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The role of APRIL in immunity and tumorigenicity

Hardenberg, G.

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

The TNF family member -A PRoliferation-Inducing Ligand- (APRIL) has been suggested to act as a co-stimulatory 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 T cell responses nor endogenous TCR responses were affected by transgenic 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 transgenic mice. Strikingly, this erythroleukemia-facilitating property of APRIL was confirmed using the erythroleukemogenic Friend-MLV. Erythroleukemia in APRIL transgenic mice was characterised by very low hematocrits and grossly enlarged spleens with increased percentage of erythroid precursors. Altogether, these results unveil new pro-erythroleukemogenic properties of APRIL.
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 and T cells and their expression profile widely overlaps [1-4]. APRIL binds to transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA), both members of the TNF receptor family. BAFF can also interact with the latter two TNF receptors, but also binds its own unique TNF receptor, named BAFF receptor (BAFF-R or BR3). A third receptor for APRIL is thought to exist as well, since 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 both 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 monoclonal anti-mouse and anti-human TACI antibodies failed to detect TACI on both 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 both 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 dendritic cells, using decoy Ig-fusion receptors, prevents this effect [8, 15, 16]. This BAFF co-stimulatory effect was shown to be dependent on BAFF-R as T cells expressing a defective BAFF-R did not respond to BAFF-induced co-stimulation, while 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 co-stimulate anti-CD3-induced T cell activation [17] and another 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 periph-
eral 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 analysed 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 CD62L+ T cells, suggesting a difference in homing. Moreover, in vivo activation of these APRIL Tg T cells using Staphylococcal Enterotoxin B (SEB) as 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 analysed for T cell activation after KLH immunisation, 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, either on a T cell receptor 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**

6-8 weeks 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 donor 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-MLV infections, APRIL-Tg mice were first backcrossed ten 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 AMC and the IGMM.

Flow cytometry

Expression of BCMA and TACI on CD4+ and CD8+ T lymphocytes was determined after blocking Fc-receptors with 2.4G2 hybridoma supernatant using the directly labeled monoclonal anti-mouse BCMA antibody (ATTO647N-conjugated Vicky-2 from Alexis Biochemicals, San Diego, CA, USA), anti-mouse TACI antibody (PE-conjugated 8F10), anti-mouse CD4 (FITC-conjugated RM4-5) and anti-mouse CD8α (FITC or APC-conjugated 53-6.7) (all from BD Biosciences, San Diego, CA, USA). Rat IgG2a anti-mouse Foxp3-APC (clone FJK-16 from 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 Strepavidin-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 CD8α (APC-conjugated 53-6.7) were used together with anti-mouse CD45.2 (biotin conjugated 104) and Strepavidin-APC (all from BD Biosciences) where appropriate. In the OVA/CT immunisation experiments, OVA specific MHC-I tetramers (a kind gift of T. Schumacher, NKI, Amsterdam) 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, labeled with 5 μM CFSE for 10 minutes 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 hours on a monolayer of adherent fibroblast APC expressing Kb-OVA (257-264) (SIINFEKL) and B7.1, as described earlier [34]. The resulting activated OT-1 or OT-1 APRIL Tg cells were then either cultured in vitro or injected intravenously (2x10⁶ cells in 200 μL 0.1% BSA/PBS) in recipient C57BL/6 wild-type mice. 72h after initiation of priming, the clonal expansion in vitro and in vivo was analysed 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, washed once and 2x10⁶ cells were injected intravenously in 200 μL 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 μg in 100 μL 0.1% BSA/PBS) subcutaneously in their left flank. 72h 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 μg/mL OVA17 peptide and analysis 72h 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 2 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 immunisations

To analyse the CD8⁺ T cell response against adenovirus, 10⁸ PFU adenovirus ts125 were injected intramuscularly in 50 μL PBS in the hind legs of APRIL Tg and littermate mice and the response was followed over time in blood or 14 days after immunisation in the spleen and peritoneal cavity with E1A specific MHC-I tetramers (kind gift of Dr T Schumacher, NKI Amsterdam). Directly ex vivo, spleen and peritoneal cavity cells were incubated for 5 h with either Ad5E1A (234-243) peptide (SGPSNTPPEI; 0.5 μg/ml), or HPV16 E7 peptide (RAHYNIVTF, 0.5 μg/ml) as control in the presence of GolgiPlug. Surface staining for CD8α (APC-conjugated 53-6.7 from BD Biosciences), 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 rep-
resent 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.

**Ovalbumin (OVA)/cholera toxin (CT) immunisations**

OVA/CT immunisations were performed by administering 500 μg OVA (Sigma Aldrich, Zwijndrecht, The Netherlands) + 1 μg CT in 50 μL PBS intranasally to APRIL Tg and littermate mice, under brief ether (Sigma) anesthesia. Secondary immunisation was performed similarly, 6 weeks after the primary immunisation. Animals were sacrificed 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 murine leukemia virus inoculation of newborn mice and leukemogenesis**

Newborn APRIL Tg mice (n=24) and control littermates (n=18) were inoculated intraperitoneally with approximately 5 x 10³ FFU wild-type Moloney-MLV and followed for induction of leukemia in thymus, spleen and lymph nodes as previously described [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 μL) collected by puncture at the retroorbital sinus of anesthetised animals with a heparinised capillary tube. Mice were sacrificed 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 while spleen enlargement scoring was as previously described [35, 36], with 1 to 2 corresponding to normal or marginally enlarged spleens less than 0.25 g. Grossly enlarged leukemic spleens scored 3 to 4, 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 previously confirmed by histology [35, 36] or FACS analyses with the Ter 119+ erythroid marker (see below). Enlarged thymus typically exceeding 1 g and lymph nodes alterations were scored according to the number of lymph nodes involved, with a maximal score of 3 (i.e. an animal with 3 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 Chi-Square test. Erythroleukemia incidence, diagnosed as described above, was analysed 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 analysed (expected) outcome, before the expected outcome is observed.

**Friend virus murine leukemia virus inoculation of adult mice and leukemogenesis**

APRIL Tg mice (n=17) and control littermates (n=10) on a BALB/c background were inoculated intravenously with 1.5x10^4 FFU of the Friend-MLV strain 57 at 2 months of age. Disease progression was followed by weekly hematocrit measurements and spleen palpation. 11 weeks after virus inoculation, 5 mice per strain were sacrificed 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 sacrificed 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 analysed (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**

Since the expression levels of the receptors for APRIL on mouse T cells are controversial [8], we set out to analyse the expression of TACI and BCMA on murine T cells in different immune compartments of naïve C57BL/6 Pep3b CD45.1+ mice using monoclonal anti-mouse antibodies. TACI was expressed on a small percentage (<2.5%) of T cells in the spleen, mesenteric lymph nodes and peripheral lymph nodes. In contrast, about 19% of CD4+ T cells and 15% of CD8+ T cells in the peritoneal cavity expressed TACI (Figure 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 (Figure 1b). The failure to detect larger numbers of BCMA-expressing T cells is not due to the antibody, as BCMA was readily
detected on B-1 and B-2 B cells in the peritoneal cavity (Figure 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 both 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 both spleen and lymph nodes, labeled with CFSE, activated for 4 hours on a monolayer of engineered

Figure 1. TACI expression on T cells in different immune compartments
TACI is expressed on T cells in the spleen, mesenteric and peripheral lymph nodes and peritoneal cavity exudate cells of C57Bl/6 Pep3b CD45.1+ wild-type mice. Stainings were repeated multiple times with similar results and similar results were obtained using different monoclonal anti-TACI and anti-BCMA antibodies. A. FACS plots show TACI expression on CD4+ and CD8+ T cells in the peritoneal cavity. The percentage of positive cells within the CD4+ or CD8+ T cells is indicated. TACI is expressed on a small percentage of CD4+ and CD8+ T cells in the spleen, mesenteric lymph nodes and peripheral lymph nodes. Histograms represent the mean ± SEM of two independent experiments with in total 6 mice. B. FACS plots show BCMA staining on CD4+ and CD8+ T cells in the peritoneal cavity. BCMA is marginally expressed on T cells in the peritoneal cavity. BMCA is marginally expressed on T cells in the peritoneal cavity. BMCA is marginally expressed on T cells in the peritoneal cavity. BMCA is marginally expressed on T cells in the peritoneal cavity. BMCA is marginally expressed on T cells in the peritoneal cavity.
APRIL facilitates viral-induced erythroleukemia, but is dispensible for T cell immunity and lymphogenesis.

Chapter 6
APCs and either kept in culture or transferred intravenously to naïve recipient mice. 72 hours after initiation of priming, clonal expansion was analysed 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 to the cells in vitro, which is in line with previous findings [34] (Figure 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 subcutaneously 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 (Figure 2b). Also here, division indices were highly comparable. Together, these findings do not support an effect of APRIL overexpression on the clonal expansion of either CD4+ or CD8+ T lymphocytes.

Figure 2. Expansion of TCR Tg T lymphocytes
A. CFSE-labelled OT-1 APRIL Tg and OT-1 cells were stimulated in vitro for 4 hours and analysed after 72 hr, 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 analysed 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).
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, because 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 immunising APRIL Tg versus littermate mice. First, we analysed the CD8+ T cell response following intramuscular immunisation with adenovirus. This immunisation 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 (Figure 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 interferon gamma (IFN-γ) was not different between the groups, indicating that APRIL also does not affect the functionality of the responding cells (Figure 3b and data not shown).

Figure 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 immunisation.

A. No significant differences in the expansion, contraction and memory phase between APRIL Tg and littermate animals was 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 ± 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 ± SEM percentage of specific IFN-γ release of CD8+CD44+ T cells of n=4 mice.
Primary and secondary T cell response to OVA/CT in APRIL Tg mice

Next, we stimulated T cells in vivo using a non-viral antigen. For this, APRIL Tg versus littermate animals were subjected to an intranasal OVA/CT immunisation. Under brief ether anesthesia, the mice inhaled a mixture of OVA and CT [37]. At different days after immunisation the absolute numbers of CD4+ and CD8+ T cells in spleen, lung and draining lymph nodes were determined (Figure 4a and data not shown). However, no significant differences (p > 0.05, Student’s T test) in absolute numbers of T cells be-

![Graphs showing the absolute number of CD4+ and CD8+ T cells in lung, and the absolute number of tetramer+ CD8+ T cells in lung after secondary immunisation.](image)

**Figure 4.** Primary and secondary response in the lung after intranasal OVA/CT immunisation 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 analysed after this primary immunisation in littersmates (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 littersmates (solid lines, filled squares) and APRIL Tg mice (dashed lines, open squares) (B). 6 weeks after the first challenge, the mice received a second immunisation. OVA-tetramer+ CD8+ T cells in lungs were analysed at different time points after secondary immunisation in littersmates (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 of one representative experiment out of three.
tween 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) (Figure 4b). 6 weeks
after the primary immunisation, animals received a secondary OVA immunisation. The
secondary response peaked slightly earlier when compared to 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 (Figure 4c). In conclusion, no significant differ-
ences in absolute numbers of T cells between APRIL Tg and littermate mice were ob-
served during the primary and secondary response after OVA/CT immunisation. 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 endog-
enous T cell repertoire. Although we 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 tumours [1, 5,
38]. Moreover, several B lymphoid malignancies have been associated with high se-
rum 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 possibil-
ity 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 lymphomagen-
esis, which they develop only after long latency, while BALB/c mice are more suscep-
tible [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 towards increased leukemogenesis (log rank
test, p=0.11) (Figure 5a).

Moreover, APRIL Tg and control littermates that developed thymic and nodular lympho-
mas showed similar patterns of 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 Chi-Square test) (Table 1). The biological relevance of this finding
remains to be determined, as it did not result in enhanced mortality (Figure 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, 7 out of 24 APRIL Tg mice compared to 1 out of 18 littermates developed this leukemia, suggesting an enhancing effect of APRIL in the development of erythroleukemia (Figure 5b).

**Erythroleukemia induced by Friend virus 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 murine leukemia virus (Friend-MLV). Friend-MLV 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 3 to 4-fold in APRIL Tg mice compared to control littermates.

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**Figure 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).
APRIL facilitates viral-induced erythroleukemia, but is dispensible for T cell immunity and lymphomagenesis (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, while only 20% of the littermates succumbed to Friend-MLV-induced erythroleukemia at that time point (p=0.039) (Figure 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 to 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 (Figure 6), APRIL also enhances the development of erythroleukemia.

DISCUSSION

Previous in vitro studies have shown that APRIL can co-stimulate T cell activation and enhance T cell survival. At present, it is however not clear via which receptor(s) APRIL delivers these co-stimulatory 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 the known TNF receptors 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 both CD4+ and CD8+ T lymphocytes in different immune compartments. Figure 6 shows Kaplan Meier survival curves after Friend-MLV infection in APRIL Tg and control littermates. Mice were infected with Friend-MLV at two months of age and followed over time. Kaplan Meier survival curve after Friend-MLV infection in control littermates (solid lines) and APRIL Tg mice (dashed lines). APRIL Tg mice show significantly increased mortality compared to littermates (log rank, p=0.039).
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 to OT-1 and OT-2 cells respectively both in vitro and in vivo. To exclude the possibility that this was due to the use of TCR Tg cells, we extended these studies with in vivo immunisation 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 it 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 immunisation 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 due to 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. Around 30% of the APRIL Tg animals developed such erythroleukemia, while only 5% of the littermates showed this malignancy. This effect on erythroleukemia formation was confirmed using Friend-MLV, a different non-acute 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 due to 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 erytroleukemia as well. Further studies on sera of patients with this disease are therefore awaited.

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The extent of splenic involvement after Moloney-MLV infection differed significantly (p=0.026 using a Pearson Chi-Square test) between APRIL Tg and littermate mice. The spleen score was stratified into two groups, one with 2 or lower and one with 3 or higher.

APRIL Tg mice show significantly increased spleen weight compared to littermates 11 weeks after Friend-MLV infection (Student’s T-test, p=0.0035).

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 to littermates, 11 weeks after Friend-MLV infection.
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