NURSELIKE CELLS EXPRESS BAFF AND APRIL, WHICH CAN PROMOTE
SURVIVAL OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS VIA A PARACRINE
PATHWAY DISTINCT FROM THAT OF SDF-1α

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Abstract

We examined expression of B cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) on chronic lymphocytic leukemia (CLL) B cells and nurselike cells (NLC), which differentiate from CD14* cells when cultured with CLL B cells. NLC expressed significantly higher levels of APRIL than monocytes and significantly higher levels of BAFF and APRIL than CLL B cells. Also, the viability of CLL B cells cultured with NLC was significantly reduced when CLL B cells were cultured with decoy receptor of B-cell maturation antigen (BCMA), which can bind both BAFF and APRIL, but not with BAFF-R:Fc, which only binds to BAFF. The effect(s) of BAFF or APRIL on leukemia cell survival appeared additive and distinct from that of stromal cell-derived factor-1 alpha (SDF-1α), which in contrast to BAFF or APRIL induced leukemia-cell phosphorylation of p44/42 mitogen-activated protein-kinase (ERK 1/2) and AKT. Conversely, BAFF and APRIL, but not SDF-1α, induced CLL-cell activation of the NF-kappa B1, and enhanced CLL-cell expression of the anti-apoptotic protein Mcl-1. However, BAFF, but not APRIL, also induced CLL-cell activation of NF-kappa B2. We conclude that BAFF and APRIL from NLC can function in a paracrine manner to support leukemia cell survival via mechanisms that are distinct from those of SDF-1α, indicating that NLC use multiple distinct pathways to support CLL-cell survival.
Introduction

B-cell chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal B-cells in the blood, secondary lymphoid tissues, and marrow.\textsuperscript{1} The leukemia cells primarily are arrested in the $G_0/G_1$-phase of the cell cycle and appear resistant to programmed cell death.\textsuperscript{2,3} Despite their apparent longevity in vivo, CLL cells typically undergo spontaneous apoptosis under conditions that support the growth of human B cell lines in vitro.\textsuperscript{4-7} This implies that the factors essential for survival are not intrinsic to the CLL B cell.\textsuperscript{4-6,8}

In vitro a subset of blood mononuclear cells from patients with CLL can differentiate into large, round, adherent cells that can attract leukemia cells and protect them from undergoing apoptosis.\textsuperscript{9} When removed from these cells, the CLL B cells experience a rapid decline in viability. Because these cells attract CLL B cells, share features in common with thymic nurse cells, and support CLL B cell survival, the adherent cells are termed nurselike cells, or NLC.

Subsequent studies found that NLC differentiated from CD14$^+$ blood mononuclear cells upon co-culture with leukemia cells in vitro. Nevertheless, despite expressing myelomonocytic antigens, NLC were found to have an expression profile of surface and cytoplasmic antigens (CD14$^{\text{low}}$, CD68$^{\text{high}}$, CD83$^{\text{negative}}$, CD106$^{\text{negative}}$) that is distinct from those of monocytes, macrophages, or blood-derived dendritic cells.\textsuperscript{10} Abundant cells with the morphology and phenotype of NLC are present in secondary lymphoid tissues of patients with CLL,\textsuperscript{10} suggesting they might also function to promote leukemia cell survival in vivo.
The mechanisms whereby NLC promote CLL cell survival are not resolved. NLC express high-levels of stromal-derived factor-1 alpha (SDF-1α), a CXC chemokine capable of inducing chemotaxis, phosphorylation of mitogen activated protein kinases (MAPK), and improved survival of CLL cells in vitro. Nevertheless, the viability of CLL B cells cultured with even high concentrations of SDF-1α is not as high as that achieved by co-culture with NLC, indicating that factors other than SDF-1α also might be responsible for promoting CLL B cells survival by NLC in vitro.

Investigators have reported that CLL cells express B-lymphocyte stimulator (BLyS), otherwise known as B cell-activating factor of the tumor necrosis factor family. BAFF is a type II transmembrane protein that can act in a membrane-bound or soluble form to promote B cell survival (reviewed by Mackay and colleagues). Moreover, in mice, disruptive mutations of either BAFF or its receptor, BAFF-R, causes profound loss of mature B cells, indicating that BAFF-BAFF-R interactions are critical for the differentiation and/or survival of mature B cells. CLL B cells also were found to express the primary BAFF receptor (BAFF-R), as well as two other receptors that can interact with BAFF, namely B-cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI). Kern and colleagues also detected expression of BAFF on the surface of CLL cells, implying that BAFF may function in an autocrine manner to support CLL B cell survival.

Two of the BAFF receptors, namely BCMA and TACI, also can bind a proliferation inducing ligand (APRIL), a factor that also can contribute to B cell survival. The third receptor for BAFF, namely BAFF-R, is specific for BAFF and cannot bind to APRIL. APRIL originally was found in tumor cells and supposedly is
expressed primarily as a secreted soluble molecule through the action of furin proteases present in the Golgi. However, Kern and colleagues reported that CLL cells also can express surface APRIL, and suggested that this factor also may function as an autocrine survival factor in this disease.

Whether the expression of BAFF and/or APRIL on CLL cells is sufficient for optimal leukemia cell survival is not known. Of note, addition of recombinant BAFF could significantly enhance leukemia cell viability, suggesting that the amount of BAFF expressed on isolated CLL cells may be insufficient to support leukemia cell survival, at least in vitro. Because of the noted dependency of leukemia B cells on accessory cells such as NLC for survival in vitro, and presumably in vivo, we examined the blood mononuclear cells, NLC, and isolated leukemia cells of patients with CLL for their relative expression of BAFF and APRIL.
Materials And Methods

Cell Preparation

After obtaining informed consent, blood samples were collected from patients at the University of California, San Diego (UCSD) Medical Center who satisfied diagnostic and immunophenotypic criteria for common B-cell CLL. Blood mononuclear cells were isolated via density-gradient centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were suspended in fetal calf serum (FCS) containing 5% dimethyl sulfoxide for storage in liquid nitrogen. The viability of the CLL cells was at least 85% at the initiation of cell culture, as assessed by their capacity to exclude propidium iodide (PI, Molecular Probes, Eugene, OR). All CLL mononuclear cell samples contained >95% CD19+/CD5+/CD3- CLL B cells, as assessed by flow cytometry using fluorochrome-conjugated monoclonal antibodies (mAbs) specific for CD19, CD5, or CD3 (BD PharMingen, La Jolla, CA). CLL cells were cultured in RPMI-1640 (Gibco BRL, Rockville, MD) supplemented with 10% FCS and penicillin-streptomycin-glutamine (culture medium) in 5% CO₂ in air at 37°C.

CD14+ blood mononuclear cells or CD19+ B cells of healthy donors were isolated from the buffy-coat of blood samples collected from adult volunteers at the San Diego Blood Bank (San Diego, CA), as described. CD14+ cells were cultured with isolated CLL B cells in culture medium at cell-densities of 1 x 10⁵/ml and 1 x 10⁷/ml, respectively. After 10 to 14 days, the plates were rinsed free of the nonadherent CLL cells. The adherent NLC were then removed for analyses, as described.
Reagents

Anti-human BAFF mAb was purchased from RDI (Flanders, NJ). Isotype control mouse IgG₁ (MOPC-21) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG₁ was purchased from BD PharMingen. Phycoerythrin (PE)-conjugated anti-human BAFF mAb was purchased from R&D Systems (Minneapolis, MN). Goat-anti-human APRIL (R15) polyclonal antibody was from Santa-Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated anti-goat IgG was from Rockland (Gilbertsville, PA). Recombinant human BAFF (rhBAFF) was a kind gift from Dr. G Zhang (National Jewish Medical and Research Center, Colorado). Recombinant human APRIL MegaLigand and BCMA-Fc were purchased from Alexis Biochemicals (San Diego, CA). BAFF-R:Fc and Control Ig were purchased from R&D Systems (Minneapolis, MN). We received the CXCR4 antagonist 4F-benzoyl-TE14011 (4F), which specifically can inhibit the activity of SDF-1α²⁰, as a gift from Dr. N. Fujii (Graduate School of Pharmaceutical Sciences, Kyoto University, Japan).

Cell Isolation

Isolated blood mononuclear cells of patients with CLL were incubated with saturating amounts of “Dynabeads” coated with anti-CD2 or anti-CD14 mAbs (Dynal A.S, Oslo, Norway). Bead-bound cells were removed with a strong magnetic field. Following depletion, less than 0.5% of cells were CD2⁺ or CD14⁺, respectively, whereas more than 99% were CD19⁺, as assessed via flow cytometry (data not shown). Peripheral normal CD19⁺ B cells were purified from the buffy-coat of blood samples collected from adult volunteers at the San Diego Blood Bank using CD19-Dynabeads
and Detatch A Bead (Dynal), following manufacturer’s instruction. The purity of the isolated B cells was >95%, as assessed by flow cytometry using a fluorochrome-conjugated anti-CD19 mAb that does not compete with the anti-CD19 mAb used for prior positive selection.

**Real-Time Quantitative RT-PCR**

Total RNA was isolated from normal CD14+ cells, NLC, normal peripheral B cells, and CLL cells before or after depletion of CD14+ cells, using RNeasy Mini Kit (QIAGEN, Valencia, CA). In other experiments, CD14+ monocytes were added to isolated CLL B cells at the indicated ratio and total RNA was made from each sample. To remove contaminating DNA, the isolated RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed with SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). For Real-time PCR, SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA) was used with 300 nmol / l forward and reverse primers in a final volume 50 µl for each reaction. Amplification primers were as follows: human BAFF 5’ ACCGCGGGACTGAAAATCT 3’ and 5’ CACGCTTATTTCTGCTGTTCTGA 3’, human APRIL 5’-CTGCACCTGGTCCCATTAAC-3’ and 5’-AAGAGCTGGTTGCCACATCA-3’, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5’ACGGATTTGCTGATTTGCGGC 3’ and 5’ TTGACGGTGCCATGGAATTTG 3’. Each sample was run in duplicate. The polymerase chain reactions were performed using GeneAmp 5700 Detection System (Applied Biosystems) with an initial incubation at 95°C for 10 minutes, followed by 40 cycles, each cycle consisting of a one minute
incubation at 60°C, followed by a fifteen second denaturation step at 95°C. For each run, serially diluted cDNA of U937 cells were used in samples run in parallel to standardize the assay. We determined the cell equivalence (CE) numbers of BAFF, APRIL, and GAPDH mRNA in each sample using the 7700 sequence detector (Applied Biosystems), using the standard curve generated from the diluted U937 cells. The unit number showing relative BAFF or APRIL mRNA level in each sample was determined as a value of BAFF or APRIL CE normalized with GAPDH CE. Melting curve analysis was performed to assess the specificity of PCR product. Following 40 cycles of PCR, samples were heated to 95°C for 30 seconds, and 60°C for 20 seconds, then heated to 95°C at a ramp rate of 0.2°C/second. Melting curves for each sample were drawn with 5700 sequence detector software (Applied Biosystems).

**Flow Cytometry**

The cells were stained with saturating amounts of antibodies for 30 minutes at 4°C in Deficient RPMI-1640 supplemented with 0.5% bovine serum albumin (FACS buffer), washed 2 times, and then analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Flow cytometry data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

**Immunofluorescence Staining**

CD14+ monocytes were cultured with CLL B cells on Lab-Tek chambered cover glass (Nalge Nunc International, Naperville, IL) for immunofluorescence staining, as described. After 14 days, the cells were prepared for immunofluorescence staining
using the Cytofix/Cytoperm Kit (BD PharMingen), as per the manufacturer’s instructions. The fixed and permeabilized cells were incubated with control antibodies, PE-conjugated anti-BAFF mAb and FITC-anti-CD19 (BD PharMingen), or goat-anti-APRIL IgG and PE-anti-CD19 (BD PharMingen). The latter was counterstained with FITC-conjugated anti-goat IgG to detect cell-bound goat antibody. Hoechst 33342 (Molecular Probes, Eugene, OR) was used to stain the nuclei. Optical sections of fluorochrome-labeled cells were captured with a Delta-Vision deconvolution microscope system (Applied Precision, Issaquah, WA) of the Digital Imaging Core of the UCSD Cancer Center.

**Immunoblot Analysis**

Cell lysates were prepared with RIPA buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton x 100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA), containing 1 mM PMSF, 0.28 TIU/ml aprotinin, 50 µg/ml leupeptin, 1 mM benzamidine, 0.7 µg/ml pepstatin. Lysates were normalized for total protein (20 µg), subjected to SDS-PAGE (4-15% gradient gels, Bio-Rad, Hercules, CA) and immunoblot assay. We incubated the blots with secondary antibodies that were conjugated with horseradish peroxidase. Blots then were prepared for enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, Buckinghamshire, UK) and subsequent autoradiography with Super RX film (Fuji, Tokyo, Japan). The mouse mAb against APRIL (APRIL8) was from Alexis Biochemicals. The mouse mAb against inhibitor of kappa B-α (IκBα) was from Imgenex (San Diego, CA). The antibodies against anti-phosho-MAP kinase Erk1/2 and anti-MAP kinase Erk1/2-CT were purchased from Upstate Biotechnology.
Antibodies against AKT or phospho-AKT (Ser473) were from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies (Mcl-1, Bcl-2, and Bax) were raised against synthetic peptides.21 Also primary antibodies included β-actin (Sigma Immunochemicals, St Louis, MO). Anti-p52 and anti-p65 antibodies were purchased from Upstate Biotechnology.

Subcellular Fractionation and Detection of Cytoplasmic or Nuclear NF-κB

For fractionation experiments, cells were collected by centrifugation and washed with PBS. The cell pellet containing $5 \times 10^6$ cells was suspended in 100 µl of hypotonic buffer (50 mM Tris (pH 7.4), 5 mM EDTA, 10 mM NaCl, 0.05% NP-40, 1 mM PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 10 µg/ml Pepstatin, 10 mM β-Glycerophosphate, 1 mM Na-Vanadate, 1 mM NaF). After 10 minutes the lysate was spun and the supernatant was collected as cytoplasmic lysates. The pellet was washed 5 times in hypotonic buffer containing 0.1% NP-40. The remaining pellet was suspended in 100 µl RIPA buffer containing protease and phosphatase inhibitors. After an appropriate amount of 3x sample buffer (200 mM Tris (pH 6.8), 30 mM EDTA, 30% Glycerol, 6% SDS) was added, the sample was boiled for 10 minutes, spun for 10 minutes and the supernatant was recovered as nucleus lysates. Anti-NF kappa B p52 and p65 were purchased from Upstate Biotechnology. Anti-SP-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
Measurement Of Cell Viability

Freshly thawed CLL B cells were cultured at the concentration of 1 x 10^6/ml under various conditions. Determination of CLL cell viability in this study was based on the analysis of mitochondrial transmembrane potential (ΔΨ_m) using 3,3'-dehexyloxacarbocyanine iodine (DiOC_6) and cell membrane permeability to PI, as described. For viability assays, 100 µl of the cell culture was collected at the indicated time points and transferred to polypropylene tubes containing 100 µl of 60 nmol/l DiOC_6 (Molecular Probes) and 10 µg/ml PI in FACS buffer. The cells then were incubated at 37° C for 15 minutes and analyzed within 30 minutes by flow cytometry using a FACSCalibur (Becton Dickinson). Fluorescence was recorded at 525 nm (FL-1) for DiOC_6 and at 600 nm (FL-3) for PI.

Statistical Analysis

Results are shown as mean ± S.D. of at least 3 samples each. For statistical comparison between groups, the Student t test or the Bonferroni t test was used. Analyses were performed using Glanzman’s “Primer of Biostatistics” software (McGraw-Hill Inc., New York, NY).
Results

Expression of BAFF mRNA and Protein on CLL Cells and NLC

We examined the peripheral blood mononuclear cells (PBMC) of patients with CLL for expression of BAFF mRNA by real-time RT-PCR. In each case, we detected expression of BAFF mRNA, consistent with earlier reports.\textsuperscript{11-13} Moreover, we found that rigorous depletion of CD14\textsuperscript{+} cells from the PBMC significantly lowered the amount of BAFF mRNA detected in each sample (59 ± 30 Units in PBMC and 29 ± 13 Units in isolated CLL B cells, n = 12, \(P < 0.001\), paired t test, Figure 1A). Furthermore, the amount of BAFF mRNA detected in CD14\textsuperscript{+} cells (320 ± 230, n = 4) or NLC (270 ± 110, n = 12) was significantly greater than that noted in the isolated leukemia B cells (\(P<0.0001\), Figure 1B) or isolated CD19\textsuperscript{+} blood B cells of healthy donors.

Small numbers of CD14\textsuperscript{+} cells present in the blood mononuclear cells isolated from patients with CLL potentially could contribute a large proportion of the BAFF mRNA detected by real-time RT-PCR assay, which uses GAPDH mRNA to normalize the assay. To evaluate this possibility we added small numbers of CD14\textsuperscript{+} blood mononuclear cells to purified CD19\textsuperscript{+} CLL B cells and examined how this affected the amount of BAFF mRNA detected in each sample (Figure 1C). For each 1\% of added CD14\textsuperscript{+} cells there was an increase in the detected amount of BAFF mRNA of 10-13 Units. At the y-intercept of each graph (Figure 1C) when the proportion of CD14\textsuperscript{+} cells was extrapolated to 0\%, we detected 30-40 Units of BAFF mRNA. We attribute this to the amount of BAFF mRNA expressed by CLL B cells themselves, as this is the amount we detected in the isolated leukemia B cells (Figure 1B; e.g. 29 ± 13 Units). This
implies that on a cell-per-cell basis, CD14+ cells apparently contain approximately 30-fold more BAFF mRNA than CLL B cells.

We next examined CLL cells and NLC for surface expression of BAFF by flow cytometry. In contrast to CLL B cells or purified normal B cells, NLC expressed high-levels of BAFF that were easily detected by flow cytometry (Figure 1D), or immunofluorescence microscopy (Figure 1E). These data indicate that NLC express large amounts of BAFF protein relative to that expressed by CLL B cells.

Expression of APRIL mRNA and Protein on CLL Cells and NLC

We also examined the PBMC of patients with CLL for expression of APRIL mRNA with the same techniques used for evaluating the expression of BAFF. In contrast to our studies on BAFF mRNA, we found that rigorous depletion of CD14+ cells from the PBMC did not lower the amount of APRIL mRNA detected in each sample tested (440 ± 308 Units in PBMC and 348 ± 228 Units in isolated CLL B cells, n = 11,
NS, paired t test, Figure 2A). This indicates that CD14+ blood mononuclear cells do not contribute significantly to the amounts of APRIL mRNA found in CLL blood mononuclear cells. Consistent with this, we found that isolated CD14+ cells had very low amounts of APRIL mRNA (52 ± 20, n=5).

In contrast, the amounts of APRIL mRNA detected in differentiated NLC was significantly higher (Figure 2B; 1595 ± 1090, n=11) than that of non-differentiated CD14+ blood mononuclear cells. Moreover, NLC had significantly greater amount of APRIL mRNA than that noted in the isolated leukemia B cells or isolated CD19+ blood B cells of normal donors (P < 0.01, Bonferroni t test, Figure 2B).

We evaluated for expression of APRIL by immunoblot analysis. As seen in Figure 2C, total lysates from NLC had higher amounts of APRIL than did CD14+ blood mononuclear cells, purified CLL B cells, or isolated CD19+ blood B cells of normal donors. NLC also were found to express high levels of APRIL relative to CLL B cells by immunofluorescence staining (Figure 2D).
Figure 2
Effect of BCMA-Fc or BAFF-R:Fc on the Viability of CLL Cells Cultured with NLC

Because NLC express both BAFF and APRIL, we examined whether these factors contributed to the capacity NLC to sustain CLL cell survival in vitro. We cultured CLL B cells with decoy receptors of BCMA (BCMA-Fc), which can bind to both BAFF and APRIL, and BAFF-R (BAFF-R:Fc), which binds to only BAFF, and compared the viability of the leukemia cells with that of such cells cultured with control immunoglobulin (control Ig). We observed that addition of BCMA-Fc to co-cultures of CLL cells and NLC significantly reduced the viability of the CLL cells relative to that of co-cultures treated with control Ig (Figure 3A). In contrast, there was no decline in leukemia-cell viability in such co-cultures when we added saturating amounts of BAFF-R:Fc (Figure 3A), which in parallel studies were found capable of inhibiting B cell
survival in co-cultures with rhBAFF or fibroblast-like synoviocytes that expressed BAFF, but not APRIL (data not shown).\textsuperscript{19}

**Additive Effects of SDF-1\textalpha{} and BAFF or APRIL on CLL B-Cell Survival**

Next we examined whether NLC or exogenous BAFF or APRIL could enhance the viability of CLL B cells in vitro. For this, we monitored the viability of CLL B cells over time when cultured with or without NLC or with or without rhBAFF or rhAPRIL. Consistent with prior studies\textsuperscript{9,10}, CLL cells cultured alone had less viability than leukemia cells cultured with NLC. The addition of rhBAFF or rhAPRIL significantly improved the viability of CLL cells cultured without NLC (Figure 3B). The viability of the CLL cells co-cultured with either rhBAFF or rhAPRIL alone was not enhanced further by the addition of rhAPRIL or rhBAFF, respectively.
Because NLC express BAFF, APRIL, and SDF-1α, we examined whether these factors together could support CLL B cell survival better than either factor alone. The viability of isolated CLL B cells was highest when co-cultured with NLC (Figure 5). However, isolated CLL B cells co-cultured with rhBAFF plus SDF-1α, or rhAPRIL plus SDF-1α, had a significantly greater viability than that of CLL B cells cultured with any one factor alone (Figure 4). Collectively, these data support the notion that BAFF or APRIL promotes leukemia cell survival via a mechanism(s) independent of that used by SDF-1α.

**Effects of rhBAFF, rhAPRIL, or SDF-1α on Signaling Pathways in CLL B Cells**

We examined the intracellular signaling pathways stimulated by rhBAFF, rhAPRIL, or SDF-1α at concentrations that can promote CLL B cell survival in vitro.
Prior studies indicated that BAFF could induce activation of the NF-κB2 in normal B cells, a pathway that appears critical for the growth and/or survival of normal B cells. Such activation involves processing of p100 to p52 with subsequent translocation of p52 to the nucleus. We found that rhBAFF could induce activation of NF-κB2 also in CLL B cells (Figure 5A). In contrast, we did not observe activation of NF-κB2 in CLL cells treated with rhAPRIL or SDF-1α, even at concentrations that could support CLL cell survival in vitro. Both rhBAFF and rhAPRIL, however, induced degradation of the inhibitor of kappa B (IκBα) and translocation of p65 to the nuclear fraction, indicating activation of the classical NF-κB pathway (Figure 5B). SDF-1α, on the other hand, did not have this activity (Figure 5).

We also examined for phosphorylation and activation of AKT, which prior studies found also could enhance CLL B cell survival. In contrast to SDF-1α, we found that rhBAFF or rhAPRIL could not induce phosphorylation of p44/42 mitogen-activated phosphokinase (MAPK, ERK1/2) or activation of AKT in CLL B cells, even at concentrations that could promote CLL B cell survival in vitro (Figure 6, and data not shown).
However, SDF-1α not only induced phosphorylation of ERK1/2, as noted previously, but also induced phosphorylation of AKT at Ser^{473} in isolated CLL B cells (Figure 6A). The capacity of SDF-1α to induce CLL-cell phosphorylation of ERK1/2 and AKT at Ser^{473} could be blocked by 4F-benzoyl-TE14011 (4F), a specific CXCR4 antagonist (Figure 6B).
NLC, BAFF, or APRIL, but not SDF-1α, Can Induce CLL-Cell Expression of Mcl-1

To examine mechanisms that might account for the effects on leukemia-cell survival, we evaluated for the expression of pro-apoptotic and anti-apoptotic proteins in CLL B cells following culture with or without NLC or with either rhBAFF or SDF-1α. We did not observe significant changes in the levels of Bcl-2, Bax, or Bcl-xL expressed by isolated CLL B cells in any of the short-term culture conditions used (Figure 7, and data not shown). On the other hand, CLL B cells co-cultured with NLC, rhBAFF, or rhAPRIL were induced to express increased levels of Mcl-1 (Figure 7 and data not shown). In contrast, SDF-1α could not induce isolated CLL B cells to express higher levels of Mcl-1,
even at concentrations that could protect CLL-cell survival in vitro (Figure 7, and data not shown).

Figure 7
Discussion

Increasing attention is being focused on cells and factors of different microenvironments that contribute to CLL cell survival. Such accessory cells include marrow stromal cells, follicular dendritic cells, and NLC. Defining the mechanisms whereby these cells contribute to the survival of CLL cells potentially could identify novel targets for treatment of this disease.

In this study, we found that NLC express high levels of BAFF and APRIL, two factors of the TNF family that play an important role in maintaining the survival of mature B cells. Because NLC are derived from CD14+ cells, expression of BAFF by NLC was anticipated, as this factor originally was found expressed by myeloid lineage cells, such as monocytes, macrophages, or dendritic cells. Moreover, we found that CD14+ cells accounted for most of the BAFF mRNA found in the blood mononuclear cells of patients with CLL and, on a cell-per cell basis, contained approximately 30-fold more BAFF mRNA than did CLL B cells, which prior studies found could also express this B-cell survival factor. From the studies reported here, it is appears that such CD14+ cells maintain high-level expression of BAFF, even after they differentiate into NLC upon co-culture with CLL B cells in vitro.

In contrast, NLC expressed significantly more APRIL than newly isolated CD14+ blood cells, which in turn contributed little to the APRIL mRNA detected in the blood mononuclear cells of patients with CLL. Moreover, the low-to-negligible amount of APRIL mRNA detected in CD14+ blood mononuclear cells appeared less than that expressed by CLL B cells, or even normal B cells. In contrast, CD14+ myeloid cells in the secondary lymphoid tissues of patients with non-Hodgkin’s lymphomas, including
CLL, apparently express high-levels of BAFF and APRIL. Conceivably, such cells may include CD14^+ cells that already have differentiated into NLC in vivo.

We investigated whether BAFF and/or APRIL on NLC could contribute to their capacity to promote leukemia cell survival in vitro. Previous studies showed BCMA-Fc could impair leukemia-cell viability over time when this decoy receptor was added to isolated leukemia cells. However, we did not observe this effect on the viability of CLL B cells cultured without NLC, even at concentrations of BCMA-Fc of 30 µg/ml (data not shown). The reason for the discrepancy between our data and others is not clear. Instead, BCMA-Fc significantly impaired the viability of CLL B cells cultured with NLC (Figure 3A). However, BAFF-R:Fc, which only can inhibit BAFF interactions with BAFF-R, failed to impair the viability of CLL cells that were cultured either with or without NLC, implying that APRIL may play an important role in the protective effect(s) of NLC on CLL cell survival. Although the studies in knock-out mice showed that APRIL appeared to be dispensable for developing normal immune systems, a recent study by Planelles found that APRIL may play a role in the pathogenesis of B1-cell malignancies, namely CLL. In this light, strategies that only interfere with BAFF/BAFF-R interactions may not be sufficient to affect CLL cell viability in vivo.

Previously, we reported that NLC also express SDF-1α, a chemokine that can trigger phosphorylation of p44/42 MAPK ERK1/2 and enhance CLL cell survival in vitro. Although some studies have suggested that the ERK pathway might not be involved in preventing spontaneous apoptosis of CLL B cells, suppression of ERK activity is seen in CLL B cells undergoing drug induced apoptosis, suggesting that this pathway is important for survival of CLL B cells.
Since SDF-1α had an additive effect on the viability of isolated CLL cells cultured with BAFF and/or APRIL (Figure 4), we reasoned that BAFF or APRIL might promote CLL cell survival via a pathway(s) that is distinct from that of SDF-1α. Consistent with this notion, we found that SDF-1α, in addition to its noted capacity to induce phosphorylation of ERK1/2 MAPK, could induce CLL B cells activation of phosphatidylinositol 3-kinase (PI3K) AKT (Figure 6), a pathway that is essential for the survival of CLL B cells.25,26 These findings are consistent with those of others who found that SDF-1α could induce activation of AKT in other types of cells besides leukemia B cells.39-42 Recently, Moreaux and colleagues reported that addition of exogenous BAFF to myeloma cells induced late activation of both ERK1/2 and AKT,42 but the direct influence of BAFF on these two pathways was not resolved. In the study presented here, it appears that neither pathway is activated in CLL cells by rhBAFF or rhAPRIL, indicating that these factors must use other mechanisms to protect CLL B cells from spontaneous apoptosis.

Some TNF super family proteins like BAFF trigger their functions by activating NF-κB. Two main pathways - the canonical and alternative pathway - regulate the activity of NF-κB.24 Activation of the canonical pathway results from degradation of the inhibitor of NF-κBα (IκBα), which is induced upon its phosphorylation by the beta subunit of the IκB kinase (IKK) complex, IKKβ.43 This leads to nuclear translocation of active NF-κB heterodimers (that are composed of p65, c-Rel or p50) where they can effect changes in gene expression. As noted for lymphoma or CLL B cells,11,12 concentrations of rhBAFF or rhAPRIL required for optimal enhancement of CLL cell survival also induced degradation of IκBα and translocation of p65 to the nucleus,
indicating that either factor can activate the canonical NF-κB pathway. Activation of the canonical NF-κB pathway in normal B cells appears secondary to the capacity of BAFF or APRIL to interact with BCMA, or BCMA and/or TACI, respectively.44

Alternative pathway activation results from processing of NF-κB2 p100 to p52, which is triggered by the phosphorylation of NF-κB2 p100 by the alpha subunit of the IKK complex, namely IKKα.43 This allows for nuclear translocation of p52 along with RelB, where this complex can influence expression of genes that are distinct from those regulated by the canonical NF-κB pathway. We noted that rhBAFF, but not rhAPRIL or SDF-1α, could induce degradation of p100 to p52 and translocation of p52 to the nucleus. Because the BAFF-R interacts with BAFF, but not APRIL, the selective activation of p100 processing by BAFF suggests that the BAFF-R may be distinct from BCMA or TACI in its capacity to activate the alternative NF-κB pathway in CLL B cells. This is similar to the interaction of BAFF with its receptor on normal B cells, which also promotes processing of NF-κB2.17,45 Moreover, studies have shown that IKKα is required for B cell maturation and formation of secondary lymphoid organs.46,47 However, because treatment of co-cultures of CLL cells and NLC with BAFF-R:Fc failed to inhibit the protective effect of NLC on leukemia cell survival, it appears that activation of the canonical pathway may obviate the requirement for activation of the alternative NF-κB pathway in CLL to promote leukemia cell survival, at least in the in vitro culture conditions used in this study.

Finally, we evaluated for expression of Bcl-2-family-member proteins that can influence the resistance or sensitivity of CLL cells to apoptosis. Prior studies found that BAFF can up-regulate expression of Bcl-2 in most B cells.14,12 BAFF induced up-
regulation of Bcl-2 was less apparent in CLL B cells, possibly secondary to the constitutive high-level expression of this anti-apoptotic protein in this leukemia. However, we found that rhBAFF, rhAPRIL, or NLC could induce CLL B cells to express high-levels of Mcl-1 (Figure 7, and data not shown). Like Bcl-2, Mcl-1 also appears to play a role in the resistance of CLL B cells to drug induced apoptosis, and patients with CLL who fail to achieve complete remission after chemotherapy tend to have high levels of Mcl-1. There are several reports that AKT or ERK1/2 regulate the expression of Mcl-1 in various types of cells. On the other hand, O’Connor reported that the persistence of plasma cells in mice was associated with a BAFF-mediated up-regulation of Mcl-1. In the present study, we found that rhBAFF or rhAPRIL, which did not activate AKT or ERK1/2, up-regulated Mcl-1 in CLL B cells. However, saturating amounts of BCMA-Fc or BAFF-R:Fc that could inhibit rhBAFF-induced expression of Mcl-1 failed to block the capacity of NLC to enhance expression of Mcl-1 in CLL B cells (data not shown), suggesting that NLC-associated factors other than BAFF and APRIL also may induce expression of this anti-apoptotic protein in CLL cells. In any case, we found that SDF-1α, which can activate AKT or ERK1/2 in CLL cells, was unable to induce CLL cells to express Mcl-1 (Figure 7). As such, these data suggest that BAFF up-regulates expression of Mcl-1 in CLL B-cell via a pathway(s) distinct from that involving activation of MAPK or AKT.

Whereas isolated CLL B cells undergo apoptosis when cultured alone, the addition of rhBAFF, rhAPRIL, and/or SDF-1α to the CLL B cells significantly enhanced their viability (Figure 4), as noted previously. Nevertheless, the viability of CLL cells cultured with SDF-1α and rhBAFF and/or rhAPRIL still was not as high as that seen
when CLL B cells were cultured with NLC, suggesting that yet additional NLC factors are involved in promoting leukemia-cell survival. In this regard, it is noteworthy that Deaglio and colleagues recently found that NLC also express high-levels of CD31 and plexin-B1, which also can contribute in part to the capacity of NLC to sustain CLL cell viability. Conceivably, strategies that can target one or more of the mechanisms whereby NLC sustain CLL cell survival could have therapeutic potential for patients with this disease.
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Figure Legends

Figure 1

Expression of BAFF mRNA and Protein

(A) Quantitative real-time RT-PCR was performed on RNA samples isolated from the blood mononuclear cells of individual patients with CLL before (left) and after (right) depletion of CD2⁺ and CD14⁺ cells. The lines connect the pre- and post-isolation levels of BAFF mRNA detected in each sample. The amount of BAFF mRNA detected is indicated in arbitrary units. The amount of BAFF mRNA detected in an equivalent number of U937 cells is 1,000 Units (data not shown). (B) Quantitative real-time RT-PCR measurement of the average amount of BAFF mRNA detected in CD14⁺ cells (n=4), NLC (n=12), purified CLL B cells (n=12) and isolated CD19⁺ blood B cells of normal donors (n=2), as indicated at the bottom of the panel (** indicates that the level of BAFF mRNA detected in NLC was significantly greater than that found in isolated CLL B cells, \( P < 0.0001 \)). (C) Reconstitution experiments in which small numbers of CD14⁺ blood mononuclear cells are added to 5 × 10⁶ isolated CLL B cells that subsequently were evaluated for BAFF mRNA in two representative patients. On the x-axis is the percent of CD14⁺ cells detected by FACS in the reconstituted cell population prior to extraction of RNA. The y-axis indicates the level of BAFF mRNA detected in Units. (D) Representative histograms depicting surface BAFF detected by flow cytometry on CD14⁺ cells, NLC, CD19⁺ CLL B cells, or CD19⁺ blood B cells of healthy donors, as indicated at the top of each graph. Shaded histograms represent the fluorescence of cells stained with a fluorochrome-labeled anti-BAFF mAb, whereas the clear histograms depict the fluorescence of cells stained with an isotype control mAb.
(E) An immunofluorescence picture of an NLC and CLL cells stained with fluorescein-labeled anti-CD19 mAb (green) and a phycoerythrin-labeled anti-BAFF mAb (red). The nuclei are labeled blue with Hoechst 33342.

**Figure 2**

*Expression of APRIL mRNA and Protein* (A) Quantitative real-time RT-PCR was performed on RNA samples isolated from the blood mononuclear cells of patients with CLL before (left) and after (right) depletion of CD2+ and CD14+ cells. The lines connect the pre- and post-isolation levels of APRIL mRNA in each sample. The amount of APRIL mRNA detected is indicated in arbitrary units. The amount of APRIL mRNA detected in an equivalent number of U937 cells is 30 Units (data not shown). (B) Quantitative real-time RT-PCR measurement of the average amount of APRIL mRNA detected in CD14+ cells (n=4), NLC (n=11), purified CLL B cells (n=11), or isolated CD19+ blood B cells of healthy donors (n=3), as indicated at the bottom of the histogram (** indicates that the mean level of APRIL mRNA detected in NLC was significantly greater than that found in isolated CLL B cells, \( P < 0.01 \)). (C) Representative immunoblot data showing the expression of APRIL by NLC, CD14+ blood mononuclear cells, CLL B cells, or isolated CD19+ blood B cells of healthy donors. Whole cell lysates were prepared as described in the Material and Methods section. The protein content was normalized to 20 µg and subjected to immunoblot analysis with antibodies specific for APRIL or β-actin, using ECL-based detection. (D) An immunofluorescence picture of NLC and CLL cells stained with phycoerythrin-labeled anti-CD19 mAb (red)
and goat IgG anti-APRIL polyclonal antibody that was detected using a fluorescein-labeled anti-goat IgG (green). The nuclei are labeled blue with Hoechst 33342.

Figure 3

**CLL Cell Survival With or Without NLC**

(A) *Inhibition of CLL-cell survival on NLC by BCMA-Fc, but not BAFF-R:Fc*  
CLL B cells were cultured with (open squares) or without (closed squares) NLC and 1 µg/ml control Ig. BCMA-Fc (closed triangles) or BAFF-R:Fc (closed circles) at 1 µg/ml was added to the wells of CLL B cells cultured with NLC at day 0. Viability was subsequently determined for each time point, as indicated on the horizontal axis. Displayed are the mean percent viability ± S.D. (error bars) of samples from each 5 patients. The percent viability of BCMA-Fc treated cultures was significantly less than that of control Ig treated cultures (* indicates P < 0.05; ** indicates P < 0.01; Bonferroni t test).  

(B) *Enhanced CLL cell survival with NLC or rhBAFF or rhAPRIL*  
1 x 10^6/ml of isolated CD19^+ CLL B cells were cultured alone (open squares), with 50 ng/ml rhBAFF (closed triangles), 500 ng/ml rhAPRIL (closed circles), both rhBAFF and rhAPRIL (open circles) or with NLC (closed squares) and evaluated over time. Displayed are the mean percent viability ± S.D. of samples from each 3 patients. The percent viability of rhBAFF-treated CLL cells or rhAPRIL treated CLL cells was significant greater than that of control treated CLL cells (* indicates P < 0.05; ** indicates P < 0.01; Bonferroni t test).
Figure 4

**Effect of rhBAFF, rhAPRIL, and/or SDF-1α on CLL-cell Survival**

CLL B cells were cultured with (open squares) or without (closed squares) NLC. SDF-1α (closed circles) rhAPRIL (closed diamonds) at 500 ng/ml, rhBAFF (closed triangles) at 50 ng/ml or both (open diamonds) were added to wells without NLC at day 0. Also SDF-1α and rhBAFF (open diamonds) or SDF-1α and rhAPRIL (open circles) were added to the cultures without NLC. The mean viability ± S.E. of replicate wells was determined for each time point indicated on the horizontal axis. A representative example of three different CLL patients is presented.

Figure 5. Activation of NF-κB in CLL B cells by rhBAFF or rhAPRIL

(A) *Processing of p100 and nuclear translocation of p52 or p65* – CLL B cells were cultured with or without SDF-1α (500 ng/ml), rhBAFF (50 ng/ml), rhAPRIL (500 ng/ml) for 24 hours. Cytoplasmic and nuclear extracts were prepared as described in Material and Methods for immunoblot analysis with anti-p100 or anti-p65 antibodies as indicated on the left side of each panel. The agent used to treat the CLL cells is indicated at the top of each panel under the label indicating whether the extract was derived from cytoplasmic (left panel) or nuclear (right panel) cell fractions. We evaluated for equal loading in each lane by stripping the blot and probing it again with antibodies specific for β-actin (for cytoplasmic extracts) or SP-1 (for nuclear extracts), as indicated on the left side of each panel. (B) *Degradation of IκBα* – Extracts of CLL cells were prepared for immunoblot analysis prior to treatment (Pre-Tx) of after a 30 minute incubation with culture medium alone (Medium), or medium supplemented with SDF-1α (500 ng/ml),
rhBAFF (50 ng/ml), rhAPRIL (500 ng/ml), or TNFα (50 ng/ml), as indicated at the top of each lane. The immunoblot was probed with antibodies specific for IkBα (top panel). We evaluated for equal loading in each lane by stripping the blot and probing it again with antibodies specific for β-actin (bottom panel).

**Figure 6**

*Activation of MAPK (ERK1/2) and AKT in CLL Cells*

(A) CLL B cells were cultured for 3 or 10 minutes with SDF-1α (200 ng/ml), rhBAFF (50 ng/ml), or media, as indicated above the sample lanes. Cell lysates were prepared and analyzed by immunoblot using antibodies specific for phosphorylated ERK1/2 (P-ERK 1/2), ERK 1/2, phosphorylated AKT (P-AKTSer473), or AKT, as indicated on the left-hand margin. Equal loading in the lanes was evaluated by stripping the blot and probing again with anti-ERK1/2 and an anti-AKT antibody. Five different CLL B cells gave similar results. In (B) the CLL cells were stimulated for 3 minutes with either media (far left lane) or SDF-1α (200 ng/ml) (right three lanes). For samples treated with SDF-1α we included the CXCR4 antagonist 4F-benzoyl-TE1401120 (4F) at 0 nM, 50 nM, or 500 nM, respectively. The samples were analyzed and the results presented as noted in Figure 6A.

**Figure 7**

*Expression of Mcl-1 in CLL B-cells by Immunoblot Analysis*

Representative immunoblot data showing up-regulation of Mcl-1 by NLC or rhBAFF. CLL B cells were cultured with or without NLC, or with SDF-1α (500 ng/ml) or rhBAFF
(50 ng/ml) for 24 hours. Whole cell lysates were then prepared. The protein content normalized to 12.5 µg, and analyzed by immunoblot analysis with antibodies specific for Mcl-1, Bax, Bcl-2, or β-actin, using ECL-based detection.
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Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1α

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