

APRIL facilitates viral-induced erythroleukemia but is dispensable for T cell immunity and lymphomagenesis

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Abstract: The TNF family member, a proliferation-inducing ligand (APRIL), has been suggested to act as a costimulatory molecule in T cell responses. However, studies addressing this role in vivo are largely lacking. Here, we evaluated the effects of APRIL on physiological T cell responses in vivo. Although receptors for APRIL are expressed on a subset of T cells, neither TCR transgenic (Tg) T cell responses nor endogenous TCR responses were affected by Tg APRIL expression in vivo. Moreover, APRIL did not significantly enhance the induction of T cell lymphomas upon Moloney murine leukemia virus (MLV) infection. This clearly contrasts current belief and indicates that APRIL does not serve a major role in T cell immunity or lymphomagenesis. However, we did observe a strong increase in erythroleukemia formation after MLV inoculation of APRIL Tg mice. Strikingly, this erythroleukemia-facilitating property of APRIL was confirmed using the erythroleukemogenic Friend-MLV. Erythroleukemia in APRIL Tg mice was characterized by low hematocrits and grossly enlarged spleens with an increased percentage of erythroid precursors. Altogether, these results unveil new proerythroleukemogenic properties of APRIL. *J. Leukoc. Biol.* 84: 000–000; 2008.

Key Words: T lymphocyte · TACI · Moloney-murine leukemia virus · Friend-murine leukemia virus

INTRODUCTION

A proliferation-inducing ligand (APRIL) and B cell-activating factor belonging to the TNF family (BAFF) are two related members of the TNF ligand superfamily, which have been implicated in B cell regulation and malignancy. Both ligands are expressed by a variety of cell types, predominantly of hematopoietic origin, such as monocytes, macrophages, dendritic cells (DC), and T cells, and their expression profile widely overlaps [1–4]. APRIL binds to transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA), both members of the TNFR family. BAFF can also interact with the latter two

TNFRs but also binds its own unique TNFR, the BAFF receptor (BAFF-R; or BR3). A third receptor for APRIL is thought to exist as well, as cell lines that do not express TACI and BCMA can still respond to APRIL [1, 5]. Recently, it has been shown that APRIL can interact with heparan sulfate proteoglycans (HSPGs), but whether these structures represent the unidentified third receptor remains to be clarified [6, 7].

The expression levels of the receptors for APRIL and BAFF on B cells vary and are dependent on the maturation and activation state of the B cell. BCMA is solely expressed on B cells and appears activation-dependent [8–10]. TACI is reportedly expressed on B and T cells, but its expression on T cells remains controversial. TACI was first described to be expressed on activated human T cells [11, 12], but a separate study rather reported decreased TACI mRNA expression upon activation of human T cells [13]. A detailed analysis with several independent anti-mouse and anti-human TACI mAb failed to detect TACI on mouse- and human-activated T cells [8]. Nevertheless, TACI was shown to be expressed on a subset of synovial CD3⁺ T cells from patients with rheumatoid synovitis [11], suggesting that TACI may be present in a subset of T cells.

BAFF has been shown to increase anti-CD3-mediated activation of mouse and human T cells in vitro [8, 14]. In addition, it has been shown that blocking BAFF produced by T cells themselves and/or DC, using decoy Ig-fusion receptors, prevents this effect [8, 15, 16]. This BAFF costimulatory effect was shown to be dependent on BAFF-R, as T cells expressing a defective BAFF-R did not respond to BAFF-induced costimulation, and T cells derived from TACI-deficient mice responded normally [8].

The role of APRIL in T cell biology is less well understood. In vitro evidence for a role of APRIL in T cells comes from a study where bacterial-produced murine APRIL was shown to costimulate anti-CD3-induced T cell activation [17] and an-

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other report in which anti-APRIL antibodies were suggested to prevent T cell stimulation in vitro [18]. More evidence for a role of APRIL in T cell biology comes from analysis of APRIL transgenic (Tg) mice, which express human APRIL in mature thymocytes and peripheral T lymphocytes. T cells derived from these mice show increased proliferation and survival ex vivo [19]. This survival is accompanied by increased Bcl-2 levels in APRIL Tg T cells. However, addition of TACI-Fc or BCMA-Fc does not abrogate the in vitro survival effect. Combined, these data have led to the idea that APRIL plays a role in T cell immunity, but there are currently no reports that have carefully analyzed the role of APRIL in vivo. APRIL Tg mice have been reported to show a small decline in absolute numbers of T cells in peripheral lymph nodes and increased numbers of CD62 ligand (CD62L)⁻ T cells, suggesting a difference in homing. Moreover, in vivo activation of these APRIL Tg T cells using Staphylococcal Enterotoxin B (SEB) as a polyclonal stimulus revealed no differences in the T cell response, except for an enhanced survival of SEB-reactive CD4⁺ T cells in vivo correlating with elevated Bcl-2 levels in T cells [19]. T cell responses in the two strains of APRIL-deficient mice have not been addressed carefully either. The first strain was analyzed for T cell activation after keyhole limpet hemocyanin (KLH) immunization but without apparent effect [20]. However, the second strain showed increased percentages of effector/memory T cells, although T cell proliferation in vitro was normal [21].

Besides its potential immune regulatory role, APRIL has been shown to sustain the survival of malignant B cells. This is observed in aging APRIL Tg mice, which develop a B1 B cell malignancy reminiscent of B cell chronic lymphocytic leukemia [22], and in patients with B cell chronic lymphocytic leukemia [23–25], non-Hodgkin lymphoma [23, 26, 27], and multiple myeloma [23, 28–30]. The role of APRIL in patients with T cell leukemia has been poorly studied, except for a report mentioning an association with Sézary syndrome [31]. However, APRIL enhances the proliferation of the T cell leukemic cell line Jurkat [1], suggesting that APRIL could enhance the growth of T cell leukemias as well. Combined, the current data therefore point to a role of APRIL in T cell immunity and lymphomagenesis, but in vivo data are largely lacking.

We therefore set out to gain a better insight in the potential role of APRIL in T cell biology. APRIL Tg mice on a TCR Tg background or on a normal T cell background responded normally to physiological T cell antigens in vitro and in vivo. In addition, retrovirally induced T cell lymphoma development is hardly affected by APRIL expression, whereas a significant effect of APRIL in the formation of erythroleukemia was observed.

MATERIALS AND METHODS

Mice

Six- to 8-week-old C57BL/6 Pep3b CD45.1⁺ wild-type mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for receptor expression analysis. For OT-1 and OT-2 experiments, heterozygous APRIL Tg mice on a C57BL/6 background [19] were crossed with heterozygous OT-1 and homozygous OT-2 mice (Jackson Laboratories), respectively, and the offspring with the desired genotype was used as donors to assess the in vitro and in vivo expansion of

APRIL Tg and littermate TCR Tg T cells. C57BL/6 and C57BL/6 Pep3b CD45.1⁺ wild-type animals (Jackson Laboratories) were used as recipients in these experiments. For Friend-murine leukemia virus (MLV) infections, APRIL Tg mice were first backcrossed 10 rounds on the BALB/c background, a mouse strain that is more susceptible to Friend-MLV-induced leukemia [32]. All experiments were performed in accordance with the animal ethical committees of the Academic Medical Center (AMC; The Netherlands) and the Institut de Génétique Moléculaire de Montpellier (IGMM; France).

Flow cytometry

Expression of BCMA and TACI on CD4⁺ and CD8⁺ T lymphocytes was determined after blocking FcRs with 2.4G2 hybridoma supernatant using the directly labeled anti-mouse BCMA mAb (ATTO647N-conjugated Vicky-2 from Alexis Biochemicals, San Diego, CA, USA) and anti-mouse TACI antibody (PE-conjugated 8F10), anti-mouse CD4 (FITC-conjugated RM4-5), and anti-mouse CD8 α [FITC- or allophycocyanin (APC)-conjugated 53-6.7; all from BD Biosciences, San Diego, CA, USA]. Rat IgG2a anti-mouse forkhead box p3-APC (clone FJK-16, eBiosciences, San Diego, CA, USA) and rat IgG2a PE (BD Biosciences) were used as isotype controls. Propidium iodide was used to exclude death cells from these analyses. Similar results were found using monoclonal anti-TACI PE clone 166010 and anti-BCMA FITC clone 161616 (both from R&D Systems Inc., Minneapolis, MN, USA). B-1 and B-2 B cells in the peritoneal cavity exudate were revealed with anti-mouse IgD (FITC-conjugated 11-26c.2a), anti-mouse CD43 (biotin-conjugated S7), and Streptavidin-PerCP (all from BD Biosciences). To improve the discrimination of cells of interest in the clonal expansion experiments, anti mouse-CD4 (APC-conjugated RM4-5) and anti-mouse CD8 α (APC-conjugated 53-6.7) were used together with anti-mouse CD45.2 (biotin-conjugated 104) and Streptavidin-APC (all from BD Biosciences) where appropriate. In the OVA/cholera toxin (CT) immunization experiments, OVA-specific MHC-I tetramers [a kind gift of Ton Schumacher, Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands] were used to determine the number of OVA-specific CD8⁺ T cells. The antibody Ter-119 (BD Biosciences; APC-conjugated TER-119) was used to stain erythroid lineage cells according to the manufacturer's instructions and as described previously [33].

OT-1 and OT-2 experiments

OT-1 and OT-1 APRIL Tg cells were purified from spleens and lymph nodes of the respective mouse strains by performing a B cell depletion with nylon wool and goat anti-mouse IgG beads (both from Polysciences, Inc., Warrington, PA, USA). Purified OT-1 or OT-1 APRIL Tg cells were resuspended at a concentration of 10⁷ cells/ml in 0.1% BSA in PBS and labeled with 5 μ M CFSE for 10 min at 37°C (Molecular Probes, Eugene, OR, USA), and the labeling was quenched with ice-cold PBS. The cells were subsequently washed with medium and activated for 4 h on a monolayer of adherent fibroblast APC expressing K^b-OVA (257–264; SHINFEKL) and B7.1, as described earlier [34]. The resulting, activated OT-1 or OT-1 APRIL Tg cells were then cultured in vitro or injected i.v. (2 \times 10⁶ cells in 200 μ L 0.1% BSA/PBS) in recipient C57BL/6 wild-type mice. Seventy-two hours after initiation of priming, the clonal expansion in vitro and in vivo was analyzed by flow cytometry.

OT-2 and OT-2 APRIL Tg cells were purified from spleens and lymph nodes of the respective mouse strains by performing a B cell depletion with M5114 hybridoma supernatant and sheep anti-mouse IgG beads (DynaL, Oslo, Norway). Purified OT-2 or OT-2 APRIL Tg cells were labeled with CFSE according to the method described above and washed once, and 2 \times 10⁶ cells were injected i.v. in 200 μ L 0.1% BSA in PBS into recipient C57BL/6 Pep3b CD45.1⁺ mice. One day later, the mice were challenged with OVA17 peptide (ISQAVHAAHAEINEAGR; 40 μ g in 100 μ L 0.1% BSA/PBS) s.c. in their left flank. Seventy-two hours after peptide administration, the clonal expansion in spleen was determined by flow cytometry. The clonal expansion in vitro was determined by pulsing CFSE-labeled OT-2 or OT-2 APRIL Tg splenocytes with 0.5 μ g/mL OVA17 peptide and analysis 72 h later by flow cytometry.

Division indices were determined with help of the proliferation platform of FlowJo software. The division index is the average number of divisions that a cell, present in the starting population, has undergone. For example, if half of the cells in the starting population divided exactly two times, and the other half did not, the division index would be 1. Statistical significance of the division indices of the different experimental groups was compared using Student's *t*-testing. *P* values less than or equal to 0.05 were considered statistically significant.

Adenovirus immunizations

To analyze the CD8⁺ T cell response against adenovirus, 10⁸ PFU adenovirus ts125 was injected intramuscularly in 50 μ L PBS in the hind legs of APRIL Tg and littermate mice, and the response was followed over time in blood or 14 days after immunization in the spleen and peritoneal cavity with EIA-specific MHC-I tetramers (kind gift of Dr. T. Schumacher, NKI). Directly ex vivo, spleen and peritoneal cavity cells were incubated for 5 h with Ad5E1A (234–243) peptide (SGPSNTPEI; 0.5 μ g/ml) or HPV16 E7 peptide (RA-HYNIVTF; 0.5 μ g/ml) as control in the presence of GolgiPlug. Surface staining for CD8 α (APC-conjugated 53-6.7 from BD Biosciences) and CD44 (FITC-conjugated IM7 from eBioscience) and intracellular cytokine staining for IFN- γ (PE-conjugated XMG1.2 from BD Biosciences) were performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. The indicated numbers represent the percentage of CD8⁺CD44⁺ T cells producing IFN- γ upon incubation with the specific peptide minus the (background) IFN- γ release upon incubation with the control peptide. Statistical analysis was performed with a Student's *t*-test. *P* values less than or equal to 0.05 were considered statistically significant.

OVA/CT immunizations

OVA/CT immunizations were performed by administering 500 μ g OVA (Sigma Aldrich, Zwijndrecht, The Netherlands) + 1 μ g CT in 50 μ L PBS intranasally to APRIL Tg and littermate mice under brief ether (Sigma Aldrich) anesthesia. Secondary immunization was performed similarly 6 weeks after the primary immunization. Animals were killed at the indicated days, and single-cell suspensions were obtained from spleen, lymph nodes, and lungs by grinding the tissues through nylon sieves. Flow cytometry was performed on the single-cell suspensions. Statistical significance was determined by Student's *t*-tests. *P* values less than or equal to 0.05 were considered statistically significant.

Moloney-MLV inoculation of newborn mice and leukemogenesis

Newborn APRIL Tg mice ($n=24$) and control littermates ($n=18$) were inoculated i.p. with $\sim 5 \times 10^3$ focus-forming units (FFU) wild-type Moloney-MLV and followed for induction of leukemia in thymus, spleen, and lymph nodes as described previously [35]. Briefly, mice were monitored for hematocrits and gross organ enlargement by palpation under anesthesia with Forène® (isofurane, Abbott, Rungis, France). Hematocrits, expressed as the percentage of erythrocytes in total blood volume, were measured from blood samples (20 μ L) collected by puncture at the retro-orbital sinus of anesthetized animals with a heparinized capillary tube. Mice were killed for ethical reasons based on the appearance of signs of illness such as gross organ enlargement, ruffled hair, hunched appearance, and lethargy. After sacrifice of moribund animals, no enlargement of lymphoid organs was scored as 0, and spleen enlargement scoring was as described previously [35, 36], with one to two corresponding to normal or marginally enlarged spleens less than 0.25 g. Grossly enlarged leukemic spleens scored three to four, corresponding to spleen weights of 0.25 g or higher, with observed weights that could exceed 2 g for the largest spleens. The combination of gross spleen enlargement and hematocrits below 30% is a hallmark of erythroleukemia with blocked erythropoiesis. This has been confirmed previously by histology [35, 36] or FACS analyses with the Ter-119⁺ erythroid marker (see below). Enlarged thymus typically exceeding 1 g and lymph node alterations were scored according to the number of lymph nodes involved, with a maximal score of three (i.e., an animal with three or more enlarged lymph nodes). Survival analysis was evaluated using Kaplan-Meier curves and log-rank tests. Size scores of the different organs were tested for significance by using a Pearson χ^2 test. Erythroleukemia incidence, diagnosed as described above, was analyzed with a Kaplan-Meier curve and log-rank testing. *P* values less than or equal to 0.05 were considered statistically significant. The term “censored” relates to the death of a mouse for reasons other than the analyzed (expected) outcome before the expected outcome is observed.

Friend-MLV inoculation of adult mice and leukemogenesis

APRIL Tg mice ($n=17$) and control littermates ($n=10$) on a BALB/c background were inoculated i.v. with 1.5×10^4 FFU of the Friend-MLV strain 57

at 2 months of age. Disease progression was followed by weekly hematocrit measurements and spleen palpation. Eleven weeks after virus inoculation, five mice per strain were killed to determine mouse and spleen weight and number of Ter-119⁺ cells. Statistical significance of these parameters was determined with Student's *t*-tests. The remaining mice were used to study survival that was determined using a Kaplan-Meier curve and log-rank testing. Animals showing typical signs of illness as described above were killed for ethical reasons. *P* values less than or equal to 0.05 were considered statistically significant. Also, here, the term censored relates to the death of a mouse for reasons other than the analyzed (expected) outcome before the expected outcome is observed. Erythroleukemia was diagnosed as described above and confirmed by FACS with typically more than 25% Ter-119⁺ splenocytes.

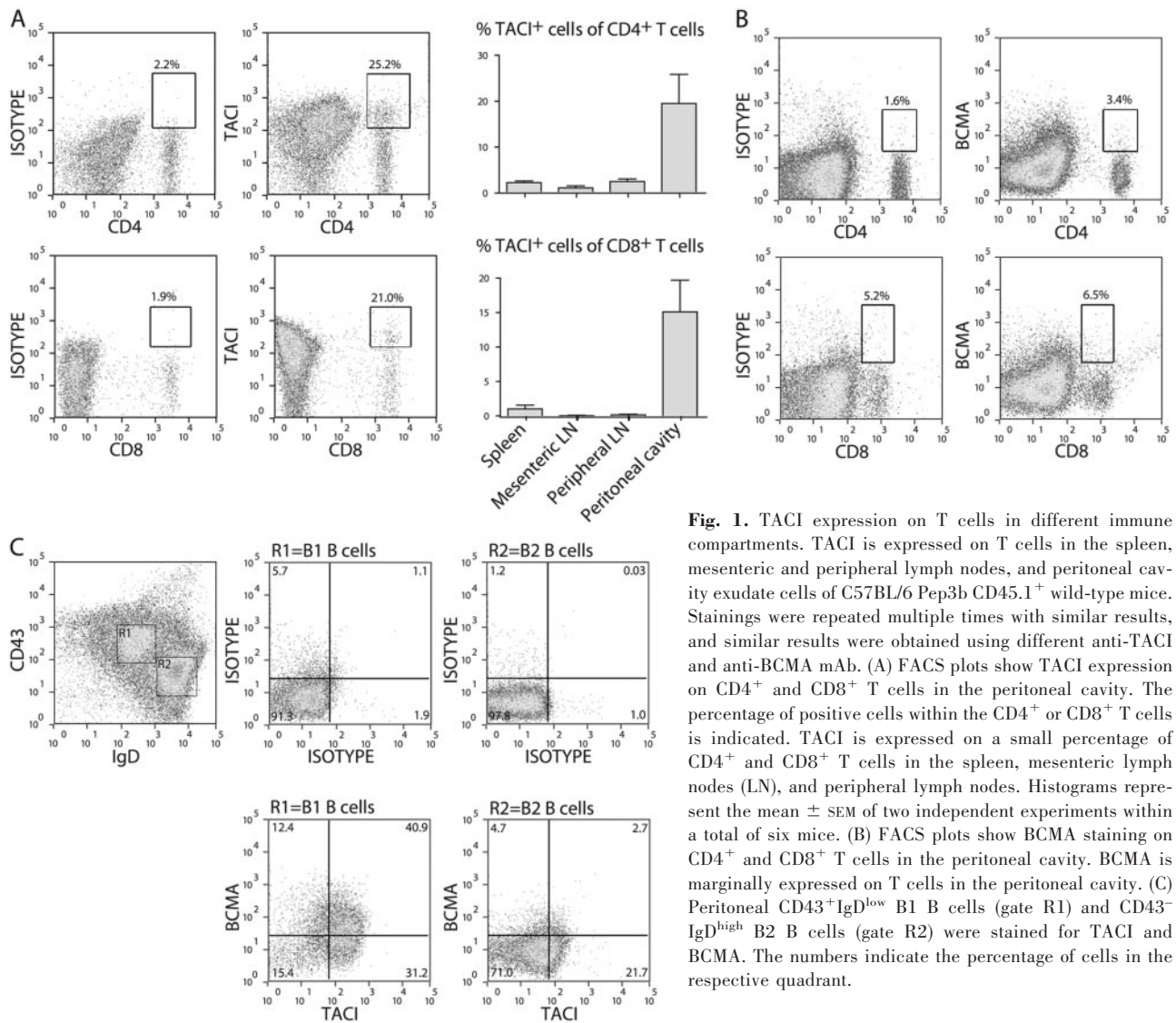
RESULTS

Expression of BCMA and TACI on mouse T cells

As the expression levels of the receptors for APRIL on mouse T cells are controversial [8], we set out to analyze the expression of TACI and BCMA on murine T cells in different immune compartments of naïve C57BL/6 Pep3b CD45.1⁺ mice using anti-mouse mAb. TACI was expressed on a small percentage (<2.5%) of T cells in the spleen, mesenteric lymph nodes, and peripheral lymph nodes. In contrast, $\sim 19\%$ of CD4⁺ T cells and 15% of CD8⁺ T cells in the peritoneal cavity expressed TACI (**Fig. 1A**). The T cells in the different compartments did not show BCMA expression, with the possible exception of the peritoneal cavity, which may contain a small subset of BCMA-expressing T cells (Fig. 1B). The failure to detect larger numbers of BCMA-expressing T cells is not a result of the antibody, as BCMA was readily detected on B-1 and B-2 B cells in the peritoneal cavity (Fig. 1C). These data provide an explanation for the apparent controversy that exists on TACI expression in T cells, as they clearly show that a subset of T cells can express TACI and possibly BCMA and that these may reside at specific locations in the body. APRIL could therefore be a relevant ligand in T cell biology.

Overexpression of APRIL does not affect TCR Tg T cell expansion in vitro and in vivo

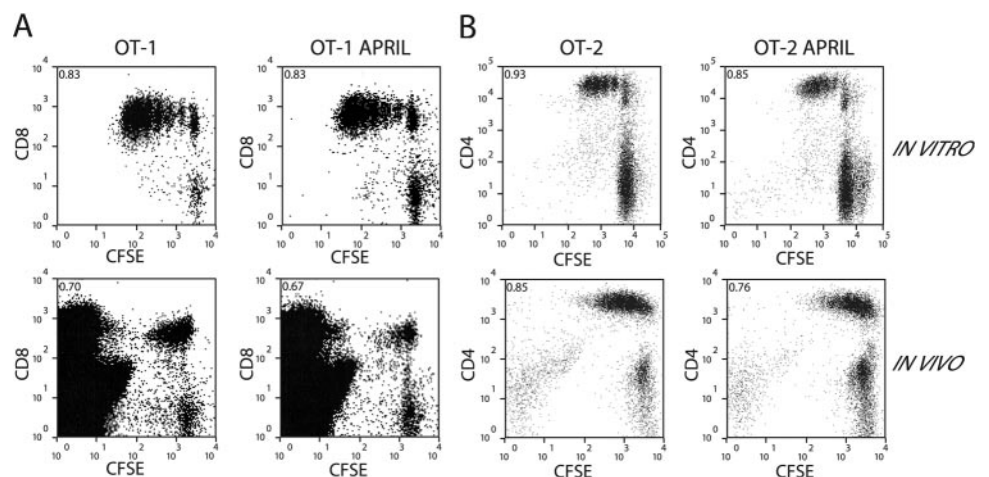
To study the effect of APRIL overexpression on the expansion of CD4⁺ and CD8⁺ T cells upon activation, the APRIL Tg mice strain was crossed with the OT-2 and OT-1 mice, respectively. First, we compared the ability of OT-1 versus OT-1 APRIL Tg cells to expand in vitro and in vivo. Therefore, these cells were purified from spleen and lymph nodes, labeled with CFSE, activated for 4 h on a monolayer of engineered APCs, and kept in culture or transferred i.v. to naïve recipient mice. Seventy-two hours after initiation of priming, clonal expansion was analyzed in vitro and in vivo. The OT-1 and OT-1 APRIL Tg cells proceeded through the same number of divisions in vitro and displayed a similar profile of CFSE dilution, indicating that in vitro proliferation and survival are not notably different. In agreement, the division index, which exemplifies the average number of divisions that the starting population has undergone, was identical for both T cell preparations. In vivo, the OT-1 and OT-1 APRIL Tg cells proliferated to a lesser extent compared with the cells in vitro, which is in line with previous findings [34] (**Fig. 2**). However, also under these conditions, the T cell expansion was not affected by APRIL, suggesting that APRIL did not affect CD8⁺ T cell expansion.



The role of APRIL in CD4⁺ T cell expansion was studied using OT-2 and OT-2 APRIL Tg cells after direct administration of specific OVA peptide to in vitro splenocyte cultures or by challenging C57BL/6 CD45.1⁺ mice s.c. with this peptide

after adoptive transfer of OT-2 or OT-2 APRIL Tg cells. Similar to the findings with the CD8⁺ TCR Tg T cells, we did not detect differences in clonal expansion between OT-2 and OT-2 APRIL Tg cells in vitro and in vivo (Fig. 2B). Also, here,

Fig. 2. Expansion of TCR Tg T lymphocytes. (A) CFSE-labeled OT-1 APRIL Tg and OT-1 cells were stimulated in vitro for 4 h and analyzed after 72 h in vitro or after adoptive transfer in the spleens of recipient mice in vivo. (B) Peptide-stimulated OT-2 APRIL Tg and OT-2 cells were analyzed for their clonal expansion in vitro and in vivo. Expansion in vivo was determined in the spleen of recipient mice (CD45.1⁺) by gating on the CD45.2⁺ cell population. FACS profiles shown are representative of at least two independent experiments, with $n = 4$ mice. Division indices, the average number of cell divisions that the T cells underwent, were determined with the proliferation platform of FlowJo software and are indicated in the upper-left corner of the dot-plots. The division indices between APRIL Tg and littermate T cells were not statistically significant ($P > 0.05$, Student's *t*-test).



division indices were highly comparable. Together, these findings do not support an effect of APRIL overexpression on the clonal expansion of CD4⁺ or CD8⁺ T lymphocytes.

CD8⁺ T cell response against adenovirus in APRIL Tg mice

The use of TCR Tg T cells could be disadvantageous in the search for a role of APRIL, as the reported low activation threshold of these cells could potentially blur subtle effects of this ligand. Therefore, we also chose to study the role of APRIL on T cells of the endogenous repertoire by directly immunizing APRIL Tg versus littermate mice. First, we analyzed the CD8⁺ T cell response following intramuscular immunization with adenovirus. This immunization generates adenovirus E1A-specific CTL responses that can be followed in blood over time using E1A peptide-specific MHC-I tetramers. No significant differences ($P > 0.05$, Student's *t*-test) were observed at any time-point between APRIL Tg and littermate mice in the priming, contraction, and memory phase of the antiviral CD8⁺ T cell response, suggesting that Tg APRIL expression does not affect a physiological CD8⁺ T cell response (Fig. 3A).

To ascertain that the response was not different in other compartments, we measured the response in the spleen and peritoneal cavity on Day 14. Also, here, we did not observe differences in the E1A-specific response between APRIL Tg and littermate mice. Moreover, the ability to release IFN- γ was not different between the groups, indicating that APRIL also does not affect the functionality of the responding cells (Fig. 3B, and data not shown).

Primary and secondary T cell response to OVA/CT in APRIL Tg mice

Next, we stimulated T cells in vivo using a nonviral antigen. For this, APRIL Tg versus littermate animals were subjected to an intranasal OVA/CT immunization. Under brief ether anesthesia, the mice inhaled a mixture of OVA and CT [37]. At different days after immunization, the absolute numbers of CD4⁺ and CD8⁺ T cells in spleen, lung, and draining lymph nodes were determined (Fig. 4A, and data not shown). However, no significant differences ($P > 0.05$, Student's *t*-test) in absolute numbers of T cells between APRIL Tg and littermate mice were observed during the primary response at any time-point. The antigen specificity of the CD8⁺ T cell response was confirmed with OVA-tetramers, but this analysis also did not reveal differences in the CD8⁺ T cell response between the Tg and littermate animals ($P > 0.05$, Student's *t*-test; Fig. 4B). Six weeks after the primary immunization, animals received a secondary OVA immunization. The secondary response peaked slightly earlier when compared with the primary response, but again, no differences between APRIL Tg and littermate mice were observed ($P > 0.05$, Student's *t*-test) at any time-point (Fig. 4C). In conclusion, no significant differences in absolute numbers of T cells between APRIL Tg and littermate mice were observed during the primary and secondary response after OVA/CT immunization. Taken together, we conclude that APRIL overexpression neither influences the response of CD4⁺ and CD8⁺ TCR Tg T cells nor the adenovirus and OVA responses of the endogenous T cell repertoire. Although we

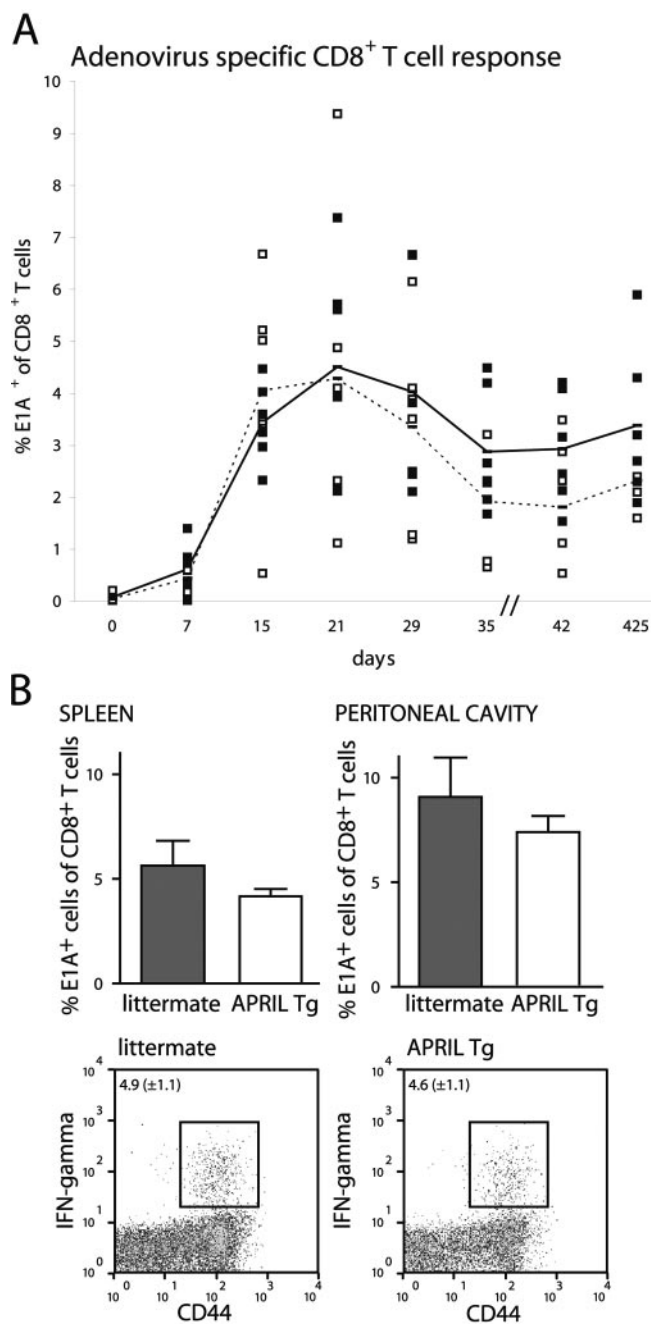


Fig. 3. CD8⁺ T cell response against adenovirus in APRIL Tg versus littermate mice. APRIL Tg (dashed lines, open squares, open bars) and littermate mice (solid lines, filled squares, filled bars) were injected intramuscularly with adenovirus ts125, and the adenovirus-specific CD8⁺ T cell response was followed over time in blood and in the spleen and peritoneal cavity on Day 14 after immunization. (A) No significant differences in the expansion, contraction, and memory phase between APRIL Tg and littermate animals were observed ($P > 0.05$, Student's *t*-test). This experiment has been performed three times with comparable results. (B) No significant differences were observed in the percentage of E1A-positive cells in the spleen and peritoneal cavity CD8⁺ T cells between APRIL Tg and littermate mice ($P > 0.05$, Student's *t*-test). Histograms represent the mean \pm SEM, with $n = 4$ mice. The IFN- γ production of the responding cells was also not different. Shown are representative dot-plots of Ad10-specific IFN- γ release in the spleen of APRIL Tg and littermate mice (gated on CD8⁺). The numbers indicate the mean (\pm SEM) percentage of specific IFN- γ release of CD8⁺CD44⁺ T cells of $n = 4$ mice.

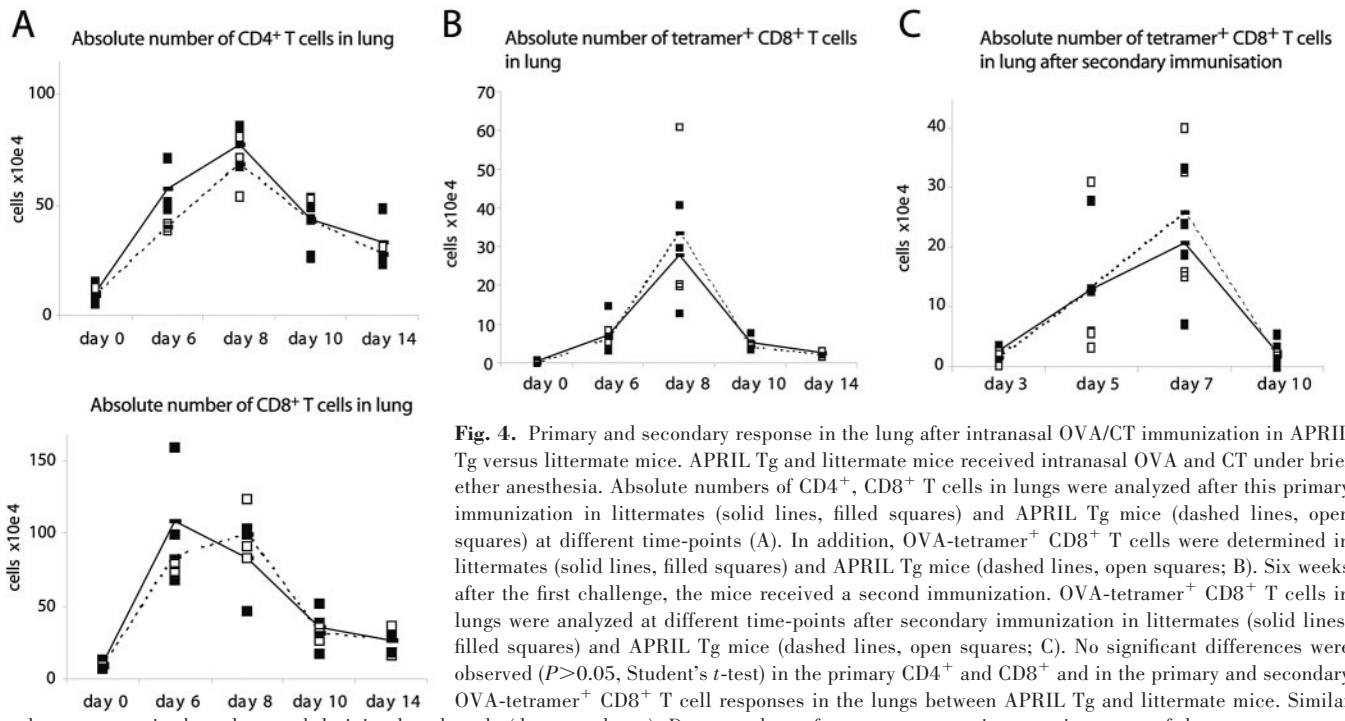


Fig. 4. Primary and secondary response in the lung after intranasal OVA/CT immunization in APRIL Tg versus littermate mice. APRIL Tg and littermate mice received intranasal OVA and CT under brief ether anesthesia. Absolute numbers of CD4⁺, CD8⁺ T cells in lungs were analyzed after this primary immunization in littermates (solid lines, filled squares) and APRIL Tg mice (dashed lines, open squares) at different time-points (A). In addition, OVA-tetramer⁺ CD8⁺ T cells were determined in littermates (solid lines, filled squares) and APRIL Tg mice (dashed lines, open squares; B). Six weeks after the first challenge, the mice received a second immunization. OVA-tetramer⁺ CD8⁺ T cells in lungs were analyzed at different time-points after secondary immunization in littermates (solid lines, filled squares) and APRIL Tg mice (dashed lines, open squares; C). No significant differences were observed ($P > 0.05$, Student's *t*-test) in the primary CD4⁺ and CD8⁺ and in the primary and secondary OVA-tetramer⁺ CD8⁺ T cell responses in the lungs between APRIL Tg and littermate mice. Similar results were seen in the spleen and draining lymph node (data not shown). Data are shown for one representative experiment out of three.

cannot formally exclude a role for APRIL in T cell immunity, our data indicate that this role is clearly not a general one.

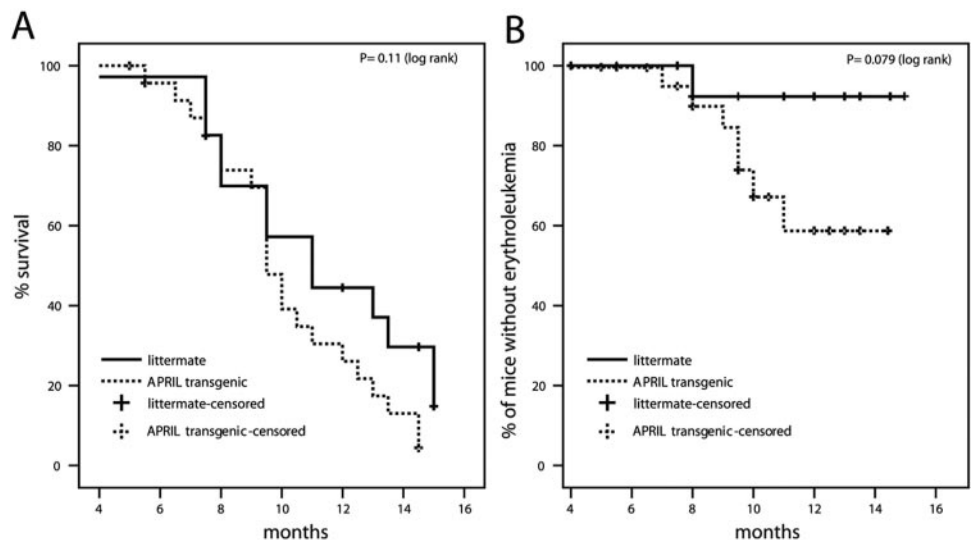
Increased Moloney-MLV-induced splenic involvement and erythroleukemia in APRIL Tg mice

Previous studies have implicated APRIL in the growth and survival of tumors [1, 5, 38]. Moreover, several B lymphoid malignancies have been associated with high serum levels of APRIL [23–25, 27–30, 39]. We have previously shown that B-1 B cells in APRIL Tg mice have an enhanced survival capacity. As a result, these mice develop B-1 B cell malignancies at an advanced age [22]. As APRIL has also been suggested to induce survival in human cutaneous T cell leukemias [31], we addressed the possibility that APRIL plays a role in T

cell malignancy as well. Moloney-MLV is a pathogenic, non-acute retrovirus that generally gives rise to T cell lymphomas in newborn mice [40]. C57BL/6 mice are relatively resistant to Moloney-MLV-induced lymphomagenesis, which they develop only after long latency, and BALB/c mice are more susceptible [41]. Newborn APRIL Tg and littermate mice, both on a C57BL/6 background, were injected with Moloney-MLV and followed for leukemia induction. At 4–5 months of age, leukemia started to develop, but no significant difference in leukemia latency and death was observed between APRIL Tg and littermate mice, although at later time-points, the APRIL Tg mice showed a trend toward increased leukemogenesis (log-rank test; $P = 0.11$; **Fig. 5A**).

Moreover, APRIL Tg and control littermates that developed thymic and nodular lymphomas showed similar patterns of

Fig. 5. Leukemia and erythroleukemia incidence in Moloney-MLV inoculated APRIL Tg mice and control littermates. Newborn mice were infected with Moloney-MLV and followed over time. Data represent a Kaplan Meier survival curve in control littermates (solid lines) and APRIL Tg mice (dashed lines; A) or the incidence of erythroleukemia formation after Moloney-MLV infection in control littermates (solid lines) and APRIL Tg mice (dashed lines; B). No statistically significant differences were observed in overall leukemia incidence and erythroleukemia formation in APRIL Tg and littermate mice (log-rank test; $P = 0.11$ and 0.079, respectively).



disease with no statistical differences observed in the disease time course, organ enlargement, and nodular involvement (data not shown). However, splenic involvement was more frequent in the APRIL Tg group ($P=0.026$ using a Pearson χ^2 test; **Table 1**). The biological relevance of this finding remains to be determined, as it did not result in enhanced mortality (Fig. 5A).

Strikingly, we did observe a clear difference in the incidence of erythroleukemia that developed in the two groups. Generally, Moloney-MLV rarely induces erythroleukemia, as tested in several mouse strains, including C57BL/6 [41]. However, seven out of 24 APRIL Tg mice compared with one out of 18 littermates developed this leukemia, suggesting an enhancing effect of APRIL in the development of erythroleukemia (Fig. 5B).

Erythroleukemia induced by Friend-MLV is more severe in APRIL Tg (BALB/c) mice

To directly examine the role of APRIL in erythroleukemia formation in more detail, APRIL Tg and littermate mice were crossed onto a BALB/c background and inoculated with Friend-MLV, which is a retrovirus that mainly induces erythroleukemia in the so-called susceptible strains of mice, including BALB/c mice [32]. A hallmark of this leukemia is an enlarged spleen combined with hematocrits that drop below 35% against 45% in control sex- and age-matched animals [42]. Here, we used adult-inoculated animals, which are less sensitive to erythroleukemia than neonatally inoculated animals and only considered hematocrits of 30% and below as a distinctive mark of erythroleukemogenesis. After inoculation of Friend-MLV in this strain of mice, we observed a dramatic, enhanced expansion of the spleen. Splenic weight was increased three- to fourfold in APRIL Tg mice compared with control littermates (**Table 2**; Student's t -test; $P=0.0035$), suggesting that APRIL significantly enhances erythoblastosis in mice. Survival analysis revealed that the majority (84%) of the APRIL Tg mice died within 24 weeks after a challenge with Friend-MLV, and only 20% of the littermates succumbed to Friend-MLV-induced erythroleukemia at that time-point ($P=0.039$; **Fig. 6**). Closer examination of the affected spleens in the APRIL Tg mice demonstrated that Ter-119⁺ cells were strongly expanded in the lymphocyte gate when compared with

TABLE 1. Splenic Involvement after Moloney-MLV Infection in APRIL Tg and Littermates

		Littermate	APRIL Transgenic	Total
Spleen score 0–2	count	15	12	27
	percentage	83.3%	50.0%	64.3%
3–4	count	3	12	15
	percentage	16.7%	50.0%	35.7%
Total	count	18	24	42
	percentage	100.0%	100.0%	100.0%
		P value		0.026

The extent of splenic involvement after Moloney-MLV infection differed significantly ($P = 0.026$ using a Pearson χ^2 test) between APRIL Tg and littermate mice. The spleen score was stratified into two groups, one with two or lower and one with three or higher.

TABLE 2. Mouse and Spleen Weight after Friend-MLV Infection in APRIL Tg and Littermates

	Mouse weight (in g)		Spleen weight (in g)	
	Littermate	APRIL transgenic	Littermate	APRIL transgenic
M1	22.2	M6 25.8	M1 0.11	M6 0.34
M2	26.4	M7 25.1	M2 0.24	M7 0.42
M3	28.2	M8 24.9	M3 0.11	M8 0.46
M4	24.5	M9 24.5	M4 0.11	M9 0.39
M5	24.9	M10 25.5	M5 0.11	M10 0.79
Average (\pm sb)	25.2 (± 2.2)	25.2 (± 0.5)	0.14 (± 0.06)	0.48 (± 0.18)
P value	0.94		0.0035	

APRIL Tg mice show significantly increased spleen weight compared with littermates 11 weeks after Friend-MLV infection (Student's t -test; $P = 0.0035$).

littermates (**Table 3**; Student's t -test; $P=0.039$). We therefore conclude that in addition to the increased severity of disease observed in APRIL Tg mice (Fig. 6), APRIL enhances the development of erythroleukemia.

DISCUSSION

Previous in vitro studies have shown that APRIL can costimulate T cell activation and enhance T cell survival. At present, it is, however, not clear via which receptor(s) APRIL delivers these costimulatory and survival signals. The APRIL-induced proliferation and survival could be mediated by TACI, HSPGs, and/or a yet-unidentified, additional receptor. More importantly, evidence for a similar role of APRIL in vivo has been lacking to date. In this study, we determined the expression of

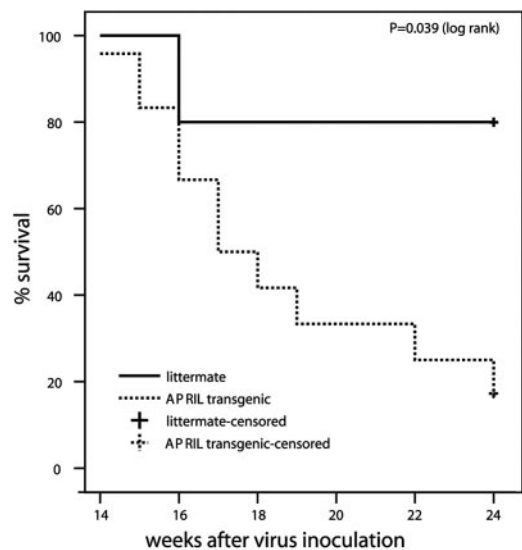


Fig. 6. Kaplan Meier survival curve after Friend-MLV infection in APRIL Tg and control littermates. Mice were infected with Friend-MLV at 2 months of age and followed over time. Kaplan Meier survival curve after Friend-MLV infection in control littermates (solid line) and APRIL Tg mice (dashed line). APRIL Tg mice show significantly increased mortality compared with littermates (log-rank test; $P=0.039$).

TABLE 3. Ter-119⁺ Cells among Spleen Lymphocytes 11 Weeks after Friend-MLV Infection in APRIL Tg and Littermates

	% Ter-119 ⁺ cells in lymphocyte gate			
	Littermate		APRIL transgenic	
M1	7.55	M6	26.45	
M2	36.26	M7	47.18	
M3	10.51	M8	28.55	
M4	13.84	M9	28.91	
M5	13.29	M10	29.23	
Average (±SD)	16.29 (±11.4)		32.06 (±8.5)	
P value	0.039			

The percentage of Ter-119⁺ cells in the lymphocyte gate was significantly higher (Student's *t*-test; *P* = 0.039) in the spleens of APRIL Tg mice compared with littermates 11 weeks after Friend-MLV infection.

the known TNFRs for APRIL on murine T cells in different immune compartments. We found expression of TACI on T lymphocytes, but this was only apparent in a subset of the cells, and variable levels of TACI were present in CD4⁺ and CD8⁺ T lymphocytes in different immune compartments. TACI expression was highest on T cells in the peritoneal cavity. In this compartment, BCMA was marginally expressed on T lymphocytes. These data provide an explanation for the discrepancies observed in TACI expression on T cells and show that a subset of T cells can express TACI. Together with a potential role of HSPGs on T cells [6], this clearly implies that APRIL-TACI interactions can play a role in T cell biology. A role for APRIL in in vitro T cell activation has been suggested previously [17–21]. However, our data do not reveal a role for APRIL in T cell responses. For instance, we did not observe differences in the expansion of OT-1 APRIL Tg and OT-2 APRIL Tg lymphocytes compared with OT-1 and OT-2 cells, respectively, in vitro and in vivo. To exclude the possibility that this was a result of the use of TCR Tg cells, we extended these studies with in vivo immunization protocols comparing T cell responses of the endogenous repertoire between APRIL Tg and littermate mice. Also, here, no differences in the primary adenovirus and primary and secondary OVA/CT response were observed. Our data therefore do not support the hypothesis that APRIL enhances T cell responses. Of course, our data do not allow us to exclude a role for APRIL overexpression in in vivo T cell responses completely, but the effect is at best subtle or restricted to a specific subset of T cells or responses. In addition, we cannot exclude that endogenous APRIL may already be sufficient to induce eventual effects. However, one report studying the T cell response after KLH immunization in APRIL-deficient mice did not reveal a role for APRIL as well [21]. In contrast to previous studies [17–19], which showed enhanced proliferation and survival in vitro and under specific conditions, better survival in vivo, we studied the effect of APRIL on antigen-specific TCR Tg and endogenous T cell responses. We therefore conclude that APRIL does not enhance T cell responses to foreign antigens.

Besides a lack of effect on normal T cell responses, we did not observe an effect on T cell lymphomagenesis in the APRIL Tg mice, although it appeared that APRIL increased splenic involvement in lymphoma-bearing mice. This could be a result

of differential homing of APRIL Tg lymphoma T cells to the spleen. Interestingly, APRIL Tg peripheral T cells have been shown to express decreased levels of CD62L and to be less-efficient in entering peripheral lymph nodes [19]. Besides this, APRIL overexpression in T lymphomas could result in increased lymphoma survival in the spleen as a result of enhanced Bcl-2 expression, a feature we observed earlier for APRIL Tg splenic T cells in vitro [19]. It remains, however, unclear why increased T lymphoma loads in the spleen do not result in increased mortality.

In contrast to the marginal effect of APRIL overexpression on T cell biology, we observed a strong increase in the development of erythroleukemia upon Moloney-MLV infection. Approximately 30% of the APRIL Tg animals developed such erythroleukemia, and only 5% of the littermates showed this malignancy. This effect on erythroleukemia formation was confirmed using Friend-MLV, a different, nonacute retrovirus known for induction of erythroleukemia. APRIL Tg mice showed increased severity of leukemia with increased expansion of Ter-119⁺ cells and shorter survival. Increased numbers of Ter-119⁺ erythroid cells could be a result of increased APRIL-TACI signaling, as a small (~1%) percentage of the Ter-119⁺ cells expresses TACI (data not shown), or as a result of HSPG and/or a yet-unknown, additional APRIL receptor [5].

Combined, our data indicate that APRIL does not play a major role in lymphomagenesis and immunity of T cells in vivo. However, we firmly established a role for APRIL in erythroleukemia formation. In analogy with B cell leukemia, it is tempting to speculate that APRIL plays a role in human erythroleukemia as well. Further studies about sera of patients with this disease are therefore awaited.

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