

APRIL promotes B-1 cell-associated neoplasm

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Summary

A tumor-supporting role for the TNF-like ligand APRIL has been suggested. Here we describe that 9- to 12-month-old APRIL transgenic mice develop lymphoid tumors that originate from expansion of the peritoneal B-1 B cell population. Aging APRIL transgenic mice develop progressive hyperplasia in mesenteric lymph nodes and Peyer's patches, disorganization of affected lymphoid tissues, mucosal and capsular infiltration, and eventual tumor cell infiltration into nonlymphoid tissues such as kidney and liver. We detected significantly increased APRIL levels in sera of B cell chronic lymphoid leukemia (B-CLL) patients, indicating that APRIL promotes onset of B-1-associated neoplasms and that APRIL antagonism may provide a therapeutic strategy to treat B-CLL patients.

Introduction

APRIL (a proliferation-inducing ligand) is a secreted protein, named for its capacity to stimulate tumor cell proliferation in vitro (Medema et al., 2003). APRIL mRNA is expressed in hematopoietic cells, and the protein has been detected in primary T cells and dendritic cells (Litinskiy et al., 2002; Pradet-Balade et al., 2002). APRIL transcripts are also observed in a variety of nonhematopoietic carcinoma lines and primary tumor samples, pointing to APRIL involvement in tumor formation and/or maintenance (Deshayes et al., 2004; Hahne et al., 1998; Kelly et al., 2000; Novak et al., 2002; Rennert et al., 2000). Concurring with this, APRIL-transfected NIH-3T3 fibroblasts show an increased tumor growth rate in nude mice (Hahne et al., 1998; Rennert et al., 2000). Moreover, sequestering APRIL with a soluble form of B cell maturation antigen (BCMA-Fc), an APRIL receptor, retards growth of colon and lung carcinomas that express APRIL endogenously (Rennert et al., 2000), supporting the idea that APRIL has an important role in tumor growth. We provide compelling evidence that APRIL promotes tumor formation, as APRIL transgenic (Tg) mice develop lymph node hyperplasia after 9 months of age. Hyperplasia is initiated by B-1 B lympho-

cyte expansion in the peritoneum and is reminiscent of human B-CLL.

Results and discussion

B-CLL, the most prevalent leukemia in Western countries, is characterized by gradual accumulation of CD5⁺ B cells, due to a survival advantage (Bannerji and Byrd, 2000; Keating, 1999). Novak et al. showed that transcripts of the TNF family member BAFF (BLyS) (Mackay et al., 2002), a fundamental survival factor for transitional and mature B cells, are expressed aberrantly in B-CLL cells (Novak et al., 2002). In addition, APRIL mRNA was detected in a small percentage (<10%) of B-CLL samples analyzed, leading to the conclusion that BAFF, and possibly APRIL, is involved in B-CLL development (Novak et al., 2002). This study was recently extended by Kern et al., who found BAFF and APRIL expression in all B-CLL samples tested, at levels comparable to those detected in normal B cells (Kern et al., 2004). To corroborate these findings, we analyzed a panel of B-CLL tumors and found that 18 of 40 B-CLL samples expressed detectable amounts of APRIL mRNA (Figure 1A and results not shown). APRIL mRNA levels were not derived from contaminat-

SIGNIFICANCE

A gradual accumulation of CD5⁺ B cells resulting from an elevated survival capacity is characteristic for B-CLL, the most common leukemia in Western countries. The underlying mechanisms that cause B-CLL are mostly unknown. Here we report the development of B-1 cell-associated tumor formation in aging APRIL transgenic mice being highly reminiscent of human B-CLL. Increased APRIL mRNA and serum levels in samples of B-CLL patients strongly suggest an implication of this cytokine in B-CLL. We therefore propose that APRIL transgenic mice are an appropriate animal model for studying the human disease.

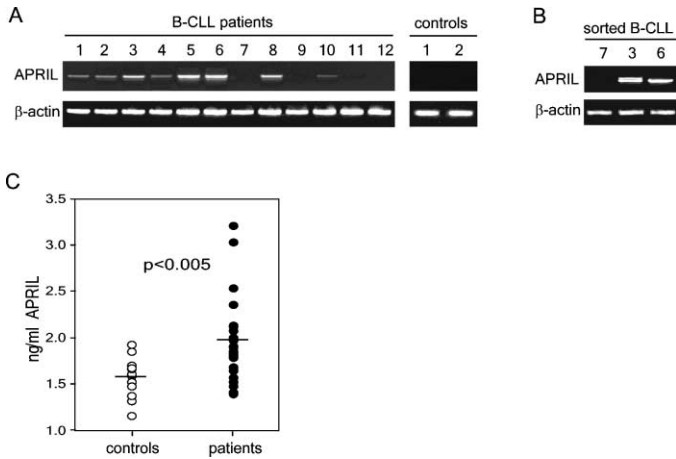


Figure 1. Elevated APRIL levels in B-CLL tumor samples and patient serum

A: PCR analysis using RNA derived from 12 B-CLL patients. Upper panel shows APRIL, lower panel shows β actin control. Picture is representative of 40 samples analyzed. Control B cell samples are derived by magnetic cell sorting from pooled buffy coats from at least 5 donors.

B: B-CLL cells were purified to near-homogeneity (>98%) by magnetic sorting and reanalyzed for APRIL mRNA expression. Sample numbers are identical to **A**.

C: Serum APRIL levels in normal healthy donors ($n = 12$) and B-CLL patients ($n = 22$) as determined by ELISA.

ing cells in the B-CLL preparations (Figure 1B), nor was APRIL mRNA detectable in naïve B cells under these conditions, indicating that a large fraction of B-CLL tumors overexpress APRIL mRNA (Figure 1A). As APRIL acts as a secreted cytokine (Lopez-Fraga et al., 2001), we used ELISA to determine whether B-CLL patients have increased circulating APRIL levels, comparing sera from patients and healthy donors. This analysis revealed a significant increase in serum APRIL levels in a patient cohort (Figure 1C). Our data therefore indicate that this cytokine is overexpressed in a large fraction of B-CLL tumors, resulting in increased APRIL serum levels in B-CLL patients.

New Zealand White mice are often used as a model for B-CLL, as they show massive expansion of $CD5^+$ B cells (also termed B-1 B cells [B-1 cells]) in older age (Hamano et al., 1998). Similarly, transgenic mice containing B-cell specific expression of the T cell leukemia-1 (TCL1) gene, an oncogene translocated in some human T cell leukemia, develop B-CLL-like characteristics due to $CD5^+$ B cell expansion (Bichi et al., 2002). Thus, murine models for B-CLL depend on the expansion of B-1 B cells. Physiologically, these cells play an important role in T cell-independent humoral responses, and a large proportion of these cells reside in the peritoneal cavity (Berland and Wortis, 2002). We recently showed that APRIL-Tg mice, which have detectable APRIL protein levels in serum, show significantly elevated type 1 and type 2 thymus-independent (TI) humoral immune responses (Stein et al., 2002) (M.H., unpublished data), suggesting that B-1 cell activity is promoted by APRIL. In addition, serum IgM concentrations in APRIL-Tg mice are increased approximately 2-fold compared to control littermates (Stein et al., 2002), probably a consequence of increased B-1 cell activity in the Tg mice (Potter and Melchers, 2000).

The lymphoid population in the peritoneal cavity consists mainly of B-2 B cells or mature B cells (IgD^{high} , $CD43^-$, IgM^+ , $B220^+$,

$CD23^+$, and $CD5^-$) as well as B-1 cells ($CD43^+$, IgD^+ , $lowIgM^{high}$, $B220^{low}$, and $CD23^-$) (Berland and Wortis, 2002). The B-1 population can be further divided into B-1a and B-1b cells, based on CD5 expression, which is present in B-1a and not in B-1b cells (Berland and Wortis, 2002). As reported (Stein et al., 2002), no change in the B-1/B-2 cell ratio was detected in the peritoneal exudate cells (PEC) of APRIL-Tg mice at 6–12 weeks of age. Analysis of older mice (9–12 months) nonetheless revealed that B-1 cells were present in a significantly higher percentage in the PEC of APRIL-Tg mice than in the ones of littermates (Figure 2A). Importantly, cellular expansion is specific for peritoneal B-1 B cells, as no effect on splenic B cell populations is observed (Table 1). Moreover, serum IgA levels, a typical sign of B-1 cell activity, are significantly increased in older APRIL-Tg mice, thus concurring with a recent report on defective IgA production in APRIL $^{-/-}$ mice (Castigli et al., 2004) (Figure 2B). Most B-1 cells in the peritoneum of older Tg mice express CD5, indicating that the expanding population is comprised of B-1a cells, thus reminiscent of the cells detected in B-CLL patients. At an intermediate age (4–5 months), enlargement of the B-1 cell pool was already evident in the APRIL-Tg mouse peritoneum (Figure 2A), suggesting that these cells accumulate slowly over time. Indeed, scoring the mice for B-1 expansion in different age groups (9–12, 13–15, >15 months) revealed that B-1 expansion gradually affects all Tg mice and increases in severity over time (Figure 2F).

Closer inspection of 9- to 12-month-old APRIL-Tg mice showed significant lymphoid organ alterations, i.e., in either mesenteric lymph nodes (mLN) or Peyer's patches (PP), in about 40% of animals analyzed. We found marked enlargement of mLN (Figure 2C), which was due to an increase in cell number (Figure 2D). No alterations in mLN were observed in age-matched littermates or in an APRIL-Tg line that lacks detectable transgene protein expression (Figure 2D) (Stein et al., 2002), demonstrating that expansion depends on transgenic APRIL expression. In addition to mLN, PP in APRIL-Tg mice were larger on average than those of littermates (Figures 2C–2E). Examination of 40 paraffin-embedded PP derived from 13 older APRIL-Tg mice (~ 3 PP/mouse) revealed that 7 PP (from 5 different mice) measured from 25 mm 2 to 122.6 mm 2 , which is larger than the maximum PP size in littermates (Figure 2E). Increased PP size was paralleled by an increase in cell numbers in these organs (not shown). In extreme cases, PP and mLN were approximately 10-fold larger than their counterparts in littermates (Figure 2C–2E). Also, for the mLN and PP alterations, we found that incidence and severity increase over time (Figure 2F), suggesting a progressive hyperplasia.

Spleen hyperplasia was also detected in older APRIL-Tg mice with increasing incidence during aging (Figure 2F). Spleen architecture was highly disorganized, such that red and white pulp areas became indistinguishable (Figure 3A). Similar disruption of T and B cell-rich areas was also observed in enlarged mLN of APRIL-Tg mice (Figure 3B). Cell infiltration, as seen in malignancy (Frederickson and Harris, 2000), was detectable in the capsule of hyperplastic spleen (Figure 3A) and in the subcapsular sinus of mLN (Figure 3C), as well as in PP, as determined by the large B cell numbers within the lamina propria of the intestinal mucosa. Nevertheless, epithelial layer destruction was not seen (Figure 3D).

Although unaffected mLN and PP of 9-month-old APRIL-Tg mice revealed no significant alteration in either T cell or B cell composition in comparison with age-matched littermates

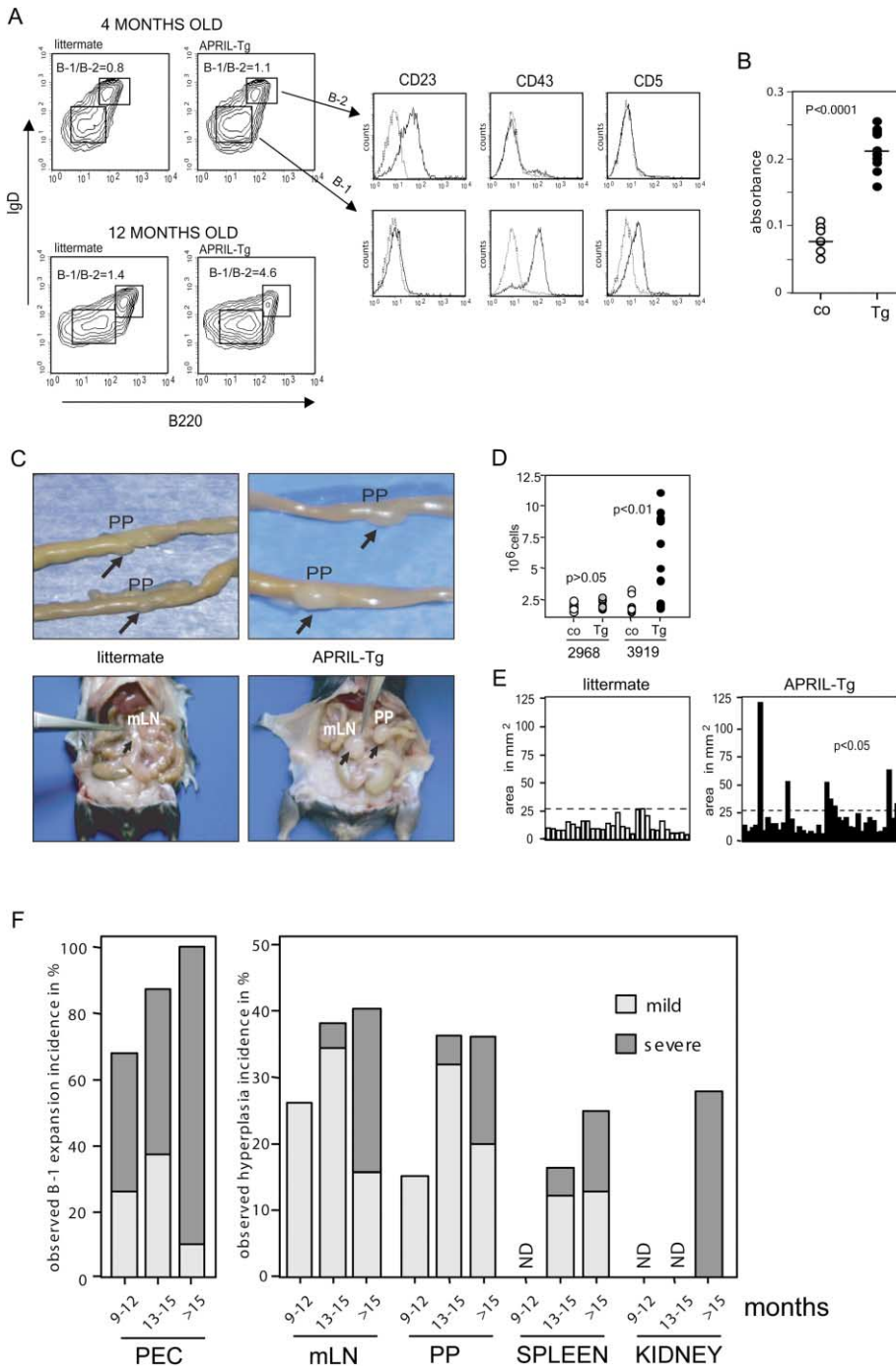


Figure 2. B-1 cell expansion, hyperplasia in lymphoid organs, and kidney alterations in aging APRIL-Tg mice

A: Peritoneal cells were isolated, and the B-1:B-2 cell ratio was determined at different ages and compared between control littermates and APRIL-Tg mice. B-1 cells were identified by CD5, CD23, CD43, IgD, IgM, and B220 cell surface staining. Representative images are shown of 10 mice analyzed.

B: IgA levels in older (9 months) APRIL-Tg and littermates ($n = 10$) was determined in the sera using a commercially available ELISA kit.

C: Images of mLN and PP with advanced hyperplasia taken from a 15-month-old APRIL-Tg mouse and an age-matched littermate.

D: Hyperplasia in APRIL-Tg mouse mLN. Cell numbers were determined in 9- to 12-month-old APRIL-Tg mice, line 3919 (black circles) expressing the transgenic protein, line 2968 (gray circles), which do not express the transgenic protein (Stein et al., 2002), and control littermates (open circles).

E: Comparison of PP size, determined by measuring paraffin-embedded organs, in control littermates (white bars, $n = 29$) and APRIL-Tg mice (black bars, $n = 40$). Dotted line indicates maximum PP size measured in controls.

F: Incidence of hyperplasia in older APRIL-Tg mice. PEC B-1 expansion, mLN, PP, and spleen hyperplasia, and kidney infiltration were determined in three APRIL-Tg mouse age groups (9–12 months, 12–15 months, and >15 months). Mice (20–45/group) were analyzed, and the total number for each group was established as 100%. For PEC (left panel), an increase in B-1 cells up to 50% is considered mild (light gray bars), whereas >50% increase is considered severe (dark gray bars). For mLN, PP, and spleen, the mild phenotype (light gray bars) represents a 2- to 3-fold increase in cellularity; a severe phenotype is defined as a >3-fold increase (dark gray bars). Kidney infiltration is considered severe at all times, as no lymphocytes are normally present. Statistical significance is indicated, as determined by ANOVA.

(not shown), the relative mLN composition of hyperplastic mLN was moderately affected. That is, B220⁺ cell percentages were slightly increased ($35.5 \pm 2.6\%$ versus $47.7 \pm 8\%$, $p < 0.005$), CD4⁺ cell percentages were unaffected ($34.0 \pm 1.9\%$ versus $34.4 \pm 5\%$, $p > 0.05$), and CD8⁺ cell percentages were slightly decreased ($28.4 \pm 2.7\%$ versus $18.3 \pm 2.7\%$, $p < 0.005$). Despite these changes in the relative numbers of lymphoid populations, the absolute number of CD4⁺, CD8⁺, and B220⁺ cells had increased, as the mLN and PP cellularity increased more than 2-fold. Thus, B and T cell populations had all expanded in enlarged mLN from Tg mice. Forward/side scatter analysis, which

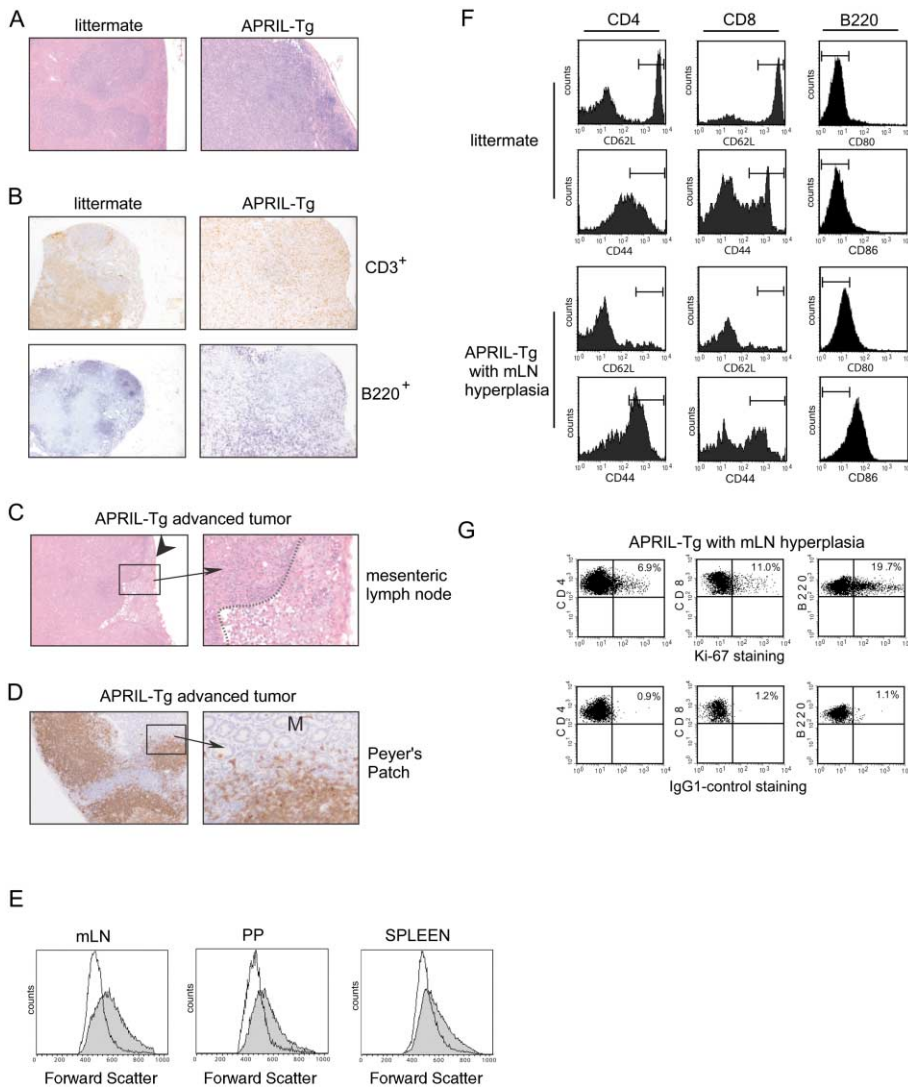
reflects cell size and can be a measure for activation, showed accumulation of enlarged blast-like cells (Figure 3E) in both T and B populations (not shown). Concomitant with this phenotype, we observed that both CD4⁺ and CD8⁺ T cells in enlarged mLN showed characteristic signs of activation, including down-regulation of CD3 and CD45RB (not shown). Specifically, we found an increase in CD62L⁻ CD44⁺ effector T cells (Figure 3F), while B220⁺ B cells displayed increased CD80 and CD86 levels, pointing to B cell activation (Figure 3F). All these populations showed a large proportion of mitotically active cells, as determined by staining for the mitosis-associated protein Ki-67

Table 1. Ectopic APRIL expression does not influence splenocyte development in 9-month-old APRIL Tg mice

	Control littermate		APRIL Tg		p value
	Cell number × 10 ⁶	S.D.	Cell number × 10 ⁶	S.D.	
splenocytes	80.6 (100%)	1.5	79.4 (100%)	3.2	p > 0.05
B cells	45.5 (56.3%)	2.5	43.2 (54.4%)	3.1	p > 0.05
mature B cells	29.7 (36.8%)	4.9	27.9 (35.2%)	3.0	p > 0.05
MZ B cells	2.5 (3.1%)	0.4	2.2 (2.8%)	0.4	p > 0.05
T1 B cells	2.6 (3.2%)	1.5	2.9 (3.7%)	0.7	p > 0.05
T2 B cells	7.4 (9.2%)	0.9	6.6 (8.3%)	1.2	p > 0.05
B1 cells	3.3 (4.1%)	0.7	4.1 (5.1%)	1.1	p > 0.05
macrophages	1.8 (2.2%)	0.7	3.4 (4.3%)	1.0	p > 0.05
granulocytes*	3.4 (4.2%)	1.0	3.0 (3.8%)	0.8	p > 0.05
dendritic cells	1.8 (2.2%)	0.1	2.0 (2.5%)	0.5	p > 0.05
T cells	17.7 (22%)	3.8	21.3 (26.8)	3.9	p > 0.05
CD4 ⁺ T cells	10.8 (13.4%)	2.1	12.2 (15.4%)	2.1	p > 0.05
CD8 ⁺ T cells	5.1 (6.3%)	2.0	7.5 (9.5%)	2.3	p > 0.05
plasma cells	1.4 (1.7%)	0.6	1.1 (1.4%)	0.5	p > 0.05

Splenocytes of 9-month-old APRIL transgenic mice and age-matched control littermates (n = 5) were analyzed for their content. Populations were defined as follows: B cells (B220⁺, IgM⁺, Thy1.2⁻); T1 (B220⁺, CD23⁻, CD21⁻, IgM^{high}); T2 (B220⁺, CD23⁺, CD21^{high}, IgM^{high}); mature B cells (B220⁺, CD23⁺, CD21⁺, IgM⁺); MZ (B220⁺, CD23⁻, CD21^{high}, IgM^{high}); B1 (B220⁺, CD23⁻, IgM⁺, CD5⁺); macrophages (Mac3⁺); granulocytes (GR1⁺), dendritic cells (CD11c⁺), T cells (Thy1.2⁺), CD4 T cells (Thy1.2⁺, CD4⁺); CD8 T cells (Thy1.2⁺, CD8⁺); plasma cells (B220^{low}+, CD138⁺, CD3⁻).

*n = 3

**Figure 3.** Histological examination of lymphoid tissues from 9- to 12-month-old APRIL-Tg mice and age-matched littermates

A: Hematoxylin/eosin (HE)-stained spleen sections from littermate and APRIL-Tg mice. Data are representative of six mice displaying splenic disorganization and cellular infiltration in the capsule.

B: Histological analysis of an mLN from a control mouse and an enlarged mLN from an APRIL-Tg mouse. CD3⁺ T cell and B220⁺ B cell distribution is shown. Data are representative of six mice analyzed.

C: HE-stained tissue sections of an advanced mLN tumor. Boxed area is enlarged to show cell infiltration into the subcapsular sinus. Dotted line indicates the subcapsular area.

D: B220⁺ cell distribution in PP from a 12-month-old APRIL-Tg mouse with an advanced PP tumor. Boxed area is enlarged to show B220⁺ cell infiltration into the adjacent mucosal layer (M). Groups of at least 10 APRIL-Tg or control mice were analyzed.

E: Forward scatter analysis of cells derived from hyperplastic spleen, mLN, and PP, representative of 30 mice analyzed.

F: CD4⁺ and CD8⁺ T cells and B220⁺ B cells of an enlarged mLN of an APRIL-Tg mouse and an age-matched littermate were stained for activation markers CD44 and CD62L (T cell) and CD80 and CD86 (B cell).

G: Analysis of lymphocytes of an enlarged mLN of an APRIL-Tg mouse using anti-Ki-67 or an isotype-matched control to determine mitotic activity.

F and **G** include representative images obtained by analyzing enlarged mesenteric lymph nodes of one APRIL-Tg mice.

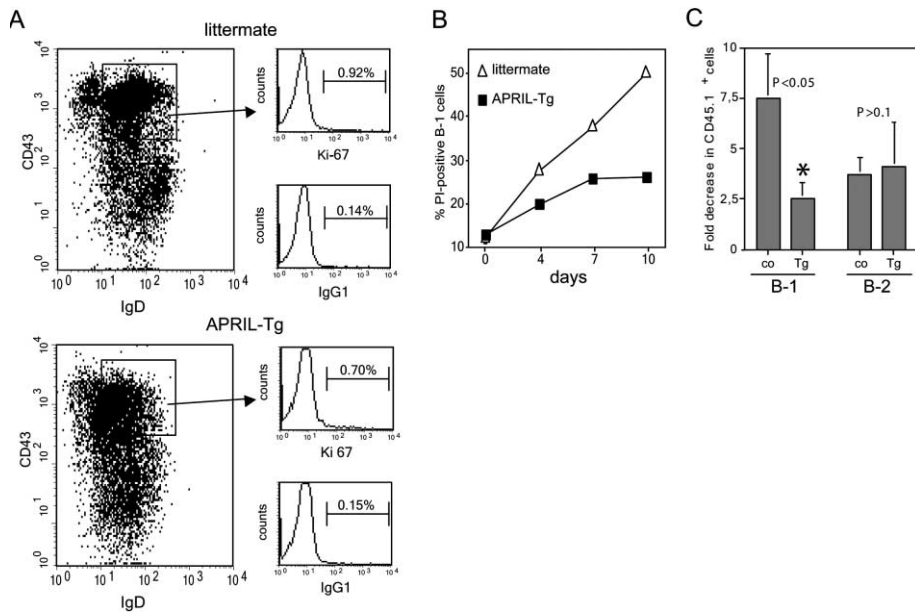


Figure 4. B-1 cell accumulation in APRIL Tg mice during aging

A: No increase in mitotic activity (anti-Ki-67) is observed in 9-month-old APRIL-Tg mouse peritoneal B-1 cells compared to those of littermates. **B:** In vitro survival of B-1 cells isolated from peritoneal cells, measured by PI exclusion. Data are representative of three independent experiments.

C: B-1 cells display increased in vivo survival in APRIL-Tg mice. SJL (CD45.1⁺) PEC cells were depleted of T cells and injected i.p. into 8-month-old APRIL-Tg mice or age-matched littermates (both CD45.2⁺). Survival of injected cells was determined after 10 days using the congenic difference (CD45⁺) and compared to the cells that could be retrieved immediately after injection (set to 100%). Decrease in cell number was determined by dividing the number of B-1 or B-2 cells present at day 10 with the amount of cells present at day 0. Five mice per group were analyzed, and statistical analysis was calculated using ANOVA.

(Figure 3G). We thus conclude that mLN expansion in APRIL-Tg mice is due to lymphocyte hyperactivation that leads to hyperplasia.

APRIL was previously shown to induce tumor cell proliferation in vitro and in vivo (Hahne et al., 1998; Rennert et al., 2000). We nonetheless found no evidence of increased B-1 cell proliferation in older APRIL-Tg mice, as measured by Ki-67 expression (Figure 4A). In addition to stimulating proliferation, APRIL can induce lymphoid and glioma tumor cell survival (Deshayes et al., 2004; Hahne et al., 1998; Stein et al., 2002; Yu et al., 2000). We therefore analyzed the survival capacity of peritoneal B-1 cells from APRIL-Tg and control mice. Approximately 50% of B-1 cells from littermates had died by 10 days in vitro culture, whereas those from APRIL-Tg mice survived far more efficiently (Figure 4B). This suggests that a survival advantage permits these cells to expand in vivo. In agreement, injection of peritoneal cells from congenic C57BL/6 SJL mice into the peritoneal cavity of APRIL-Tg and littermates revealed that B-1 cells survive 3-fold better when exposed to APRIL (Figure 4C). This survival is specific for B-1 cells, as B-2 B cells do not display a survival advantage in APRIL-Tg mice (Figure 4C). Moreover, in vitro treatment with death agonists such as VP-16, doxorubicin, and staurosporin displayed an increased survival capacity of transgenic B-1 B cells in comparison to the ones of control mice. As APRIL binds specifically and with high affinity to B-1 cells (not shown), it is likely that this survival capacity is mediated directly by APRIL.

As only 40% of the older mice show mLN hyperplasia, we reevaluated their PEC and found that B-1 cell expansion was most pronounced in APRIL-Tg mice with hyperplasia (Figure 5A). These B-1 cells were CD5⁺ and therefore of the B-1a subtype, although they showed decreased IgM and B220 expression, probably due to increased activation (Figure 5A). Migration of peritoneal B-1 cells to mLN was reported to require a greater activation level than that needed for self-renewal (Fagarasan et al., 2000). Concurring with B-1 cell migratory capacity, enlarged mLN revealed a striking increase in CD5⁺, CD19⁺, IgM^{dim},

B220^{dim}, and CD23⁻ B cells (Figure 5B). Although B-1 cells normally express high levels of IgM, peritoneal B-1 expansion is accompanied by a downregulation of surface IgM, a possible sign of an increased activation status (Fagarasan et al., 2000). Intriguingly, the CD5⁺, CD19⁺, IgM^{dim}, B220^{dim}, and CD23⁻ B cells present in the hyperplastic mLN are phenotypically identical to the expanding B-1 cells in the peritoneum (Figure 5A). This suggests that peritoneal B-1 cells accumulate gradually in the mLN of APRIL-Tg mice and are instrumental in initiating mLN hyperplasia.

To confirm this hypothesis, we tested whether B-1 cells from APRIL-Tg mice with clear signs of mLN activation could transfer the mLN hyperplastic phenotype to 8-week-old non-Tg mice that contain a congenic difference (SJL). Six mice received intraperitoneal injections of purified B-1 cells from hyperplastic mLN. Six weeks later, four of these mice appeared normal (Figure 5C), whereas two mice showed massive mLN activation, as indicated by T and B cell activation markers (Figure 5C) and Ki-67 staining (not shown). Importantly, we could only detect around 1%–2% CD45.2⁺ cells in the affected mLN, indicating that the active B and T cells were not derived from the injected cells, but were endogenous SJL cells. The difference between responding and nonresponding mice could be the result of slower tumor growth onset in the latter; alternatively, mouse-intrinsic factors may be decisive in hyperplasia development. It is nonetheless evident that these B-1 cells can induce the mLN hyperplastic phenotype in young non-Tg animals, indicating that the peritoneal B-1 cells are instrumental in the lymph node hyperplasia observed.

As described, some older APRIL-Tg mice showed splenic alteration (Figures 2F and 3A), suggesting that the hyperplastic phenotype is not confined to mLN and PP, but spreads throughout the lymphoid system. This became more evident in several 18-month-old APRIL-Tg mice analyzed, which displayed a more dramatic expansion of B-1 cells in the mLN (up to 10⁷ B-1a cells), as well as greatly enlarged spleens (>8-fold). In these mice, approximately 50% of splenocytes were CD5⁺, IgM^{dim},

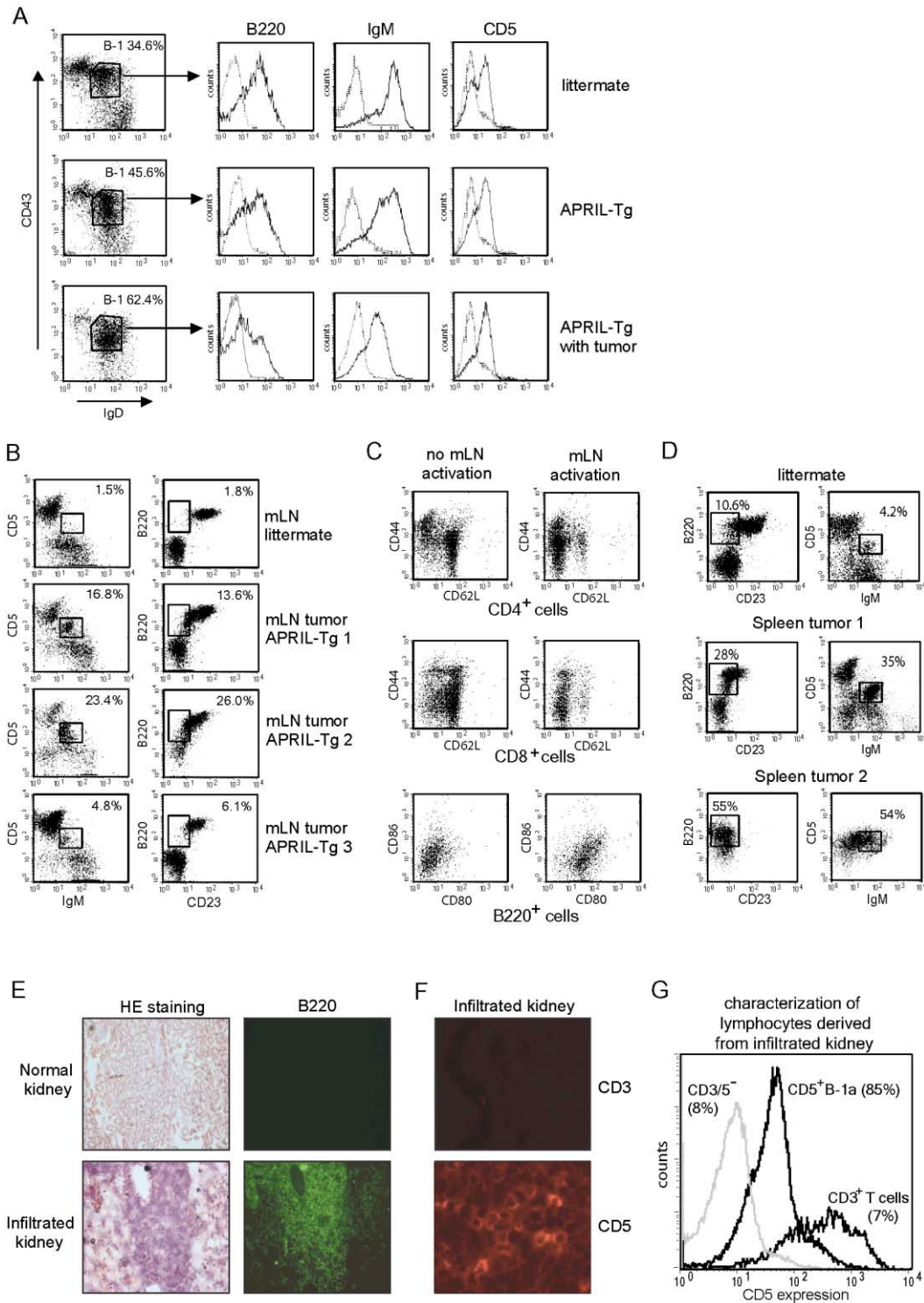


Figure 5. Accumulation of B1 cells in mLN and infiltration of B-1 cells into nonlymphoid tissue in older APRIL Tg mice

A: B-1 cell expansion is most pronounced in APRIL-Tg mice showing mLN and PP enlargement. Representative data are shown for 10 mice analyzed.

B: Increased numbers of B-1 cells are detected in enlarged mLN. B-1 cells (boxed) are $IgM^{low}CD5^{dim}$ and $CD23^{-}B220^{dim}$.

C: Transfer of pure peritoneal B-1 cells from an APRIL-Tg mouse with significant B-1 expansion results in mLN lymphocyte activation. Left panels show a nonactivated mLN, right panels show an activated mLN.

D: B-1 cell infiltration in enlarged spleen of two separate APRIL-Tg mice (18-month-old). A spleen from an age-matched littermate is used as a control. Percentages indicate B-1 cells in boxed areas ($IgM^{low}CD5^{dim}$ and $CD23^{-}B220^{dim}$).

E: HE and anti-B220 staining of infiltrating lymphocytes in kidney sections from an 18-month-old APRIL-Tg mouse. Multiple areas of cell infiltration from blood vessels are detected. Sections from control mice show no cell infiltration and reveal normal tissue architecture.

F: Kidney infiltrating cells lack CD3 expression (top), but a fraction of the cells are $CD5^{+}$ (bottom). An enlargement is shown of an area containing infiltration of $B220^{+}$ cells. Several sites of $B220^{+}$ cell infiltration were detected in the kidney; a representative image is shown.

B220^{dim}, CD23⁻ (Figure 5D), and CD19⁺ and CD3⁻ (not shown), indicating massive B-1a cell expansion in the spleen. Moreover, clear macroscopic alterations were evident in kidney and liver (not shown) in these mice, and immunohistochemical analysis of these nonlymphoid organs showed extensive lymphoid cell infiltration (Figure 5E and not shown). Infiltrating cells were B220⁺, CD3⁻, TCR $\alpha\beta$ ⁻ (Figure 5E and not shown), indicating that infiltrating cells are B cells and not T cells. Most of the B220⁺, CD3⁻, TCR $\alpha\beta$ ⁻ cells appeared to be CD5⁺, though not all of them (Figure 5F). To analyze whether this is due to the presence of a mixed population of cells or due to the efficiency of the antibody in immunohistochemistry, we decided to further identify the cells present in the infiltrate. For this, we made use of a novel technique that allows cells to be isolated from snap-frozen tissue sections and to be processed for FACS staining (Baeten et al., 2002). This analysis revealed that the majority of lymphoid cells (85%) present in the kidney, which includes the blood vessels, are CD5⁺ and CD3⁻, while only a minority are T cell (7% CD5⁺/CD3⁺) or do not stain for CD5 or CD3 (8%) (Figure 5G). We therefore conclude that the cells infiltrating the kidney are B220⁺ (Figure 5E), CD5⁺ (Figures 5F and 5G), and for the larger part CD3⁻ (Figures 5F and 5G), and are thus malignant B-1a tumor cells. In summary, the B-1 cell population expansion observed in aging Tg mice leads initially to tumor formation within the lymphoid system; these cells eventually metastasize and infiltrate nonlymphoid tissues such as mucosa, kidney, and liver.

B-1 cells appear to arise mainly, but not exclusively, during fetal/neonatal B cell lymphopoiesis (Stein et al., 2002). In contrast to B-2 cells, B-1 cells have self-renewal capacity and thus maintain normal numbers during adulthood (Potter and Melchers, 2000). Several reports suggest that B-1 cells are susceptible to oncogenic mutation and progression to B cell neoplasia (Potter and Melchers, 2000). Inherent or induced differences in individual B-1 cell clone division rates can lead in time to the development and expansion of dominant clones within the B-1 cell population (Stall et al., 1988), which are detectable in older animals (>18 months) of most, if not all, mouse strains (Stall et al., 1988). These clonal populations arise in the peritoneal cavity and eventually invade lymphoid tissue (Stall et al., 1988). We observed B-1 cell expansion in 4- to 5-month-old APRIL-Tg mice, whereas B-1 cell numbers in 6-week-old Tg mice were similar to those of age-matched littermates (Stein et al., 2002). Our findings suggest that APRIL promotes and accelerates age-related B-1 cell expansion, which appears to be a result of increased survival capacity rather than increased mitotic activity. This mirrors the status of human B-CLL cells, which are characterized by low proliferative activity and progressive accumulation of clonal B lymphocytes blocked in the G₀/G₁ cell cycle phases (Andreuff et al., 1980).

The highest incidence of tumor formation in APRIL-Tg mice was observed in mLN and PP, and less frequently in spleen. The mLN is considered to be the first lymphoid organ affected

by initially nonmalignant lymphoid abnormalities that can progress to malignant lymphoma (Frederickson and Harris, 2000). Further analysis is needed to understand the mechanisms by which B-1 cells induce lymphocyte hyperplasia in mLN and PP in older APRIL-Tg mice. Differences in B and T cell composition do not account for the hyperplasia observed in older APRIL-Tg mouse mLN, which appears to be the consequence of a mutual activation loop between B and T cells. B-1 cells present in hyperplastic mLN express large amounts of IL-10 and TNF α (not shown), which could be involved in the activation of resident mLN lymphocytes (O'Garra and Howard, 1992). In fact, a recent study in NZB IL-10 knockout mice has well documented the crucial role for IL-10 in the development and expansion of malignant B-1 cells (Czarneski et al., 2004). Moreover, an increased CD4⁺:CD8⁺ T cell ratio was detected in PP and mLN of older APRIL-Tg mice, possibly the result of such an activation loop. In contrast, the CD4⁺:CD8⁺ T cell ratio was decreased in the blood of tumor-bearing APRIL-Tg mice (not shown). It is noteworthy that inverse CD4⁺:CD8⁺ T cell ratio evolution has also been observed in lymphoid organs and blood of B-CLL patients (Terstappen et al., 1990).

We observed an expansion of peritoneal B-1 cells in 4-month-old APRIL-Tg mice that was more pronounced in 9- to 12-month-old animals. About 40% of Tg mice at this age displayed expansion of T and B cell populations in mLN or PP. We propose that APRIL is a supporting factor for B-1 cell stimulation, and that its overexpression results in increased B-1 cell survival and activity. Although APRIL can support B cell activation *in vitro*, an *in vivo* effect on B cells has not yet been described (Mackay et al., 2002). In contrast, BLyS, the closest homolog of APRIL, supports B cell survival *in vivo* and *in vitro* (Mackay et al., 2002). It is highly unlikely, though, that the effects observed in APRIL-Tg mice are mediated by BLyS, as no increases in BLyS mRNA are observed in the spleen (not shown). Moreover, the APRIL-Tg mice do not display typical signs of BLyS expression, such as splenic B cell expansion (Mackay et al., 2002) (Table 1). mRNA for two the known APRIL receptors, TACI and BCMA, is expressed at comparable levels in all B cell subsets (not shown). Nevertheless, APRIL binds with much higher affinity to B-1 B cells as compared to the splenic subsets (not shown), suggesting the possible involvement of a third APRIL receptor. Though the receptor(s) mediating the stimulatory effect of APRIL on B-1 cells remains to be identified, it is evident that APRIL imposes an *in vivo* survival advantage on PEC B-1 cells, while B-2 cells are not affected (Figure 4). How this survival advantage leads to B-1 cell accumulation in mLN and lymphoid organ hyperplasia is still an enigma. We believe that activation of B-1 cells by antigenic stimulation would allow these cells to migrate to the mLN. In line with this hypothesis is the observation that NP-LPS, a thymus-independent type 1 antigen that is known to activate B-1 cells, increases the number of B-1 cells present in the mLN significantly even at 6 weeks after NP-LPS administration (not shown). The accumulation of B-1 cells in the mLN

G: Kidney-infiltrating cells are mainly CD5 positive and are not T cells. 30 μ m cryosections of the infiltrated kidney shown in **E** and **F** were selected on the basis of infiltration. Cells were retrieved for FACS analysis and stained for H-2K^b, CD5, and CD3. H-2K^b expression was used to select lymphocytes, and CD5 and CD3 expression was measured to determine the incidence of B-1a cells in the infiltrated kidney. Shown is the CD5 expression in the three H-2K^b-high lymphocytes populations visible in the infiltrated kidney as determined by CD3 and CD5 staining. Percentages indicate the relative contribution of the population to the total lymphocytes present in the kidney.

may affect the lymphoid environment by producing cytokines and could thus lead to mLN hyperplasia. All mice that display spleen and kidney infiltration display mLN hyperplasia as well, suggesting that it is a prerequisite for the eventual malignant phenotype. It is very likely that this malignant transformation requires a second hit, similar to what has been suggested for the TCL1-induced lymphoma formation (Virgilio et al., 1998).

Age-related B-1 cell neoplasia in mice has been compared to human B-CLL, as both diseases develop during aging as a result of slowly expanding CD5⁺ B cell populations. The NZW and TCL1 Tg mouse models for B-CLL show B-1 cell expansion as a result of a survival benefit (Bichi et al., 2002; Hamano et al., 1998). By analogy with our data for APRIL-Tg mice, the TCL1 Tg mice show initial gradual increases in the peritoneal CD5⁺ B cell population; later stages are characterized by spreading of these cells to lymphoid organs. A role for B-1 cell tumor formation in humans is persuasively supported by the detection of APRIL overexpression in a large fraction of B-CLL tumors tested and of increased APRIL serum levels in these patients. Results from our APRIL-Tg mice strongly suggest a role for this TNF ligand in B-1 cell tumor development. Increased APRIL expression in B-CLL patients indicates that APRIL transgenics are an appropriate mouse model for study of the human disease.

APRIL-Tg mice express the transgene in T cells, which results in elevated APRIL serum levels. APRIL thus acts systemically in the Tg mice (Stein et al., 2002), affecting B-1 cell survival. Since APRIL acts as a secreted factor, elevated APRIL levels may also be the result of production by non-tumor cells. It is thus reasonable to assume that determination of APRIL mRNA levels in patient tumor cells may underestimate true APRIL involvement in B-CLL. It may be of interest to determine APRIL production by so-called nurse-like cells, which are described to supply B-CLL tumor cells with a survival factor *in vitro*, and reported to exist in B-CLL patients as well (Tsukada et al., 2002). The increased APRIL mRNA and serum levels in B-CLL patients, combined with the B-1-associated tumorigenesis in APRIL-Tg mice, substantiate the involvement of this cytokine in B-CLL and may yield new therapeutic strategies aimed at preventing APRIL-induced tumor cell survival.

Experimental procedures

Mice

Generation of APRIL-Tg mouse lines 3919 and 2968 has been described (Stein et al., 2002). Mice used in these experiments had been backcrossed at least seven times onto the C57BL/6 background. Mice were housed under specific pathogen-free conditions at the CNB (Madrid) or the CPV-LUMC (Leiden). Animal experiments were performed in compliance with national and institutional guidelines. Experiments were approved by the animal ethical committee of the Leiden University and authorized by the respective French authorities (Départementale des services vétérinaires de Préfecture De l'Herault), permit number B 34-172-16.

Flow cytometric analysis

Cells were stained with appropriate antibodies and analyzed on a Becton-Dickinson flow cytometer. Freshly prepared lymphocytes were resuspended in FACS buffer (1% BSA, 0.05% sodium azide in PBS). Labeled anti-mouse -H-2K^b (AF6-88.5), -CD3 (145-2C11), -CD4 (L3T4), -CD8 (Ly-2), CD45R/B220 (RA3-6B2), -IgD (11-26c.2a), -IgM (R6-60.2), CD69 (H1.2F3), CD62L (Mel-14), -CD44 (IM7), -CD45RB (16A), -CD23 (B3B4), -CD24 (HSA, 30F1), -CD43 (1B11), -CD21 (7G6), CD5 (53-7.3), CD80 (16-10A1), CD86 (GL1), -Mac3 (M3/84), -Ly6C (Gr1) (RB6-8C5), -CD11c (HL3), and -CD138 (281-2) antibodies, as

well as Ki-67 kits, were purchased from BD Pharmingen and used according to manufacturer's specifications.

ELISA

APRIL serum immunoglobulin was quantified by standard ELISA techniques using BCMA-FC (1 µg/ml; Sigma-Aldrich) for capture and anti-APRIL antibody (1 µg/ml) (Lopez-Fraga et al., 2001; Pradet-Balade et al., 2002; Stein et al., 2002) combined with on human IgG absorbed α-rabbit-peroxidase secondary antibody (Jackson laboratory) for detection. Specificity of APRIL detection was verified by including wells coated with an irrelevant Fc-hybrid protein as control. Serum dilutions used for quantification were within the linear absorbance range at 492 nm, and recombinant APRIL (R&D Systems) was used for standardization. Sera tested included samples from untreated and treated B-CLL patients; this revealed no significant difference between these two groups. The use of B-CLL samples for RNA analysis and sera protein detection was approved by the Medical Ethical Committee of the Amsterdam Medical Center (AMC).

Serum IgA levels were determined using an ELISA kit (BD/PharMingen) according to manufacturer's instructions.

Reverse transcriptase and PCR

B-CLL samples were directly subjected to RNA isolation using the Qiagen RNeasy isolation procedure. Total RNA (2 µg) was reverse transcribed using AMV-reverse transcriptase, then analyzed for APRIL expression by PCR using CCAGCCTCATCTCCTTTCTTGC as forward and TCACAGTTTCACAAACCCAGG as reverse primer. β actin served as an internal control and was amplified using GGCATCGTGATGGACTCCG as forward and GCTGGAAGGTGGACAGCGA as reverse primer. Three B-CLL samples were chosen and purified to homogeneity by negative selection for CD14 and CD3 (the impurities detected in these samples) using CD14-PE and CD3-PE (BD PharMingen) and anti-PE magnetic beads (Miltenyi Biotech). Control B cell samples were isolated from pooled buffy coats (Bloodbank Leiden, >5 donors) by positive selection with CD19-FITC (BD PharMingen) and anti-FITC magnetic beads (Miltenyi Biotech). After purification of normal B cells or B-CLL samples, RNA was immediately isolated as above.

In vitro and in vivo survival

Peritoneal cells were depleted of T cells using CD3-FITC staining and magnetic anti-FITC beads, then cultured *in vitro* (10⁵ cells/well). Cells were collected at different time points, stained for CD43 to discriminate B-1 from B-2 B cells, and cell death measured using PI-exclusion by FACS analysis. Percentages are calculated as number of CD43⁺PI⁺/total number of CD43⁺ cells.

For *in vivo* survival assays, peritoneal cells were isolated from C56BL/6 SJL mice, which contain a congenic marker (CD45.1) that allows their detection in the C56BL/6 background of the transgenic strain. SJL PEC cells were depleted of T cells using Dynal beads and subsequently injected intraperitoneally into 8-month-old littermates and APRIL-Tg (mice containing detectable levels of APRIL in their serum [Stein et al., 2002]). PEC cells of a group of recipient mice were analyzed immediately after injection to determine the amount of B-1 and B-2 cells retrievable from the PEC, and 10 days later from a separate group of 5 mice to determine the *in vivo* survival. B-1 and B-2 cells were identified with CD43 and IgD and the fold decrease in cell number was determined by dividing the absolute number present at day 10 by the absolute number present at day 0.

Histopathology

Lymphoid organs were formalin-fixed (5%) and paraffin embedded; deparaffinized sections (4 µm) were hematoxylin/eosin stained. For immunostaining, sections were deparaffinized and treated with distilled water containing 3% H₂O₂ in 70% methanol, followed by microwave treatment in 10 mM sodium citrate buffer, blocked using a biotin blocking kit (Dako, Glostrup, Denmark), and incubated with 20% normal goat serum. Sections were incubated with biotin-anti-B220 (Southern Biotechnology) antibody (overnight, 4°C) and developed with the StrepABCComplex/HRP kit (Dako). Phosphatase activity was developed using BCIP/NBT substrate system (Dako) and analyzed by conventional microscopy. Tissue sections were analyzed by Leica Q-Win software to determine Peyer's patch size.

Immunofluorescence

Kidneys were snap-frozen in liquid nitrogen, and 5 μ m sections were stained with hematoxylin/eosin and for B220, CD5, and CD3 (BD Pharmingen). Detection was direct with FITC-label (B220) or indirect with streptavidin-conjugated-Alexa fluor 546 (Molecular Probes). Background staining was blocked using the endogenous biotin blocking kit (Molecular Probes). Normal spleen served as a positive control for all antibodies used. Isolation of lymphoid cells from snap-frozen tissue for FACS analysis was performed as described by Baeten et al. (2002). In brief, 30 μ m sections were fixed in paraformaldehyde (1%) and collagenase-treated for 30 min (5 mg/ml). Afterward, cells were stained with anti-H-2K^b, -CD3, and -CD5 to determine the identity of infiltrating cells.

Adoptive transfer of peritoneal B-1 cells

Peritoneal cells from an advanced stage tumor-bearing mouse were cultured overnight to deplete peritoneal macrophages by adherence; the resulting culture was >95% B-1 cells. Cells (10^7) were subsequently injected intraperitoneally into 8-week-old C57BL/6 mice. After 6 weeks, mice were sacrificed and mLN were isolated and analyzed for CD4⁺, CD8⁺ T cell and B220⁺ B cell activation using CD44, CD62L, CD80, and CD86.

Statistical analysis

The statistical significance of the data was determined using ANOVA analysis of variance. P values > 0.05 are considered nonsignificant, p values < 0.05 as significant, p values < 0.01 as very significant, p values < 0.0001 as extremely significant.

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