B-cell activating factor and v-Myc myelocytomatosis viral oncogene homolog (c-Myc) influence progression of chronic lymphocytic leukemia

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Mice bearing a v-Myc myelocytomatosis viral oncogene homolog (*c-Myc*) transgene controlled by an Ig-alpha heavy-chain enhancer (i*Myc*$^{C\alpha}$ mice) rarely develop lymphomas but instead have increased rates of memory B-cell turnover and impaired antibody responses to antigen. We found that male progeny of i*Myc*$^{C\alpha}$ mice mated with mice transgenic (Tg) for CD257 (B-cell activating factor, BAFF) developed CD5$^+$ B-cell leukemia resembling human chronic lymphocytic leukemia (CLL), which also displays a male gender bias. Surprisingly, leukemic cells of *Myc/Baff* Tg mice expressed higher levels of *c-Myc* than did B cells of i*Myc*$^{C\alpha}$ mice. We found that CLL cells of many patients with progressive disease also expressed high amounts of *c-MYC*, particularly CLL cells whose survival depends on nurse-like cells (NLC), which express high-levels of BAFF. We find that BAFF could enhance CLL-cell expression of *c-MYC* via activation the canonical IκB kinase (IKK)/NF-κB pathway. Inhibition of the IKK/NF-κB pathway in mouse or human leukemia cells blocked the capacity of BAFF to induce *c-MYC* or promote leukemia-cell survival and significantly impaired disease progression in *Myc/Baff* Tg mice. This study reveals an important relationship between BAFF and *c-MYC* in CLL which may affect disease development and progression, and suggests that inhibitors of the canonical NF-κB pathway may be effective in treatment of patients with this disease.

nurselike cells | nuclear factor κ-light-chain enhancer of activated B cells, IκB kinase inhibitor | prognostic factors | leukemia-cell survival

The cellular proto-oncogene v-Myc myelocytomatosis viral oncogene homolog (*c-MYC*) was first identified as a cellular proto-oncogene in Burkitt lymphoma, a high-grade mature B-cell malignancy that expresses high levels of *c-MYC* as a result of chromosomal translocations (1). Chromosomal alterations resulting in increased expression of *c-MYC* also were found in other B-cell malignancies, including diffuse large B-cell lymphoma (2) and multiple myeloma (3). In rare cases of chronic lymphocytic leukemia (CLL), *c-MYC* translocations were found associated with progressive disease and poor prognosis (4).

Mouse models with dysregulated expression of *c-Myc* at various stages of B-cell development have been generated, including *Eμ-Myc* (5), *iMyc*$^{E\mu}$ and *iMyc*$^{C\mu}$ mice and mice bearing a *c-Myc* transgene controlled by an Ig-alpha heavy-chain enhancer (*iMyc*$^{C\alpha}$ mice) (6). These transgenic strains develop either immature B-cell lymphomas (*Eμ-Myc* mice) (5), Burkitt lymphoma-like disease (*iMyc*$^{E\mu}$ mice) (6), or plasmacytic malignancies (*iMyc*$^{E\mu}$ mice) (6). Apparent exceptions are *iMyc*$^{C\alpha}$ mice, in which only a small proportion of aged animals ($\leq 9\%$) develop lymphomas (7). Instead, these mice have poor antibody responses caused by the high turnover rates of plasma cells and memory B cells. B-cell activating factor of the tumor necrosis family (BAFF or CD257) failed to slow the turnover rates of plasma cells. Effect(s) of BAFF on memory B cells of these animals was not examined. BAFF interacts with three B-cell receptors, B-cell maturation (BCMA), activator and calcium-modulator and cyclophilin ligand interactor (TACI), and BAFF-R (BR3), and triggers activation of IκB kinase (IKK)/NF-κB (8). Consequently, *Baff* transgenic (*Baff*-Tg) mice have reduced turnover rates of mature and marginal-zone B cells (9, 10), and aged mice develop a lupus-like autoimmune disease (9, 11).

In a prior study (12), we found that *Eμ-TCL1/Baff*-Tg mice had accelerated rates of leukemogenesis because of reduced rates of spontaneous B-cell apoptosis relative to *Eμ-TCL1*-Tg mice. Conceivably, the progeny of *iMyc*$^{C\alpha}$-Tg mice mated with *Baff*-Tg mice might have reduced turnover rates of memory B cells because of increased expression of Myc from the *iMyc*$^{C\alpha}$ transgene. Reduced rates of cell death in the setting of perpetual cell proliferation could give rise to leukemia resembling human CLL, which apparently is derived from memory-type, antigen-experienced B cells (13, 14).

Results

Myc/Baff-Tg Mice Develop Aggressive CLL-Like Disease. To evaluate the oncogenic effect of the *c-MYC*–BAFF interaction, we crossed *iMyc*$^{C\alpha}$-Tg (*Myc*) mice, in which *c-MYC* is under control of the Ig Eα enhancer (7), with *Baff*-Tg mice, in which BAFF is produced by the liver (9). Male *Myc/Baff*-Tg mice developed lymphocytosis at age 3 mo because of increased blood B-cell number relative to that noted for WT, single-Tg mice, or female double-Tg mice (Fig. S1 *A–C*). Expansions of a CD5$^+$CD3$^-$B220^{low}IgM^{low} B-cell population in blood and spleens were observed in most male *Myc/Baff* mice, whereas most female double-Tg mice did not have such cells (Fig. 1*A* and Fig. S1 *D and E*). At age 8 mo, 78% of male *Myc/Baff* mice (14 of 18) and 9% of females (1 of 11) had circulating CD5$^+$CD3$^-$B220^{low} cells that were not detected in *Myc* or *Baff* mice even at 18 mo of age.

CD5$^+$CD3$^-$B220^{low} B cells were products of a monoclonal expansion of mature B cell-like cells, as revealed by Southern blot analysis of splenocytes from mice age 8 mo or older (Fig. 1*B*). Wright–Giemsa staining of blood smears showed excessive numbers of well-differentiated lymphocytes admixed with smudge cells, a feature resembling human CLL (Fig. S2*A*). The median life span of male *Myc/Baff* mice was 10 mo, significantly shorter than that of *Myc* or *Baff* mice (Fig. 1*C*).

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The authors declare no conflict of interest.

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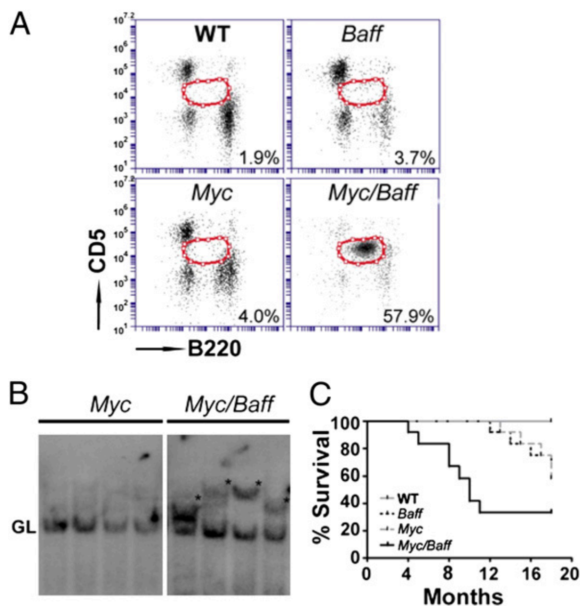

Fig. 1. Male *Myc/Baff* mice develop a monoclonal $CD5^+CD3^-B220^{low}$ cell population resembling CLL. (*A*) Blood was collected from 8-mo-old WT, *Baff*-Tg, *Myc*-Tg, and *Myc/Baff*-Tg mice and was stained with CD5–allophycocyanin (APC) and B220-FITC antibodies and analyzed by flow cytometry. Shown is a representative dot plot from 1 of 12 mice per genotype. (*B*) Early clonal expansion of B cells in *Myc/Baff*-Tg mice was determined by Southern blot analysis of genomic DNA from splenocytes of mice age 8 mo or older using a J_H probe. Monoclonal bands are marked by an asterisk. GL, germline bands. (*C*) Survival (Kaplan–Meier) plots of cohorts of 12 age-matched male mice of the indicated genotypes.

Increased ^{18}F-labeled 2-fluoro-2-deoxy-D-glucose (FDG) uptake is observed at tumor-infiltrating sites in ≈33% (19 of 57) of CLL cases (15). To examine FDG uptake in leukemic mice, we performed an FDG-PET scan on 12-mo-old *Myc* and *Myc/Baff* male mice. *Myc/Baff* mice showed three times greater splenic FDG uptake than *Myc* mice (Fig. 2*A*), suggesting elevated leukemic cell metabolism. Splenomegaly was obvious in *Myc/Baff* mice after 4 mo of age, and the average weight of *Myc/Baff* spleens at 8 mo was 2.6-fold higher than *Baff* or *Myc* spleens and 5.2-fold higher than WT spleens (Fig. 2*B*). H&E staining of splenic sections of 8-mo-old mice exhibited significantly enlarged white pulp in *Myc/Baff* mice with loss of normal splenic architecture resulting from a diffuse infiltration of mature lymphocytes (Fig. 2*C*). As in the blood smears (Fig. S2*A*), the majority of splenocytes in *Myc/Baff* mice showed features typical of well-differentiated B cells, with very sparse cytoplasm and round nuclei (Fig. S2*B*). These data suggest that *Myc/Baff* mice develop a $CD5^+$ B-cell lymphoproliferative disease that closely resembles human CLL.

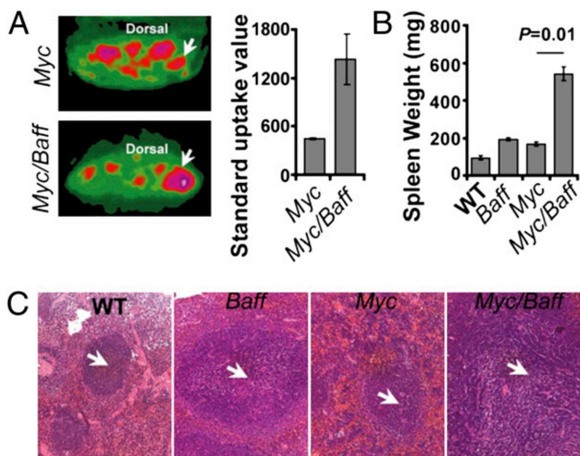

Fig. 2. *Myc/Baff* mice exhibit splenomegaly, increased splenic FDG uptake, and disrupted microarchitecture. (*A*) Representative FDG-PET scans of *Myc* and *Myc/Baff* mice. The arrows indicate spleen locations (regions of interest, ROI) (*Left*). Average FDG intensity of the ROI, was determined for two 1-y-old mice of each genotype (*Right*). (*B*) Average spleen weight of 8-mo-old mice of the indicated genotypes. Data are means ± SEM ($n = 3$). (*C*) Paraffin-embedded spleen sections from 8-mo-old male mice of the indicated genotypes were stained with H&E ($n = 3$, magnification: 200×). Arrows indicate the center of the white pulps that are disrupted in *Myc/Baff* mice.

BAFF Enhances Expression of Antiapoptotic Genes and Protects B Cells from Apoptosis Associated with Dysregulated Expression of c-Myc. Transcriptome comparison between $CD5^+CD3^-$ leukemia cells and *Myc* $B220^+$ B cells respectively obtained from *Myc/Baff* and *Myc* transgenic mice ($n = 3$ at 8 mo of age) revealed that 319 genes were differentially expressed (with a twofold increase or decrease in *Myc/Baff* $CD5^+CD3^-$ leukemic cells relative to *Myc* $B220^+$ B cells; $P \leq 0.05$ assuming unequal variance); 149 genes were up-regulated, and 170 genes down-regulated. Among the genes altered in *Myc/Baff* $CD5^+CD3^-$ leukemia cells were those with relevance to human CLL, including elevated expression of the antiapoptotic Bcl-2 family members *Bcl2*, *Mcl1*, and *A1*, decreased expression of death receptor *Fas*, marked elevation in *IL-10*, *IL-5 receptor α*, *IL-6 signal transducer*, and *TNF super family member 8*, as well as signatures of aggressive CLL, such as *Ctla-4* and *Zap70* (Fig. S3). We confirmed these findings by quantitative PR-PCR (qRT-PCR) analyses (Fig. 3*A* and Fig. S3*B*). Protein expression of

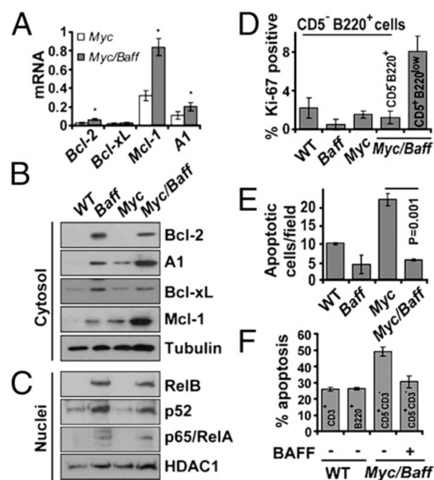

Fig. 3. BAFF elevates expression of survival and proliferation genes in leukemic cells. (*A*) $B220^+$ B cells and $CD5^+CD3^-B220^{low}$ leukemic cells were isolated from 8-mo-old *Myc* and *Myc/Baff* littermates, respectively. RNA was extracted and analyzed by qRT-PCR for expression of the indicated antiapoptotic genes. Results are means ± SEM ($n = 3$). $*P < 0.05$. (*B* and *C*) Splenic $B220^+$ cells from WT, *Baff*, and *Myc* mice and $CD5^+CD3^-B220^{low}$ leukemic cells from 8-mo-old *Myc/Baff* mice were divided into cytosolic (*B*) and nuclear (*C*) fractions that were gel separated and analyzed by immunoblotting. Results are representative of three independent experiments. (*D*) Blood mononuclear cells from 4-mo-old mice of the indicated genotypes were stained for CD5-APC, B220-phycoerythrin (PE), and Ki-67–FITC. The histogram shows the average percentages ± SEM of $Ki-67^+$ cells ($n = 5$). (*E*) Paraffin-embedded spleen sections from 8-mo-old mice of the indicated genotypes were subjected to TUNEL staining. Numbers of apoptotic cells per field were determined from five age-matched mice per group. Results are means ± SEM. (*F*) Primary mouse T and B cells were isolated from WT spleens, and leukemic cells were isolated from *Myc/Baff* mice. Cell viability was determined by PI and DIOC6 staining of triplicate samples. Results are means ± SEM ($n = 3$).

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Bcl-2 family members, including Bcl-2, Bcl-xL, Mcl-1, and A1, was examined by immunoblot (Fig. 3*B*). We also looked for differences in expression of predefined gene sets by Gene Set Enrichment Analyses (GSEA) (16). One hundred twenty-two gene sets were significantly enriched, and 181 gene sets were significantly down-regulated in *Myc*/*Baff* CD5$^+$CD3$^-$ leukemic cells compared with *Myc* B220$^+$ B cells ($P < 0.01$). Among gene sets that were enriched in *Myc*/*Baff* CD5$^+$CD3$^-$ leukemia cells were five apoptosis-related and 18 stress-induced (including UV, chemical, virus infection) gene sets, further underscoring the antiapoptotic role of BAFF (Fig. S3*C*). As a major target for BAFF signaling, the NF-κB gene set also was enriched in *Myc*/*Baff* CD5$^+$CD3$^-$ leukemia cells (Fig. S3*C*). This finding is supported by immunoblot analysis of nuclear extracts, which showed that B cells from *Baff* mice or leukemia cells from *Myc*/*Baff* mice exhibited elevated levels of nuclear RelA, RelB, and p52, relative to B cells from WT or *Myc* mice (Fig. 3*C*). Proliferation gene sets also were elevated in *Myc*/*Baff* CD5$^+$CD3$^-$ leukemic cells (Fig. S3*C*).

Because only double-Tg mice developed leukemia, we investigated whether constant BAFF exposure increased leukemia-cell proliferation or survival. Staining blood mononuclear cells for Ki-67 was performed to examine for proliferating T cells (CD3$^+$CD5$^+$), B cells (CD3$^-$B220$^+$), or leukemia cells (CD5$^+$CD3$^-$B220^{low}). T or B cells in all four genotypes generally had relatively low proportions of proliferating cells, ranging from 0.1 to 3.0% Ki-67$^+$ cells. The one notable exception was the relatively high average proportion of Ki-67$^+$ cells (8.1%) noted among the CD5+CD3-B220^{low} cells of *Myc*/*Baff* transgenic mice ($n = 5$) (Fig. S4*A* and Fig. 3*D*).

TUNEL staining of splenic sections revealed that *Myc* mice had four times more apoptotic cells in their spleens than *Myc*/*Baff* mice, suggesting that BAFF promotes B-cell expansion, at least in part, by inhibiting apoptosis of leukemic cells (Fig. S4*B* and Fig. 3*E*). Compared with normal T or B cells, the CD5$^+$CD3$^-$B220^{low} cells of *Myc*/*Baff* mice had higher proportions of cells undergoing apoptosis; these proportions could be reduced by incubation with BAFF (Fig. 3*F*). Collectively, these data suggest that BAFF promotes leukemogenesis in part by inhibiting apoptosis associated with dysregulated expression of *c-MYC*.

BAFF Stimulates *c-Myc* RNA and Protein Expression. Array analysis revealed that *c-Myc* mRNA was elevated in leukemia cells from *Myc*/*Baff* mice relative to that in B cells from *Myc* mice (Fig. 4*A*, *Left*). These findings were confirmed by qRT-PCR analysis (Fig. 4*A*, *Right*), suggesting that constitutive BAFF signaling enhances *c-Myc* transcription or mRNA stability. c-Myc protein also was elevated in *Myc*/*Baff* leukemia cells relative to levels in B cells from single-Tg *Myc* mice (Fig. 4*B*).

Similar results were observed in human primary CLL cells. BAFF treatment induced *c-MYC* RNA in most (8 of 10) CLL cells expressing low levels of *MYC* and in only a few (two of eight) high-*MYC* expressors (Fig. S5*A*). BMS345541, a specific IKKβ inhibitor, blocked the capacity of BAFF to induce c-*MYC* in CLL cells (Fig. 4*C*). IKK-dependent elevation of c-MYC was confirmed at the protein level (Fig. 4*D*). BMS345541 also could inhibit BAFF-induced IκBα degradation or RelA nuclear translocation (Fig. S5*B*). Consistently, silencing IKKβ with shRNA in primary CLL cells blocked BAFF-induced expression of c-MYC (Fig. 4*E*). In addition, ChIP revealed that BAFF induced binding of RelA/p65 to two of the predicted NF-κB consensus-binding sites of *c-MYC* promoter in two independent human primary CLL samples (Fig. 4*F*, and Fig. S5*C*).These data suggest that BAFF prevents CLL cells from undergoing apoptosis through activation of NF-κB which, in addition to up-regulating antiapoptotic genes, also up-regulates expression of *c-MYC*.

Transfer of CD5$^+$CD3$^-$ Leukemic Cells to *Baff* Mice Results in Rapid Disease Progression Involving Lymph Nodes and Marrow. Although we observed extensive expansion of blood CD5$^+$CD3$^-$ B cells in male *Myc*/*Baff* mice as early as 4 mo of age, (oligo)clonal disease was observed in mice at 8 mo of age or older (Fig. 1*B*). To determine the stage at which CD5$^+$CD3$^-$ B cells can be transferred

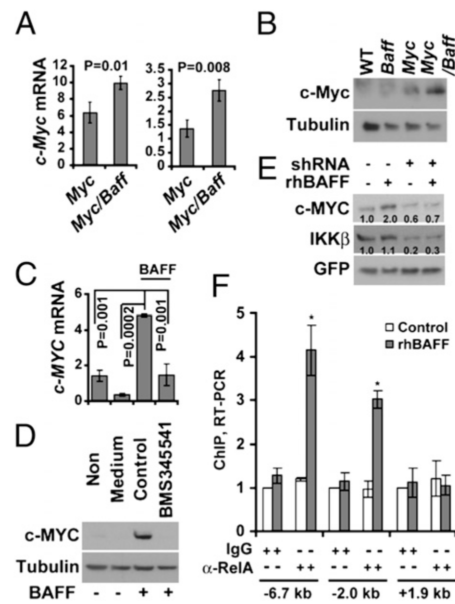

Fig. 4. BAFF enhances *c-MYC* mRNA expression through the IKK/N-κB pathway. (*A*) *c-MYC* mRNA expression was examined by microarray (*Left*) and qRT-PCR (*Right*) analyses of B220$^+$ cells and CD5$^+$CD3$^-$B220^{low} cells isolated from three 8-mo-old *Myc* or *Myc*/*Baff* male mice, respectively. Results are means ± SEM ($n = 3$). (*B*) Primary B cells of the indicated genotypes and leukemic cells from *Myc*/*Baff* mice were isolated by magnetic separation and analyzed for *c-MYC* expression by immunoblotting. (*C*) Primary CLL cells were pretreated with BMS345541 (1 μM) for 20 min and then were incubated with recombinant human (rh) BAFF (200 ng/mL). Total RNA was isolated 6 h later, and *c-MYC* mRNA was measured by qRT-PCR. Results are averages ± SEM of three independent experiments with *P* values indicated. (*D*) Primary CLL cells were cultured and treated as in *C*. Cell lysates were collected 12 h later and analyzed for *c-MYC* expression by immunoblotting. (*E*) Primary CLL cells were infected with lentivirus-encoding GFP and shRNA specific for IKKβ mRNA or a scrambled control. GFP$^+$ cells were isolated by flow cytometry and treated with BAFF. Protein lysates were analyzed by immunoblotting, quantified with Image J, and normalized to GFP expression. (*F*) Primary CLL cells were treated with BAFF for 2 h before ChIP analysis was performed on the *c-MYC* promoter. Specific primers flanking the predicted NF-κB consensus-binding sites were used for real-time PCR.

successfully into syngeneic recipients, we purified CD5$^+$CD3$^-$ B cells from spleens of 6- and 12-mo-old *Myc*/*Baff* mice and infused 10^6 cells into syngeneic *Baff* recipients. Blood mononuclear cells were monitored by flow cytometry every other week for the appearance of a CD5$^+$CD3$^-$ population. CD5$^+$CD3$^-$ B cells from 6-mo-old donors did not manifest clonal expansion in *Baff* mice, although we were able to detect CD5$^+$CD3$^-$ B cells in the blood of *Baff* recipients from 2 wk to 9 mo after cell transfer (Fig. S6*A*). However, transfer of CD5$^+$CD3$^-$ leukemic cells from 12-mo-old donors resulted in CLL-like disease in *Baff*-Tg recipients (Fig. S6*B*), which developed enlarged spleens 5 wk after transfer (Fig. S6*C*). CD5$^+$CD3$^-$ leukemic cells were found in spleen, lymph nodes, and marrow of *Baff* recipients (Fig. S6*D*), which died 5–8 wk after transfer.

***c-MYC* Expression in CLL Correlates with Disease Progression and Dependency on Nurse-Like Cells.** We interrogated the Affymetrix array data obtained by analysis of CLL cells of 166 patients and observed a broad distribution in relative levels of *c-MYC* mRNA expression (Fig. 5*A*). Differences in *c-MYC* RNA and protein were confirmed by qRT-PCR and immunoblot analyses (Fig. S7). We examined the relationship between *c-MYC* expression and the time from diagnosis to initial therapy, a parameter that correlates with disease progression (18). The median time from diagnosis to initial treatment for the patients whose CLL cells

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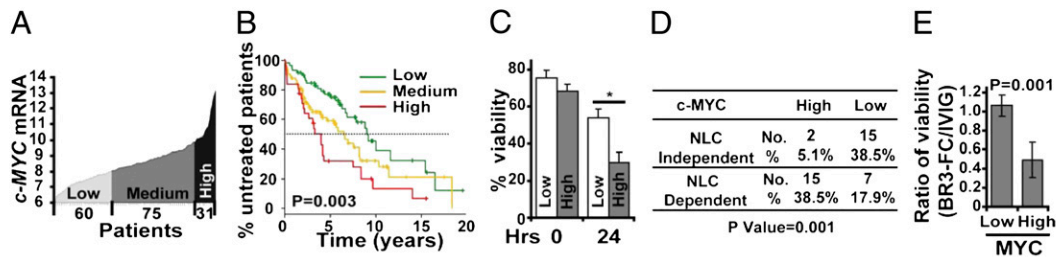

Fig. 5. Elevated *c-MYC* expression is associated with CLL progression and NLC dependency. (*A*) Relative *c-MYC* mRNA expression in leukemia cells from a cohort of 166 CLL patients. The *y* axis represents the normalized $\log_2$-transformed mRNA levels from Affymetrix GeneChips. (*B*) Elevated *c-MYC* expression is associated with more rapid disease progression. Patients were classified into three groups (low: 6–8; medium: 8–9.4; high: above 9.4) by a recursive partitioning method based on $\log_2$-transformed *c-MYC* mRNA from the Affymetrix array, and their progression from CLL diagnosis to the start of treatment was plotted as a function of time. (*C*) Primary CLL cells (five each with high or low c-MYC expression) were cultured in vitro, and their viability was determined by DIOC6 and PI staining. Results are means ± SEM (n = 5 cells per group). (*D*) High c-MYC expression is associated with NLC dependency. c-MYC protein levels in 39 primary CLL specimens were examined by immunoblotting, quantified with Image J, and normalized to tubulin expression. Patients were classified into two groups by recursive partitioning method based on c-MYC protein expression in leukemic cells. The cells were grown in the absence and presence of NLC, and NLC dependency was determined as described in *Methods*. Association between c-MYC and NLC dependency was determined by χ^2 analysis. (*E*) BAFF is required for NLC to support the viability of high c-MYC expressors. Primary CLL cells with either high or low c-MYC expression determined by qPT-PCR and immunoblotting were cocultured with NLC with either a control Ig (IVIG) or a decoy BAFF receptor (BR3-FC fusion protein). Viability was determined by DIOC-6 and PI staining. Results (means ± SEM; n = 5) depict the ratio of viable cells observed in BR3-FC–treated cultures vs. IVIG-treated cultures.

expressed the highest amounts of *c-MYC* mRNA was 4.01 y (n = 31), significantly shorter than that of patients with CLL cells that contained intermediate amounts of *c-MYC* mRNA (6.51 y, n = 75) or patients with CLL cells that had low levels *c-MYC* mRNA (9.01 y, n = 60) (Fig. 5*B*). These data suggest that elevated *c-MYC* expression in CLL cells is associated with progressive disease requiring early therapy.

Consistent with previous findings that *c-MYC* promotes proliferation as well as apoptosis, human CLL cells that expressed high levels of c-MYC exhibited accelerated death in culture relative to CLL cells with low c-MYC (Fig. 5*C*). The relatively high turnover rates of cells that express c-MYC might be mitigated by survival factors supplied by accessory cells in lymphoid tissues that comprise the leukemia cell microenvironment. The lymphoid tissue contains nurse-like cells (NLC), which express and secrete BAFF, a proliferation-inducing ligand (APRIL), and other factors that can enhance the resistance of CLL cells to apoptosis (19, 20). When CLL cells were cocultured with NLC, CLL cells that expressed high levels of c-MYC had a marked dependency on NLC for survival, whereas CLL cells with low levels of c-MYC were less NLC-dependent (Fig. 5*D*). Nearly all (15 of 17) the CLL samples that expressed high levels of c-MYC were dependent on NLC for survival, whereas only 32% (7 of 22) of CLL samples that expressed low levels of c-MYC displayed such dependency (P = 0.001) (Fig. 5*D*). Treatment with a BR3-Fc fusion protein, a BAFF decoy receptor (21), inhibited the capacity of NLC to protect CLL cells from apoptosis, particularly CLL cells that express high levels of c-MYC (Fig. 5*E*). These results reveal that CLL cells that express high levels of c-MYC have an enhanced dependency on BAFF and NLC for survival.

Inhibition of IKK Signaling Decreases Viability of Mouse and Human Leukemic Cells and Reduces Disease Burden. Inhibition of classical NF-κB signaling can trigger rapid death of human CLL cells in vitro (8). Congruently, inhibition of IKKβ reduced the viability of both mouse leukemic cells and human CLL cells cocultured with NLC (Fig. S8*A*). The IKKβ inhibitor BMS345541 was more cytotoxic for leukemic cells than for normal T cells or B cells (Fig. S8*A*). Human CLL cells expressing a high level of c-MYC also appeared more sensitive to BMS345541 than CLL cells with low levels of c-MYC when cocultured with NLC (Fig. S8*B*).

To examine the antileukemia effect of IKKβ inhibition in vivo, we transferred 2×10^6 $CD5^+CD3^-$ leukemic cells into *Baff*-Tg mice (Fig. S8*C*). BMS345541 was administered by oral gavage at 4 wk after cell transfer. Treatment with BMS345541 reduced leukemia cell counts in blood (Fig. S8*D*). Control mice treated with solvent containing solutions without BMS345541 died 5–7 wk after transplantation. Although a $CD5^+CD3^-$ population still could be detected in the blood and spleen of BMS345541-treated mice, the spleens of these mice were significantly smaller than those of the control-treated mice (Fig. S8 *E* and *F*). Infiltration of leukemia cells in the marrow, mesenteric, and inguinal lymph nodes also was inhibited by treatment with BMS345541 (Fig. S8*F*). BMS345541 inhibited NF-κB nuclear translocation (Fig. S8*G*). The mice died 7–9 wk after cessation of therapy with BMS345541 because of recurrent disease (Fig. S8*H*).

Discussion

We developed a mouse model for CLL by crossing $iMyc^{C\alpha}$ mice with *Baff-Tg* mice to generate *Myc*/*Baff* mice that develop a CLL-like B-cell malignancy. The leukemia developed by *Myc*/*Baff* mice resembles human CLL in several respects. The disease manifests a monoclonal lymphoproliferation of mature, well-differentiated $CD5^+CD3^-$ B cells that cause lymphocytosis and diffuse infiltration of spleen, lymph nodes, and marrow. Unlike other mouse models of CLL (22, 23), the leukemia that develops in *Myc*/*Baff* mice displays a male gender bias, similar to observations in human CLL. Conceivably, this mouse model might be used in future studies to interrogate the mechanism(s) accounting for the gender bias in human CLL, which remains unexplained.

The role of BAFF in *Myc*-induced leukemogenesis and progression relies in part on its ability to enhance B-cell resistance to apoptosis (Fig. 3). In parallel, primary human CLL cells that expressed high levels of *c-MYC* had enhanced susceptibility to spontaneous apoptosis relative to that of CLL cells with low-level *c-MYC* expression, and this enhanced susceptibility to apoptosis could be offset by exogenous BAFF. On the other hand, we found that BAFF also up-regulated the expression *c-MYC* through activation of NF-κB in primary leukemia B cells (Fig. 4); this finding is different from the only reported link between BAFF and *c-MYC* expression in a Burkitt lymphoma cell line harboring the t(8;14) translocation that removes the endogenous *c-MYC* promoter (24). Inhibition of IKK/NF-κB signaling either pharmacologically or by shRNA blocked the capacity of BAFF to up-regulate expression of *c-MYC*. Therefore, this study demonstrates that BAFF induces c-*MYC* expression through its endogenous promoter in CLL cells. However, we cannot exclude the contribution of other factors, such as alteration in expression of transcription factors or microRNAs (25), which might be required for BAFF to enhance expression of *c-MYC* in CLL cells.

Although it is known that BAFF levels in plasma are not elevated in CLL patients relative to levels in normal individuals (26), BAFF concentrates on the cell surface of NLC at much higher amounts than on CLL cells (19). NLC are found in the

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spleen and secondary lymphoid tissues of CLL patients (20), where they presumably protect CLL cells from apoptosis (19, 20). However, the role played by NLC in CLL cell proliferation is not defined. Pseudofollicles inside secondary lymphoid tissues such as spleen and lymph node are the presumed sites for CLL cell proliferation and disease progression (27). Therefore, it is conceivable that BAFF-mediated IKK/NF-κB activation can upregulate expression of *c-MYC* within these pseudofollicles and thereby contribute to disease progression. Moreover, primary CLL cells that express high levels of *c-MYC* had enhanced susceptibility to apoptosis relative to CLL cells that expressed low levels of *c-MYC* in vitro, suggesting that CLL cells with enhanced proliferative potential might have a greater dependency on microenvironmental survival factors, such as BAFF. Congruently, in vitro, CLL cells expressing high levels of *c-MYC* had a greater dependency on NLC, which could provide such survival signals, such as BAFF. In addition, NLC express high levels of APRIL (19, 20), which also may induce activation of the canonical NF-κB pathway in CLL (8). APRIL, together with other TNF family members that may be expressed in the proliferation centers (e.g., CD154/CD40L) (28), also may play a role in enhancing the expression of *c-MYC* in CLL cells. In any case, this study demonstrates how BAFF, acting as an exogenous factor in the CLL microenvironment, can promote leukemogenesis in concert with c-MYC.

Finally, we noted heterogeneity of c-MYC expression in CLL (Fig. 5*A*). Patients who had leukemia cells expressing high levels of c-MYC had a significantly higher risk for disease progression (Fig. 5*B*). These patients' leukemia-cell samples did not have chromosomal translocations by G-banding analyses or features of Richter's transformation. CLL cells that had high levels of c-MYC expression were more dependent for survival on NLC, which express high levels of BAFF (19) (Fig. 5 *D* and *E*). These studies therefore reveal not only an association between expression of *c-MYC* and aggressive disease in CLL but also a dynamic interplay between the expression of *c-MYC* and dependency on BAFF through activation of the canonical NF-κB pathway. We reason that inhibitors the canonical NF-κB pathway may be useful in the treatment of patients with CLL.

Methods

Mice. *iMyc*$^{C\alpha}$-Tg and *Baff*-Tg mice maintained in the C57BL/6 background were obtained from S. Janz (University of Iowa, Iowa City, IA) and L. Gorelik (Biogen Idec), respectively. Mice were housed under conventional barrier protection in accordance with University of California, San Diego (UCSD) and National Institutes of Health guidelines, and mouse protocols were approved by the UCSD Institutional Animal Care and Use Committee. Survival data were obtained by observing cohorts of 12 male mice of each genotype.

Primary Human CLL Cells. CLL cells were collected from blood samples of consenting patients who satisfied diagnostic and immunophenotypic criteria for CLL. Viability of CLL cells was assayed by double staining with 3, 3′-dihexyloxacarbocyanine iodide (DIOC6) and propidium iodide (PI). Expression of *c-MYC* in CLL cells was determined by immunoblotting, quantitated by Image J, and normalized to tubulin expression. BR3-Fc (Genentech) was used at 200 ng/mL, BAFF (Peprotech) at 200 ng/mL, and BMS345541 at 5 μM, unless otherwise noted.

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