIL, like BLyS (18), is expressed on T cells, we purified TCR Tg $CD4^+$ T cells from DO11.10 mice. These T cells can be activated in vitro using an appropriate OVA peptide. Moreover, these T cells can be directed toward a Th1 or Th2 phenotype depending on the cytokines present during activation (27). Using this model system, we analyzed APRIL and BLyS expression by RT-PCR. As reported previously, BLyS expression was upregulated during T cell activation (Figure 1a) (4). Similarly, APRIL expression was detected in activated Th1 or Th2 cells, but not in naive cells (Figure 1a), pointing to a role for this molecule in T cell–dependent immunity.

To study this finding in further detail, we generated a Tg mouse strain expressing human APRIL under the control of the lck distal promoter, which directs transgene expression to mature thymocytes and peripheral T lymphocytes (Figure 1b) (26). We confirmed in ELISA assays that binding of recombinant human APRIL to mouse TACI and BCMA is comparable to its binding to the corresponding human receptors (not shown), which concurs with a recent report that human APRIL has the same binding capacities for human and mouse BCMA (31). In four independent Tg mouse lines, we detected RNA expression of the transgene (Figure 1c). Only two of these transgenes displayed detectable levels of the transgene protein in T cells (Figure 1d), but in no other types of cells tested (not shown). Moreover, sera from 8-month-old mice of the Tg 3919 line showed detectable circulating APRIL levels at concentrations of 1–5 μg/ml (Figure 1e). Tg mice were born at the expected mendelian ratio, and histological analysis at 3–12 weeks of age revealed no gross abnormalities in either of the Tg strains. In addition, semiquantitative RT-PCR analysis of RNA derived from APRIL Tg mouse spleens revealed no alterations in BLyS, TACI, or BCMA RNA expression levels (not shown). Line 3919 was used for subsequent experiments, but similar observations were made in line 3923.

T and B cell composition of lymphoid organs. As recombinant APRIL results in increased spleen weight due to B cell accumulation (18), we compared the spleens of 12 Tg mice and their corresponding non-Tg littermates at 6–12 weeks of age. No significant difference was observed in spleen weight (Figure 2a) or total number of spleen cells

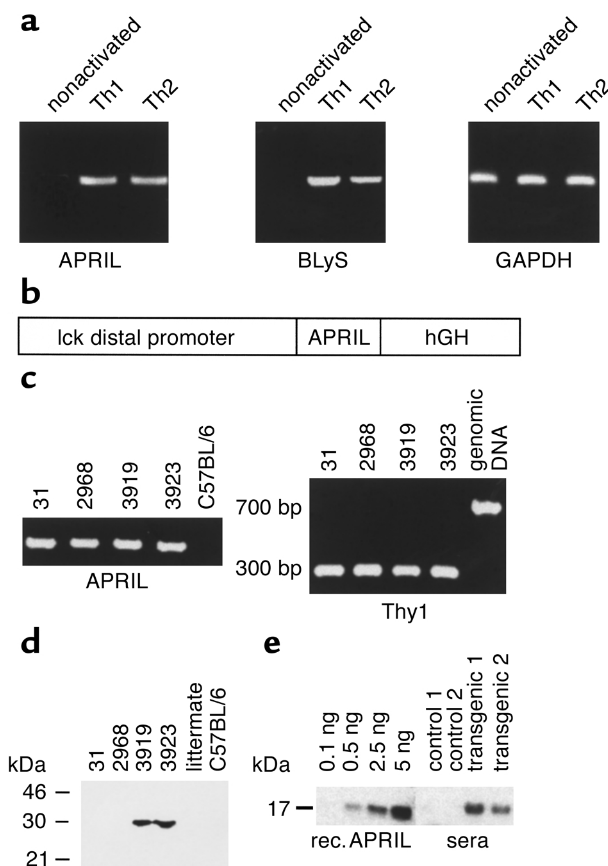

Figure 1

(**a**) APRIL expression in activated, but not in naive, T cells. Activated T cells have increased APRIL mRNA expression levels. DO11.10 spleen CD4+ T cells were isolated and activated in vitro toward a Th1 or Th2 phenotype. RNA was prepared from naive, Th1, and Th2 cells and used to analyze the expression of APRIL, BLyS, and GAPDH by RT-PCR. Generation and characterization of APRIL Tg mice. (**b**) Schematic diagram of constructs used for peripheral T cell–specific expression of the human APRIL transgene. (**c**) mRNA expression of APRIL in T cells of four different founders. RNA was prepared from purified T cells derived from spleen and lymph nodes of four Tg mice and analyzed by RT-PCR for APRIL RNA expression levels. To ensure that the amplified human APRIL signal was not due to contaminating genomic DNA, cDNA probes were amplified for Thy1 using primers that amplify a 300-bp fragment of cDNA and a 700-bp fragment of genomic DNA (genomic mouse DNA was used as control). (**d**) Protein expression of APRIL in Tg mouse T cells. Cell lysates were prepared from purified T cells derived from spleen and lymph nodes of four Tg mice, a control littermate, and a C57BL/6 mouse and analyzed in Western blot using anti-human APRIL antibodies (8). (**e**) Secreted APRIL circulates in serum of APRIL Tg mice. Sera (1 μl) of control and Tg mice were resolved under nonreducing conditions and immunoblots developed with anti-human APRIL antibodies. Recombinant (Rec.) APRIL protein (0.1–5 ng) added to control serum was used as standard.

(Figure 2b), and immunohistological analysis of APRIL Tg mouse spleen architecture showed no apparent abnormalities (not shown). Furthermore, no alteration was observed in spleen weight or total cell number in older Tg mice tested (age 4–9 months) (not shown). We next analyzed the B and T cell composition in Tg mouse secondary lymphoid organs by FACS analysis. No variation was observed in the percentage of B220+ B cells in spleen, mesenteric lymph nodes, or Peyer's patches (Figure 2c). In contrast, a small increase in the percentage of B220+ B cells was observed in peripheral lymph nodes in all Tg mice tested (n = 10) (Figure 2c). The percentages of both CD4+ and CD8+ T cells were proportionally decreased (Figure 2d), resulting in a significant decrease in absolute T cell numbers in Tg mouse peripheral lymph nodes (Figure 2e). Further analysis of T cells revealed a striking increase in CD62L– CD4+ and CD8+ T cells in secondary lymphoid organs, of which only a small percentage showed CD44 upregulation (Figure 3). Whereas a fivefold increase has been observed in CD62L– CD44^{high} effector T cells in BLyS Tg mice (19), APRIL Tg mice revealed an accumulation of CD62L– CD44^{low} T cells, which do not correspond to classically activated T cells. In agreement, there was no reproducible upregulation of CD25 or downregulation of CD45RB in the T cells in the mice analyzed (not shown).

Ectopic APRIL expression prolongs T cell survival in vivo and in vitro, correlating with increased Bcl-2 levels. Recombinant APRIL stimulates B and T cell proliferation in vitro (18). In agreement, purified T cells from APRIL Tg mice showed increased thymidine incorporation following a 2-day stimulation with anti-CD3 in combination with anti-CD28, as well as after stimulation with anti-CD3 alone (Figure 4a). The increased in vitro proliferation of Tg T cells correlated with increased IL-2 production in CD8+ T cells after in vitro stimulation (Table 1).

To determine whether the increased in vitro T cell proliferation observed can also be detected in vivo, we analyzed T cell activation using injection of SEB, which interacts predominantly with Vβ8+ T cells (32). Treatment of mice with SEB leads to systemic activation of all Vβ8+ T cells. SEB injection leads to an initial increase in the percentage of both Vβ8+ CD4+ and Vβ8+ CD8+ T cells. This initial proliferation is followed by a deletion phase, with clear decreases in Vβ8+ T cell numbers, which eventually reach levels lower than those found before SEB treatment. The decrease in Vβ8+ CD4+ cells is due to apoptosis of SEB-responsive cells (32, 33); Figure 4b shows a representative experiment tracking the percentage of Vβ8+ T cells in PBLs from APRIL Tg mice and littermates following SEB administration. Both Vβ8+ CD4+ and Vβ8+ CD8+ T cells of APRIL Tg mice show expansion similar to that of corresponding non-Tg littermates at day 3, indicating that APRIL does not affect the expansion phase of the T cell response in vivo. In contrast, a significantly delayed deletion of Vβ8+ CD4+ T cells was observed in comparison with SEB-reactive cells of littermates; this was especially evident when the percentage of Vβ8+ CD4+ T cells was compared 7 days after SEB injection. Despite the clear delay in deletion of CD4+ T cells, no significant difference was observed in the CD8 response (Table 2). This lack of effect on the CD8+ T cell response was confirmed by analyzing the CD8 response induced after infection with adenovirus. Using this approach, we

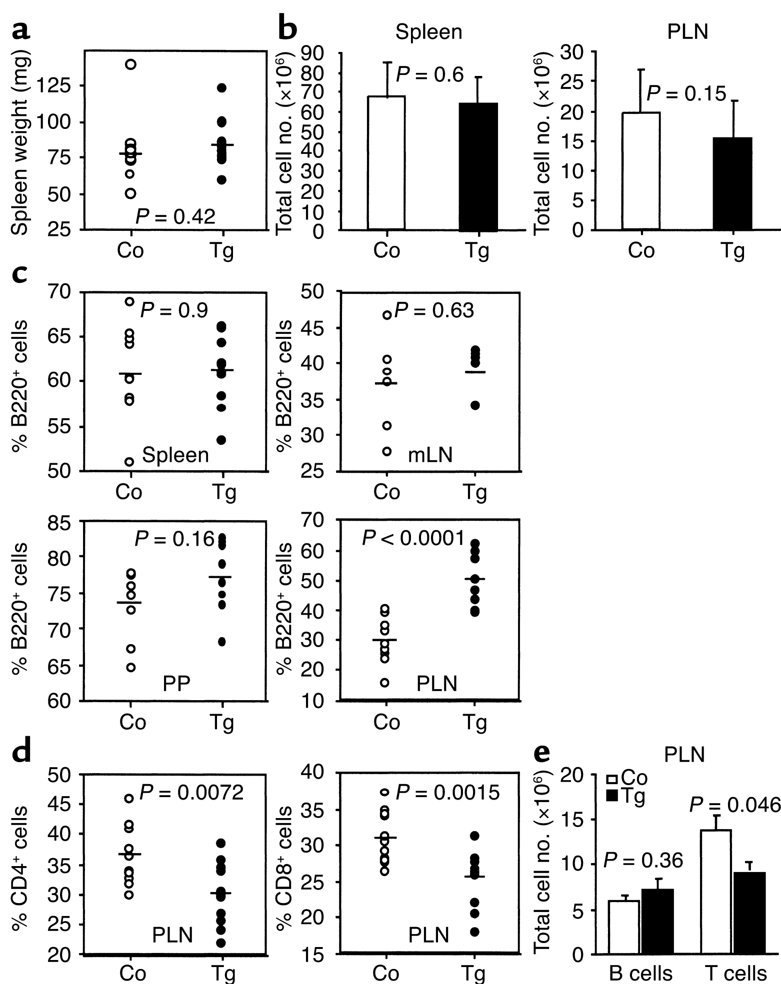

Figure 2
B and T cell homeostasis in APRIL Tg mice. (**a**) Unaltered spleen weight in APRIL Tg mice. Spleen weight of mice was measured at the age of 6–12 weeks (n = 12). (**b**) Unaltered splenocyte and peripheral lymph node cell number in APRIL Tg mice. Splenocyte number was determined in 6- to 12-week-old mice (n = 11). Data represent the mean ± SD. (**c**) Evaluation of B cell percentage (B220⁺ cells) in spleen, mesenteric lymph nodes (MLN), Peyer's patches (PP), and peripheral lymph nodes (PLN) (n = 10). (**d**) Percentage of $CD4^+$ and $CD8^+$ T cell populations in peripheral lymph nodes of APRIL Tg mice (n = 10; 6–12 weeks old). (**e**) Comparison of absolute B and T cell numbers in peripheral lymph nodes (n = 10). Data are the mean ± SM. The statistical significance of the data was determined using ANOVA. $P > 0.05$ is considered insignificant, $P < 0.05$ significant, $P < 0.01$ very significant, and $P < 0.0001$ extremely significant. Co, control.

observed no effect of ectopic APRIL expression in the $CD8^+$ T cell response to adenovirus (not shown).

To analyze this delayed T cell deletion in further detail, we tested whether ectopic APRIL-expressing T cells have an increased survival capacity in vitro. To this end, purified T cells were cultured for 3 days under various conditions, and survival was determined by measuring PI uptake. Both $CD4^+$ and $CD8^+$ T cells isolated from Tg mice showed significantly increased survival compared with those of littermates (Figure 4, c and d). This difference became evident only when T cells received suboptimal or no stimulus. Increased survival of purified T cells also became evident by quantification of live and dead cell gates by forward/side scatter (Table 3). Increased survival of APRIL Tg T cells was not blocked by BCMA-Fc or TACI-Fc (Figure 4f). In addition, the increased survival of nonactivated T cells could not be mimicked by the addition of recombinant APRIL to T cell cultures (Figure 4e). Taken together, this suggests that the presence of APRIL in in vitro cultures is not sufficient to increase survival of unstimulated T cells, but that Tg T cells receive this survival signal in vivo.

The augmented survival of B cells observed in BLyS Tg mice has been associated with increased Bcl-2 expression levels (19). Bcl-2 expression is especially effective when

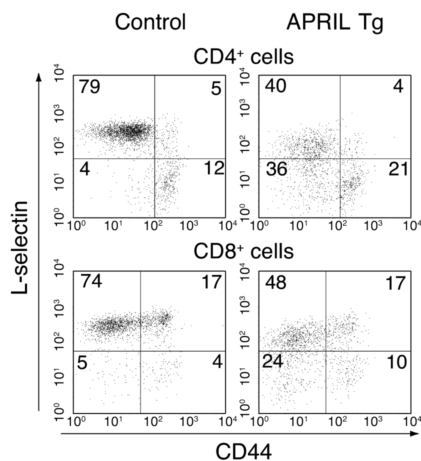

Figure 3
Decreased CD62L (L-selectin) expression in splenic T cells is not accompanied by CD44 upregulation. CD62L versus CD44 expression is shown gated on CD4 and CD8 populations. Downregulation of CD62L was seen in T cells of blood and secondary lymphoid organs; it was already apparent in Tg mouse spleen at 2 weeks of age and was maintained throughout their lives (not shown).

Table 1
Cytokine production of in vitro activated Tg T cells

	IL-2			IFN-γ		
CD4	Mean	SD	*P* value	Mean	SD	*P* value
Control	17.3	2.8	0.275	7.9	0.6	0.5307
Tg	25.9	3.4		6.6	3.1	
CD8	Mean	SD	*P* value	Mean	SD	*P* value
Control	16.9	1.4	0.0115	27.7	2.6	0.942
Tg	26.5	0.4		26.6	3.1	

T cells were cultured for 3 days in the presence of anti-CD3 and anti-CD28 and subsequently analyzed for cytokine production by intracellular FACS staining. The statistical significance of the data was determined using ANOVA.

cells are deprived of survival factors (34, 35). Moreover, Bcl-2 has been shown to delay the deletion of SEB-primed $CD4^+$ T cells (33). We therefore determined Bcl-2 expression in the APRIL Tg mice and control littermates by FACS analysis. Ex vivo $CD4^+$ and $CD8^+$ T cells, but not B cells, displayed a twofold increase in Bcl-2 levels in Tg mice compared with non-Tg littermates, suggesting that elevated Bcl-2 levels may be responsible for the prolonged survival of these cells (Figure 5a).

The increased proliferation, survival, and Bcl-2 expression may be due to the presence of the relatively large $CD62L^-$ T cell fraction in the Tg animals, which might represent activated T cells. To exclude this possibility, we analyzed $CD62L^+$ T cells, which represent the naive T cell fraction. Like unsorted T cell populations, Tg mouse $CD62L^+$ T cells showed increased T cell proliferation following anti-CD3/anti-CD28 activation, as well as prolonged survival in 3-day cultures (Figure 5, c and d). In addition, an increase in Bcl-2 expression was detected on sorted Tg mouse $CD62L^+$ T cells compared with those of littermates (Figure 5b). This indicates that the survival and proliferation effects described are not due to the increase in $CD62L^-$ T cells in the Tg mice but are a direct result of APRIL expression on naive T cells.

APRIL Tg mice display normal B cell development in spleen. Only mature B cells can recirculate and enter lymphoid follicles of spleen and lymph nodes. Two spleen B cell populations, termed type 1 (T1) and type 2 (T2) transitional B cells, were identified as precursors for mature B cells (36). BLyS preferentially supports the survival of T2 B cells (37). To analyze whether Tg expression of APRIL had a similar effect on the B cell compartment, we determined the composition in the spleen. T1 and T2 transitional B cells and marginal zone B cells can be distinguished on the basis of IgM, CD21, and CD23 expression (36). The T1 and T2 transitional B cell subpopulations are both IgM^{bright}, whereas only T2 cells are $CD21^{bright}$ $CD23^+$, which also distinguishes them from $CD21^{bright}$ $CD23^-$ marginal zone B cells (36). Triple staining of spleen B cells from mice Tg for APRIL showed no significant difference in the percentage of T1, T2, mature, or marginal B cells (Figure 6). T1 B cells are the precursors of T2 and mature B cells (36), and T1 B cells are already detectable in 1-week-old mice, whereas T2 and mature B cells only become clearly detectable in mice at about 3 weeks of age (36). To exclude that APRIL influences the kinetics of B cell development, we followed T1 and T2 B cell formation in mice at various ages; we found that T1 and T2 B cell development is comparable between APRIL Tg mice and littermates (Figure 6). Notably, B cells in peripheral lymph nodes of APRIL Tg mice correspond to mature (IgM^+, $CD23^+$, $CD21^{bright}$, and HSA^+) B cells (not shown). The increased constitutive IgM levels in sera of APRIL Tg mice suggested an alteration in the B1 cell population (38). B1 cells are characterized by B220 and IgM expression, but by a lack of CD23 expression; CD5 expression analysis allows a further subdivision into B1a ($CD5^+$) and B1b ($CD5^-$) subpopulations. We thus determined the percentages of these cells in the peritoneum. As shown in Table 4, no significant differences were found upon analysis of APRIL Tg T cells compared with those of littermates.

Ectopic APRIL expression moderately alters the T cell–dependent humoral immune response. APRIL stimulates IgM production by peripheral blood B cells in vitro (25). We therefore compared serum IgM concentrations between APRIL Tg mice and littermates. IgM serum concentrations of Tg mice showed an approximately twofold increase, whereas IgG levels were comparable between Tg mice and littermates (Figure 7, a and b). The twofold difference in IgM serum levels between Tg mice and littermates persisted in older Tg mice (at age 4–9 months; not shown). No increase was found in anti-DNA antibodies in Tg mouse serum (Figure 7c). As ectopic APRIL expression resulted in increased serum IgM levels, we tested whether the humoral response is also modulated in APRIL Tg mice. Mice were challenged with a modified version of

Table 2
P values for data in Figure 4b

P values	$V\beta8^+$ $CD4^+$	$V\beta8^+$ $CD8^+$
Day 0	0.0913	0.1814
Day 3	0.8303	0.2358
Day 7	0.0135	0.2089
Day 10	0.0121	0.0509
Day 13	0.0134	0.5435

Two-tailed *P* values comparing the respective percentages of $V\beta8^+$ cells of Tg and control mice. The statistical significance of the data was determined using ANOVA.

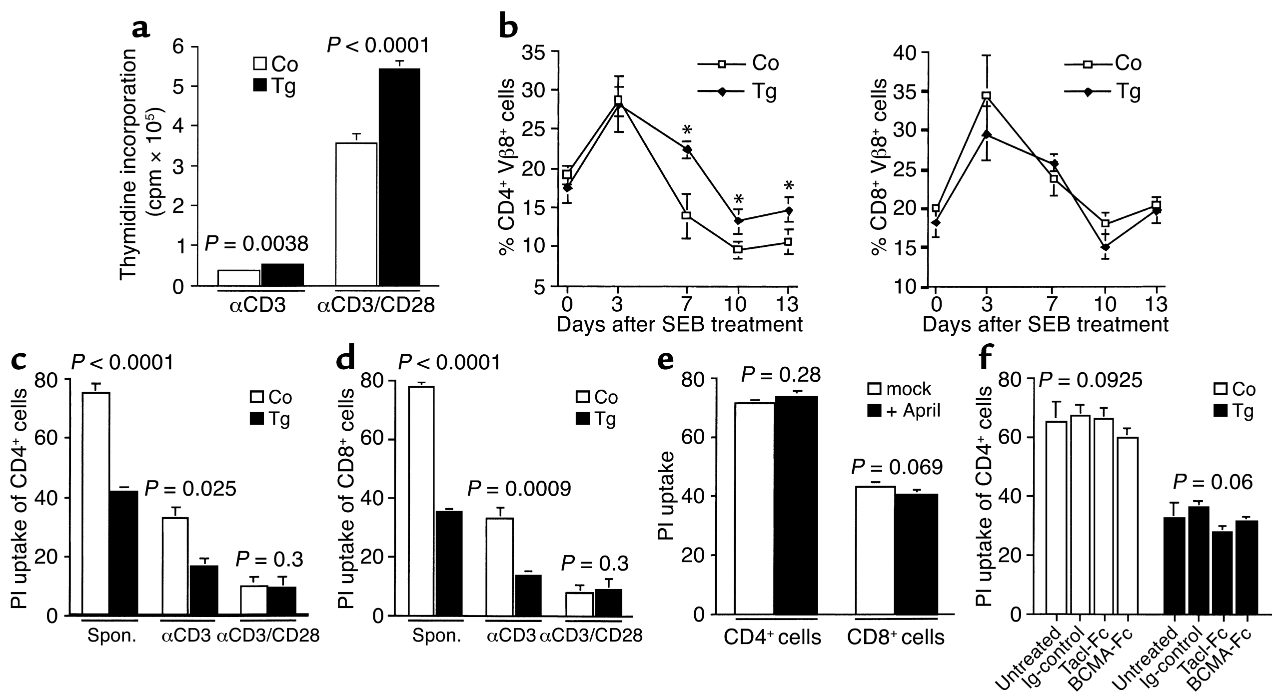

Figure 4
Increased proliferation of T cells and survival of T cells from APRIL Tg mice in vitro. (**a**) Increased proliferation of Tg T cells after 2 days' culturing with anti-CD3 alone, or anti-CD3 in combination with anti-CD28. (**b**) T cell response in APRIL Tg mice in vivo: Delayed deletion of superantigen-responsive Vβ8+ CD4+ cells in APRIL Tg mice. PBLs were stained for CD4, CD8, and Vβ8 on the days indicated (n = 4). This is one representative experiment of four performed. ANOVA confirmed that the differences for CD4+ cells at days 7, 10, and 13 are statistically significant (Table 2). *$P < 0.05$. The Vβ6+ subpopulation of CD4+ and CD8+ cells was unresponsive at all time points analyzed (not shown). (**c** and **d**) Survival of purified T cells cultured for 3 days alone, with anti-CD3, or with anti-CD3 plus anti-CD28. Cells were stained for CD4 (**c**) or CD8 (**d**) and PI as described in Methods. (**e**) Purified T cells of C57BL/6 mice were cultured for 2 days without stimulation, alone or in the presence of 5 μg/ml recombinant APRIL, and PI-stained. (**f**) Purified CD4+ T cells were cultured for 3 days without stimulation, alone or in the presence of 50 μg/ml of TACI-Fc, BMCA-Fc, or control Ig. Similar results were obtained for CD8+ T cells. All data in **a** and **c**–**f** are the mean ± SD of triplicate determinations of a representative experiment of at least three performed. The statistical significance of the data was determined using ANOVA.

Ankara-strain vaccinia virus (MVA) expressing β-galactosidase (gal), a T-dependent antigen (30, 39). Antibody (IgM and IgG) levels against virus and antigen were measured 14 days after virus inoculation. Serum samples of APRIL Tg mice showed increased levels (approximately twofold) of IgM anti-virus antibodies compared with serum samples of control mice, while IgG induction was similar in these mice (Figure 7d). The IgG2a/IgG1 ratio revealed a Th1 response in both Tg and control mice (not shown). A second immunization (boost) with MVAgal virus resulted in a moderate but nonsignificant increase in IgG in the Tg compared with that of non-Tg littermates (Figure 7d). Similar data were obtained for the antibody response to the recombinant antigen (not shown).

We also analyzed the humoral response in APRIL Tg mice to a wild-type strain of vaccinia virus (a recombinant form of the Western Reserve strain), which showed a pattern similar to that observed for MVA, i.e., a twofold increase in antigen-specific IgM production within 15 days of immunization (not shown).

Elevated TI-2 humoral response in APRIL Tg mice. Polyvalent antigens such as pneumococcal polysaccharides and Ficoll-conjugated antigens are so-called T cell–independent type 2 (TI-2) antigens, as they were originally described to induce T cell–independent immune responses (40). TACI-deficient mice revealed depressed responsiveness to TI-2 antigens, manifesting the importance of that receptor for developing TI-2 responses (22). APRIL is a known TACI ligand; we therefore evaluated the role of APRIL in the generation of the TI-2 humoral response. Immunization of APRIL Tg mice with a model TI-2 antigen, NP-Ficoll, resulted in an increase in IgG response compared with that of non-Tg littermates (Figure 8a). In addition, the IgM response was significantly elevated on

Table 3
Survival of cultured T cells determined by forward scatter and side scatter

	T cells	FSC-low	SD	*P* value
Spontaneous death	Control	77.6	1.7	0.0009
	Tg	35.0	1.2	
Anti-CD3 stimulation	Control	33.1	3.4	0.0009
	Tg	14.0	1.5	

Purified T cells were cultured as described for Figure 4, c and d. Forward scatter (FSC) versus side scatter plots were used to determine the percentage of cells in the FSC-low region, which corresponds to dead cells. The statistical significance of the data was determined using ANOVA.

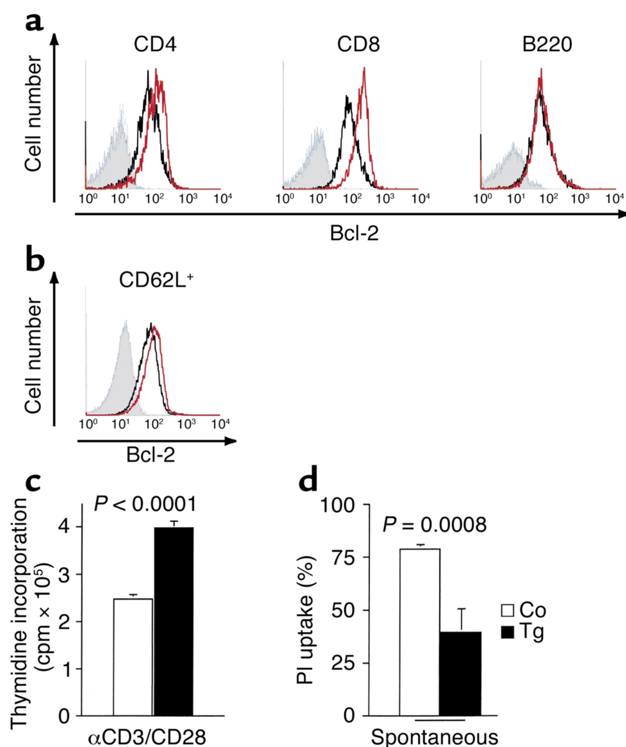

Figure 5
T cells of Tg mice have increased Bcl-2 levels. (**a**) Increased Bcl-2 expression in CD4+ and CD8+ T cells, but not B cells of APRIL Tg mice. Ex vivo CD4+ and CD8+ T cells and B220+ B cells from peripheral lymph nodes were analyzed for Bcl-2 expression as described in Methods. Staining in cells of control littermates is shown in black (gray shadow for isotype control, no shadow for Bcl-2 staining); blue shows isotype control, and red shows Bcl-2 in cells of Tg's. One representative staining of four is shown. The average increase in Bcl-2 mean fluorescence intensity was 1.5 ± 0.19–fold in Tg CD4 cells (mean ± SD, $n = 4$), 1.8 ± 0.4–fold in CD8 cells, and 1.1 ± 0.05–fold in B220+ cells. (**b**) Increased Bcl-2 expression in sorted CD62L+ T cells of Tg mice. Staining in cells of control littermates is shown in black (gray shadow for isotype control, no shadow for Bcl-2 staining); blue shows isotype control, and red shows Bcl-2 in cells of Tg's. One representative staining of three is shown. (**c**) T cell proliferation of sorted CD62L+ T cells after 2 days' culture with anti-CD3 in combination with anti-CD28. (**d**) Survival of sorted CD62L+ T cells cultured for 3 days without stimulation. Data in **c** and **d** are the mean ± SD of triplicate determinations of a representative experiment of at least three performed. The statistical significance of the data was determined using ANOVA.

day 8 (Figure 8a). The increased IgG response is particularly evident for IgG1, IgG2a, and IgG2b, as they remained significantly elevated even into day 14 after TNP-Ficoll treatment (Figure 8b). IgG3 levels were also significantly increased at day 8, but we observed a leveling at later time points (Figure 8c).

Discussion

The TNF-related ligands APRIL and BLyS and their cognate receptors BCMA and TACI form a two-ligand/two-receptor system implicated in B and T cell stimulation. APRIL and BLyS are both expressed in hematopoietic cells, and the functional differences between APRIL and BlyS within this two-ligand/two-receptor system have remained largely undefined to date. Analysis of APRIL Tg mice revealed that APRIL acts as an in vitro survival factor for T cells and affects both the humoral and the T cell response in vivo.

T cell survival and proliferation in vitro. T cells derived from APRIL Tg mice show significantly increased proliferation as measured by thymidine incorporation, which became especially evident after activation with anti-CD3/anti-CD28. This concurs with reports proposing a role for APRIL as an in vitro T cell stimulator (18, 41); we also observed that activated T cells divide more rapidly upon the addition of recombinant APRIL (not shown). The elevated production of IL-2 in in vitro activated Tg T cells probably contributes to their increased proliferation. The proliferative effect may be mediated by TACI, an APRIL receptor, whose cell surface expression is upregulated in activated T cells (42); alternatively, nonactivated T cells may express a new, yet-undefined APRIL receptor. Supporting the existence of an additional APRIL receptor is the fact that the Jurkat human leukemia T cell line, which is highly susceptible to APRIL stimulation, does not contain detectable levels of TACI or BCMA mRNA (42, 43) (M. Hahne, unpublished data). It has also recently been reported that fibroblast and epithelial cell lines express a new, still-unidentified APRIL receptor (44).

In addition to the effect on proliferation, we observed a highly significant increase in in vitro survival of ectopically APRIL-expressing T cells under conditions in which cells are deprived of survival factors and receive no or suboptimal activation signals. T cell survival coincides with a twofold increase in Bcl-2 levels in these cells. This would explain the protection observed against apoptosis induced by growth factor deprivation, as

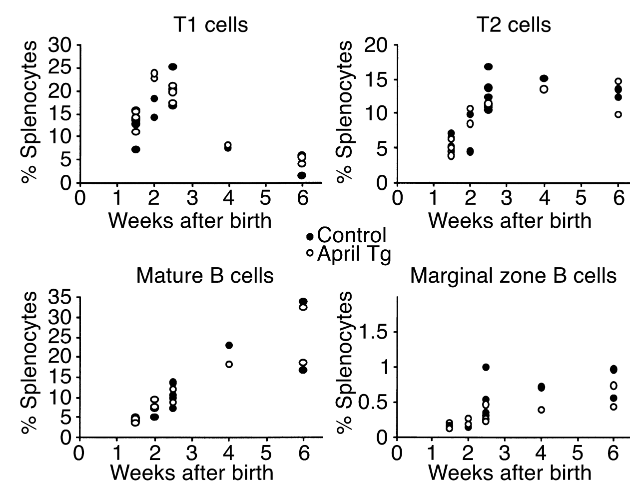

Figure 6
Ectopic APRIL expression does not influence B cell development in spleen. Splenocytes were analyzed for their content of T1 (IgM^{bright} CD21$^-$ CD23$^-$), T2 (IgM^{bright} CD21^{bright} CD23$^+$), mature (IgM$^+$ CD21^{bright} CD23$^+$), and marginal zone (IgM^{bright} CD21^{bright} CD23$^-$) B cells ($n = 20$).

Table 4
No altered percentages of peritoneal B cell populations in APRIL Tg's

	B2		B1a		B1b	
	Co	Tg	Co	Tg	Co	Tg
Mean	10.5%	13.2%	7%	6.2%	5.3%	7.2%
SD	2.5	3.8	1.8	1.3	1.5	2.0
P value	0.2609		0.4666		0.1570	

FACS analysis of peritoneal exudate B cells of 6-week-old mice (*n* = 5). B220$^+$ cells were classified into B2 (CD23$^+$) and B1 (CD23$^-$) B cells. B1 cells were further separated into B1a (CD5$^+$) and B1b (CD5$^-$) cells.

Bcl-2 Tg T cells display comparable survival in such assays (34). Nevertheless, we cannot exclude the implication of other survival-supporting molecules in APRIL Tg T cells. In contrast to the effect of APRIL on proliferation, this survival signal cannot be blocked by the addition of TACI-Fc or BCMA-Fc in vitro. It is therefore likely that the survival signal is already in place prior to isolation of the cells. Indeed, we observed that the addition of recombinant APRIL failed to upregulate Bcl-2 and to protect T cells from dying in vitro (not shown), suggesting either that APRIL-induced survival is an indirect effect of in vivo APRIL expression and not directly mediated by an APRIL receptor on the T cell, or that an APRIL receptor–mediated survival signal synergizes with other factors present in vivo but not in vitro.

T cell homeostasis in APRIL Tg animals. Despite increased survival of APRIL Tg T cells in vitro, we observe no conspicuous difference in total T cell numbers in these mice. This correlates with previous observations that Bcl-2 Tg T cells survive in vitro but do not display perturbed T cell homeostasis (34). Nevertheless, we also detected increased in vitro T cell proliferation, which is not mirrored by an increase in T cell numbers or by a difference in the in vivo growth kinetics of SEB-activated T cells. Whether this reflects a difference in signal requirements between in vitro and in vivo proliferation, similar to that reported for IL-2–induced T cell proliferation (45), is presently unclear.

Two interesting differences observed in the Tg T cell pool are the reduction in T cells in peripheral lymph nodes and an increase in the number of CD62L$^-$ T cells, the latter of which was already apparent at 2 weeks of age. These events are likely to be connected, as CD62L participates in lymphocyte recruitment from the blood into lymph nodes (46). It has been reported that CD62L expression is not essential for lymphocyte homing to Peyer's patches, mesenteric lymph nodes, or spleen but is required for homing to peripheral lymph nodes (47), which are the only secondary lymphoid organ that displays a decreased T cell count in the APRIL Tg mice. It is thus possible that the decreased percentage of T cells in peripheral lymph nodes of APRIL Tg mice is the consequence of diminished T cell homing capacity due to their decreased CD62L expression.

We do not observe complete T cell activation in APRIL Tg mice, whereas full T cell activation was previously reported for mice that had received five consecutive injections of recombinant APRIL (18). This is probably due to the levels attained using these different approaches, also reflected by the differential effects on the B cell compartment. Moreover, injection of BLyS into mice or ectopic BLyS expression results in an increased percentage of activated T cells (3, 19). Whereas ectopic BLyS expression results in an increase in CD62L$^-$CD44^{high} effector T cells (19), we observed a marked accumulation of CD62L$^-$ CD44^{low} T cells in APRIL Tg mice. We thus conclude that CD62L$^-$ T cell accumulation in APRIL Tg mice is not linked to increased Tg T cell activation. Moreover, the increased

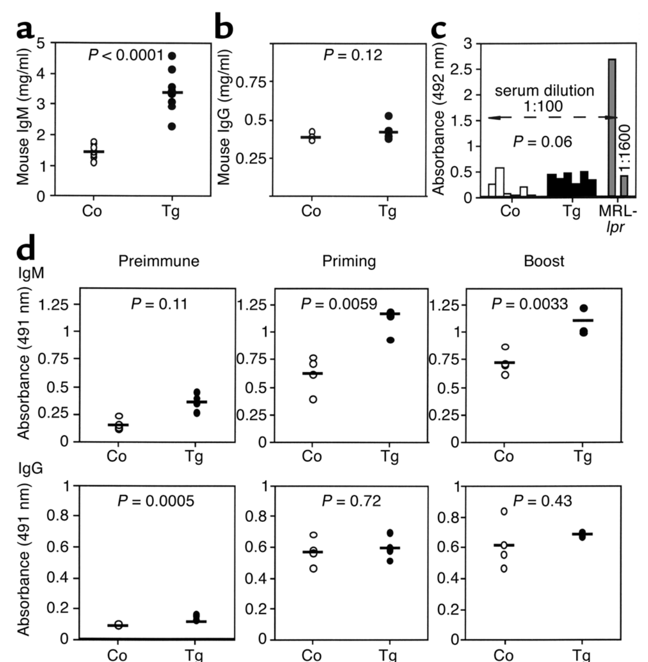

Figure 7
Moderate alteration of serum Ig levels and T cell–dependent humoral responses in APRIL Tg mice. Comparison of IgM (**a**) and IgG (**b**) levels in APRIL Tg mice (*n* = 10 in **a**; *n* = 6 in **b**). (**c**) Anti-DNA autoantibodies were not elevated in APRIL Tg mice. For comparison, pooled sera of five MRL-*lpr* mice were used at the same dilution (1:100) as those of APRIL Tg mice and littermates, and at a 1:1,600 dilution to obtain a comparable OD value. (**d**) T cell–dependent humoral response to a modified version of attenuated Ankara-strain vaccinia virus (rMVA) in APRIL Tg mice (*n* = 4). Serum IgM and IgG levels from 8-week-old mice before immunization (preimmune), 15 days after immunization (priming), and 15 days after second immunization (boost). Statistical significance was determined using ANOVA.

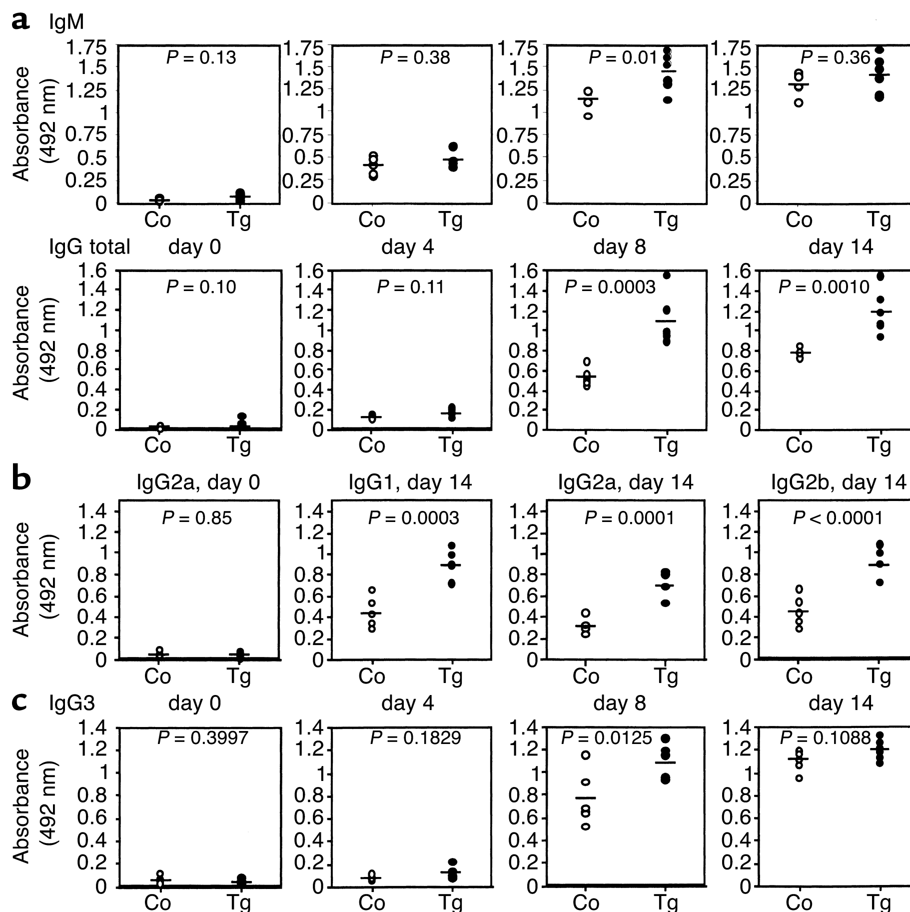

Figure 8

TI-2 humoral response in APRIL Tg mice. Groups (n = 6) of APRIL Tg mice and littermates were immunized with 30 μg NP-Ficoll, a TI-2 antigen, and serum Ig levels were determined by ELISA. (**a**) IgM and total IgG response to NP-Ficoll. (**b**) Distribution of IgG1, IgG2a, and IgG2b isotypes. For comparison, IgG2a levels of preimmune sera are shown; similar values were obtained for IgG1 and IgG2b levels in preimmune sera. (**c**) IgG3 response to NP-Ficoll. The statistical significance of the data was determined using ANOVA.

in vitro survival and proliferation capacity of Tg T cells is not linked to the increased CD62L$^-$ T cell numbers, as these features are also detected when sorted CD62L$^+$ Tg T cells are analyzed.

It has been suggested that APRIL is involved in in vitro T cell proliferation, as anti-APRIL antibodies prevent primary activation in vitro (M. Lenardo, personal communication), although this does not exclude a role for BLyS, which was recently shown to provide in vitro costimulation as well (26). Again, these in vitro observations remain to be evaluated in vivo, and the receptor through which these effects are mediated remains unknown, as no defect in T cell–dependent immunity in TACI$^{-/-}$ mice has been reported (22).

Ectopic APRIL expression attenuates the deletion kinetics of SEB-reactive CD4$^+$ T cells. We found that APRIL attenuated deletion of stimulated CD4$^+$ T cells after SEB treatment. Two independently regulated deletion pathways have been described that regulate the deletion phase of SEB-activated CD4$^+$ T cells (48). One mechanism is due to survival factor deprivation, which can be blocked by Bcl-2, whereas the other pathway is mediated by Fas-induced apoptosis (48). The retarded deletion phase indeed reflects previous observations in Bcl-2 Tg mice, confirming the importance of this molecule in the survival of APRIL Tg T cells. In contrast, deletion of SEB-reactive CD8$^+$ T cells is regulated by perforin (49), and this decline is not prevented by constitutive expression of Bcl-2 (50), even though in vitro survival is observed. These data are therefore consistent with APRIL-induced Bcl-2 expression. Similarly, adenovirus-specific CD8$^+$ T cell responses are not altered in APRIL Tg mice.

Differential APRIL and BLyS function in B cell development. Tg animals that overexpress BLyS have increased numbers of mature B cells, resulting in enlarged spleens, Peyer's patches, and lymph nodes (6, 7, 19). We detected no increase in size or weight of secondary lymphoid organs in these mice, nor was B cell accumulation observed in spleen, mesenteric lymph nodes, or Peyer's patches, although an increased percentage of mature B cells was detected in peripheral lymph nodes. This increase was not reflected in absolute cell numbers, however, but was due to a decrease in T cells in the lymph nodes. A possible explanation for this difference may be that the BLyS expression levels in BLyS Tg mice, in which the transgene is under the control of an antitrypsin or

actin promoter, are much higher than those in APRIL Tg mice (6, 19). The observation that in vivo administration of relatively large amounts of recombinant APRIL leads to B cell accumulation in the spleen, resulting in increased spleen weight (18), supports this idea. Exposure to large amounts of APRIL protein may not be physiologically relevant, however, and may induce signals in addition to those that we observed with ectopic APRIL expression. An alternative and, in our view, more likely explanation for the difference between APRIL and BLyS Tg mice is that APRIL and BLyS serve different functions within the immune system. This is supported by the fact that the development of most peripheral B cell populations is severely affected in BLyS-deficient mice, indicating that APRIL cannot compensate for the missing B cell survival signals (20, 21). The recent identification of a specific receptor for BLyS (BAFF-R or BR3) on B cells supports this conclusion. Disruption of this receptor in A/WySnJ mice results in a lack of peripheral B cells. This, combined with the surprising observation that TACI-deficient mice actually accumulate B cells (22, 23), whereas deletion of the BCMA gene has no effect on lymphocyte homeostasis (20, 24), indicates that the in vivo survival signals elicited by BLyS are mediated via this novel receptor. Our results support this conclusion, as ectopic expression of APRIL, with its potential to bind to BCMA or TACI but not BR3, does not affect B cell development and homeostasis.

APRIL plays a regulatory role in humoral responses. Although ectopic APRIL expression did not alter either B2 or B1 B cell numbers, we observed a significant (about twofold) increase in serum IgM levels in APRIL Tg mice, while serum IgG levels were unaffected. $TACI^{-/-}$ mice were shown in one report to have the opposite phenotype, i.e., decreased serum IgM but not IgG concentrations (22). This, combined with the observation that APRIL can promote IgM secretion in vitro (18), suggests that APRIL/TACI interactions may have a role in this secretion. Significantly elevated (up to tenfold) serum IgM and IgG concentrations were also found for BLyS Tg mice (6, 7, 19); furthermore, these mice show greatly increased autoantibody production, even at the age of 2–4 months (6, 7, 19). These differences between ectopic APRIL and BLyS expression can be attributed to the capacity of BLyS not only to bind to TACI and promote IgM secretion, but also to bind to BR3 and promote massive expansion of the B cell compartment. This B cell hyperplasia results in increased serum Ig concentrations, autoantibody production, and B cell responses. Under the conditions of our study, APRIL does not promote B cell hyperplasia and thus does not induce these responses.

In addition to an increase in serum IgM levels, we observed an elevation of T cell–dependent humoral responses in APRIL Tg mice. This increase in antiviral B cell responses is only observed for IgM, whereas IgGs are induced similarly in Tg mice and littermates. It thus appears that APRIL promotes B cell secretion of IgM but is incapable of inducing a class switch in a T cell–dependent response, and of promoting IgG secretion.

Unlike the effect on the T cell–dependent humoral response, APRIL Tg mice had a significantly increased IgG response to a TI-2 antigen at all time points tested. An increased IgM response was also seen 7 days after immunization but equalized later after immunization. Marginal zone B cells play an important role in responding to TI-2 antigens (38, 51). Marginal zone macrophages retain TI-2 antigens and are thus suggested to participate in the generation of TI-2 responses (52). As APRIL is expressed in monocyte/macrophages (53), this raises the possibility that endogenous APRIL secreted by macrophages within the marginal zone mediates a stimulatory signal to B cells during a TI-2 humoral response. APRIL Tg mice displayed, nonetheless, no alterations in marginal zone B cells, as is also the case for $TACI^{-/-}$ mice (22).

$TACI^{-/-}$ mice are reported to have a block in the TI-2 humoral response (22, 23), indicating a crucial role for TACI in this response. TACI is expressed on B cells; based on our observations, we suggest that the APRIL/TACI interaction participates in TI-2 humoral response as well. $TACI^{-/-}$ mice fail to develop IgM or IgG responses after challenge with TI-2 antigens. As ectopic APRIL expression results in elevated IgM and IgG levels during TI-2 responses, it is likely that TACI receives this stimulatory signal from APRIL. Whether BLyS contributes to TACI signaling is not clear. Sequestering BLyS by BR3-Fc injection is reported to affect TI-2 IgG responses, although IgM responses were not examined (15); this suggests a role for BLyS in this response. This role may nonetheless be exerted through BR3, since A/WySnJ mice, which lack functional BR3, develop a normal IgM response to the TI-2 antigen NP-Ficoll, while IgG responses are severely affected (54). Taken together, the data appear to indicate that the combined action of BLyS and APRIL through BR3 and TACI is essential in the maintenance of, and/or class switching in, the response against TI-2 antigens.

In conclusion, our data provide evidence that APRIL signals survival of T cells, and that this affects the deletion phase of a $CD4^+$ T cell response in vivo. In addition to an effect on the T cell response, APRIL mice display an increase in T cell–dependent IgM responses and TI-2 IgM and IgG responses. As BLyS plays a critical role in B cell responses, BLyS-blocking reagents are presently discussed to treat autoimmunity in humans (55). Nevertheless, further experiments are needed to elucidate whether APRIL and BLyS signals are redundant or are elicited under distinct circumstances. In contrast, the effect of APRIL on T cell survival is unique. Whether this survival signal is also crucial for the T cell response is a question that must await the generation of APRIL knockout mice. Our data nonetheless show that APRIL is involved in both T and B cell responses.

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