Flow Cytometry

Surface markers were stained with anti-CD19 (FITC), anti-CD5 (PerCP-Cy5.5), anti-B220/CD45R (APC), anti-CD3 (PE-Cy7), anti-IgM (FITC), anti-CD43b (PE), CD23 (PE-Cy7), anti-CD138 (PE), anti-CD268/BAFF-R (APC) and anti-CD267/TACI (PE) which were purchased from eBioscience (Vienna, Austria). Anti-BCMA (FITC) was purchased from R&D systems (Abingdon, UK). For measuring apoptosis, cells were stained with 40 nM 3,3′ dihexyloxacarbocyanine iodide (DiOC6; Invitrogen, Cat. No. D-273) and propidium iodide (PI; Sigma, Cat. No. P4864) for 30 min at 37°C as previously described1. All antibodies were used according to manufacturer’s specifications. Flow cytometry was performed on a FACSCalibur or FACSCanto II (BD) and data analyzed with FlowJo software (TreeStar Inc).

In vitro stimulation of TCL1-Tg derived leukemic cells.

For in vitro stimulation, 2x10^5 cells were plated in a U shaped 96 well plate in 100 μl of RPMI-1640 complemented with 8% FCS, Glutamine and Penicillin/Streptavidin and β-mercaptoethanol 50 μM. 100 μl of APRIL-WT, MOCK (empty vector) or APRIL-R206E (a BCMA-specific APRIL variant2) conditioned media was added to the plated cells and incubated at 37 °C. Conditioned media was prepared by transfection of 293T cells with plasmid DNA coding for the different variants of APRIL, followed by collection of supernatant following 4 days of culture. The amount of APRIL was evaluated by ELISA. (Supplemental Figure 4). Activity was checked by measuring cell death in 2 modified Jurkat cell lines that express either extracellular TACI - intracellular FAS receptor or extracellular BCMA - intracellular FAS receptor (Supplemental Figure 5) as has recently been described2. For APRIL stimulation we used 950-600 ng/ml. For BAFF stimulation, 200 ng/ml of purified recombinant human BAFF (a kind gift from Dr G. Zhang, National Jewish Medical and Research Center, Denver, CO) was added to MOCK medium.
Histopathology and Immuno-histochemistry

Spleens fixed in 10 % buffered formalin for 48 hr and embedded in paraffin. Sections (4 μm thick) were cut, deparaffinized and stained with hematoxylin and eosin (H&E) according to standard protocols and analyzed. H&E and immunohistochemistry were performed on consecutive sections. For immunostaining, the sections were heated for 15 min at 60°C, followed by xylene and rehydration steps through a graded ethanol series and PBS, the Antigen retrieval was performed with citrate buffer pH=6 for 15 min at 98°C. Endogenous peroxidase was blocked with 1% H₂O₂ in PBS for 15 min. In order to block non-specific staining we used Ultra-V block (TA-125-UB, Immunologic, Duiven, The Netherlands). We stained for CD3 (clone SP7, dilution 1:1000, Neomarkers, Fremont, CA, USA); B220 (MCA1258 GT/RAT9-6B2 dilution 1:7000, Serotec, Puchheim, Germany); CD5 (53-7,3, dilution 1:1000, BD Biosciences). CD3 antibody was detected with Brightvision anti-rabbit HRP (DPVR110AP, Immunologic). B220 antibody was followed by rabbit-anti-rat (6130-01, 1:3000 diluted in 10% normal mouse serum, Southernbiotec, Uden, The Netherlands) for 30 min at RT. Followed by Brightvision anti-rabbit HRP. CD5 antibody was followed by rabbit-anti-FITC Ab (4510-7804, 1:1000, Bioconnect, Huissen, The Netherlands) 30 min at RT and then detected with Brightvision anti-rabbit HRP.

Clonality analysis and spectratyping of B cell populations

CD5⁺ and CD5⁻ B cell populations were obtained after FACsorting splenocytes from TCL1-Tg and TCL1xAPRIL double-Tg based on CD5 and B220 expression. Total RNA was extracted using the RNeasy isolation kit from QIAGEN. V_{H}-DJ_{H} gene rearrangements from B cell populations were amplified using PCR primers specific for the J558 VH region gene together with a primer specific for the C_{μ} constant region gene. Using a FAM-conjugated C_{μ} constant region or a J_{H} gene-specific primer in a run-off reaction, PCR products were labeled and subsequently analyzed on a capillary sequencer (ABI3100; Applied Biosystems, Leusden, NL) by fragment-length analysis. Sequences of primers were published earlier ³.
BAFF ELISA
We used a commercial kit to measure mouse BAFF levels, BAFF Quantikine ELISA KIT cat. num. MBLYS0 R&D systems. We used 20 μl of mice serum and we followed the standard protocol described by the user’s manual.

ELISA to quantify APRIL levels in 293T supernatants
96-wells flat bottom are coated with anti-Flag(M2) Sigma at 1 μg/m in coating buffer and left overnight at 4 °C. Blocked with PBS/BSA (BSA 1%) for 1 hour at 37 °C. The different preconditioned media are diluted in serial dilutions ½ in PBS/1% BSA and incubated for 2h at 37 °C. To detect APRIL we used Aprily-5-bio (ALX-804-801, Alexis) at 1 μg/ml in PBS/BSA, incubated for 1h at 37 °C and detected using Strept-Avidin-HRP (Jackson-Immuno) to 1μg/ml in PBS/BSA

Statistics
Data are expressed as mean +/- SEM. Statistical analyses were performed using SPSS version 19. Statistical analysis was performed using a 2-tailed unpaired Student t-test. Comparison of more than three samples was performed by non-parametric one-way ANOVA followed by Tuckey’s multiple comparison correction. Overall survival was tested using Kaplan-Meier analysis with a log-rank test. Differences were considered significant when p values < 0.05 (*), < 0.01 (**) and < 0.005 (***)

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. CD5⁺ leukemic B cells derived from TCL1-Tg mice respond to APRIL and BAFF in vitro. Leukemic cells are clearly identified by FACS as B220⁺ dull and CD5⁺ int in 8-12 month old TCL1-Tg mice (> 70% CD5⁺CD19⁺B220⁺ dull) A) Effect of APRIL and BAFF on leukemic cell viability over time. Error bars represent SEM (n=6) B) The anti-APRIL antagonistic antibody hA.01A fully prevents APRIL-mediated leukemic cell stimulation.

Supplemental Figure 2. Splenic sections of TCL1-Tg mice show mild enlargement of the marginal zones at four months of age and distorted splenic architecture at eight months of age. A) Splenic section of a TCL1-Tg mouse (4 months old) presenting an enlarged marginal zone. Pictures show highly compact cell areas which lack B220 expression, indicating an
abnormal expansion of the marginal zone cells. 100X magnification. **B)** Representative consecutive spleen sections of TCL1-Tg mice stained for H&E, CD3 and B220. Spleens from TCL1-Tg report increased disruption in splenic architecture with indistinguishable B and T-cell areas, at 8 month of age. 40X magnification.

**Supplemental Figure 3.** hAPRIL in the double-Tg does not affect mouse BAFF (mBAFF) levels. BAFF ELISA on serum of mice. Serum levels of BAFF are comparable in wild type, TCL1-Tg and double-Tg mice (n=3). Serum levels of BAFF-Tg mice are shown as control.

**Supplemental Figure 4.** Quantification of APRIL levels in the different conditioned media used in TCL1-Tg in vivo stimulation. ELISA using anti-Flag coated plates to compare the amount of the different APRIL mutants added onto the TCL1-Tg cells in vitro. The detection of APRIL is with APRILY-5 Ab. Different bars represent limiting dilutions 1 in 2 of each conditioned media.

**Supplemental Figure 5.** APRIL-R206E variant is able to signal via BCMA but not TACI. Binding activity of the different APRIL variants were tested on BCMA:Fas or TACI:Fas Jurkats cells. The ligand binding is directly associated with induced cell death measured by % of DNA fragmentation.


Supplemental Figure 1. CD5⁺ leukemic B cells derived from TCL1-Tg mice respond to APRIL and BAFF in vitro
Supplemental Figure 2. Splenic sections of TCL1-Tg mice show mild enlargement of the marginal zones at four months of age and distorted splenic architecture at eight months of age.

A. 

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B. 

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Supplemental Figure 3. hAPRIL in the double-Tg does not affect mBAFF levels
Supplemental Figure 4. Quantification of the different conditioned media used in TCL1-Tg \textit{in vitro} stimulation

![Graph showing OD490 nm for APRIL-WT, APRIL-R205E, and MOCK conditions.]}
Supplemental Figure 5. APRIL-R206E variant is able to signal via BCMA but not TACI

Jurkat-BCMA:Fas

%DNA fragmentation

Jurkat-TACI:Fas

%DNA fragmentation

MOCK       APRIL-WT      APRIL-R206E

MOCK       APRIL-WT      APRIL-R206E