BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone

Jérôme Moreaux, Eric Legouffe, Eric Jourdan, Philippe Quittet, Thierry Rème, Cécile Lugagne, Philippe Moine, Jean-François Rossi, Bernard Klein, and Karin Tarté

Identification of growth factors in neoplasias may be a target for future therapies by blocking either growth factor receptor interaction or the induced pathway. Using gene expression profiling, we identified overexpression of 2 receptors for a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF) in malignant plasma cells compared with normal plasma cells. APRIL and BAFF are involved in a variety of tumor and autoimmune diseases, including B-cell malignancies. We confirmed the expression of BAFF and APRIL receptors (B-cell maturation antigen [BCMA], transmembrane activator and calcium modulator and cyclophilin ligand interactor [TACI]), and BAFF-R in a majority of 13 myeloma cell lines and in the purified primary myeloma cells of 11 patients. APRIL and BAFF were potent survival factors for exogenous cytokerine-dependent myeloma cell lines and were autocrine growth factors for the RPMI8226 and L363 autonomously growing cell lines. These factors activated nuclear factor (NF)–κB, phosphatidylinositoll-3 (PI-3) kinase/AKT, and mitogen-activated protein kinase (MAPK) kinase pathways and induced a strong up-regulation of the Mcl-1 and Bcl-2 antiapoptotic proteins in myeloma cells. BAFF or APRIL was also involved in the survival of primary myeloma cells cultured with their bone-marrow environment, and protected them from dexamethasone (DEX)–induced apoptosis. Finally, the serum levels of BAFF and APRIL were increased about 5-fold in patients with multiple myeloma (MM) as compared with healthy donors. Altogether, these data suggest that APRIL/BAFF inhibitors may be of clinical value in MM. (Blood. 2004;103:3148-3157)

© 2004 by The American Society of Hematology

Introduction

Multiple myeloma (MM) is a clonal B-cell neoplasia characterized by the accumulation of malignant plasma cells within the bone marrow, in close contact with stromal cells. Several autocrine or paracrine soluble factors can promote myeloma cell survival and proliferation.1 Interleukin 6 (IL-6), which is mainly produced by cells of the tumor microenvironment, is a major myeloma growth factor.2 Interferon alpha (IFN-α), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and heparin-binding epidermal growth factor–like growth factor (HB-EGF) can also promote the survival or proliferation of myeloma cells.3–7 The inhibition of myeloma cell growth factors may have clinical applications, eventually in combination with other drugs. For example, anti–IL-6 monoclonal antibody (MoAb) may lead to tumor regression in some advanced myeloma patients.8,9

In order to identify new myeloma cell growth factors, we recently compared gene expression profiles of myeloma cells with those of normal plasmablasts and peripheral blood B cells.10–12 Interestingly, the TACI (transmembrane activator, B-cell maturation antigen, and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen) genes coding for 2 receptors of B-cell activating factor (BAFF, also called BLyS)13,14 were highly expressed in malignant plasma cells.10,11 BAFF is a tumor necrosis factor (TNF) family member essentially expressed by monocytes, macrophages, dendritic cells, and some T cells.15 It is produced as both a membrane-bound and a proteolytically cleaved soluble protein.13,14 A third receptor for BAFF, called BAFF-R, was recently identified.16 The expression of BCMA and BAFF-R is B-cell–specific, whereas TACI is also found on a subset of activated T cells.15 Finally, BAFF shares significant homology with a proliferation-inducing ligand (APRIL), which is expressed at a low level by normal lymphoid and myeloid cells, and at a high level by a variety of human cancers.17,18 APRIL, which is directly secreted without cell-surface expression, binds to BCMA and TACI but not to BAFF-R.15

Several studies have indicated that BAFF is a survival factor for immature, naive, and activated B cells.15 The production of BAFF by myeloid dendritic cells in response to innate immune signals was shown to promote T-cell–independent immunoglobulin class switching and to sustain survival of extrafollicular plasmablasts.19 BAFF-transgenic mice develop mature B-cell hyperplasia with autoimmune manifestations, especially production of autoantibodies.15 Moreover, dysregulation of the BAFF pathway seems to be involved in autoimmune in humans.15 On the contrary, BAFF-R–deficient mice and mice treated with TACI-Fc or BCMA-Fc display severe loss of mature B cells.16

The role of APRIL is less well characterized. Recent reports have shown that APRIL provides survival and activation signals to normal B and T cells,20–22 In addition, APRIL is highly expressed in several tumor tissues and stimulates growth of tumor cells in vitro and in vivo.17

From Institut National de la Santé et de la Recherche Médicale (INSERM) U475 and Unité de Thérapie Cellulaire, Centre Hospitalier Universitaire (CHU) Montpellier, Hôpital St Eloi, and the Service d’Hématologie et Oncologie Médicale, CHU Montpellier, Hôpital La Pèreyronie, Montpellier, France; and the Service de Médecine Interne B, CHU de Nîmes, France.


Supported by grants from the Ligue Nationale Contre le Cancer (équipe labellisée), Paris, France.

Reprints: Bernard Klein, INSERM U475, 99 rue Puech Villa, 34197 Montpellier Cedex 5, France; e-mail: klein@montp.inserm.fr.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.
The signal transduction pathways driven by BAFF and APRIL are not fully characterized. The activation of nuclear factor (NF)-κB by TACI, BCMA, and BAFF-R is consistent with the antiapoptotic role of BAFF, since NF-κB enhances the transcription of several cell survival genes. Depending on the B-cell maturation stage, BAFF was reported to induce the antiapoptotic proteins Bcl-2, A1, and Bcl-XL and to reduce the proapoptotic protein Bak. BAFF also activates Jun kinase (JNK), Elk-1, p38 kinase, activating protein 1 (AP-1), and NF-AT in various models.

The striking roles of BAFF, APRIL, and their receptors in normal B-cell homeostasis and in several tumor models raise the possibility that they may be involved in the pathogenesis of B-cell malignancies. Recent studies reported the aberrant expression of BAFF and APRIL by tumor B cells isolated from a subset of patients with chronic lymphoid leukemia, suggesting the existence of an autocrine survival loop in this disease. In vitro, a BCMA-Fc fusion protein is able to enhance apoptosis of B-cell chronic lymphocytic leukemia (B-CLL) cells. In addition, patients with follicular non-Hodgkin lymphomas have increased levels of soluble BAFF in their serum, and BAFF seems to favor B-lymphoma cell survival.

In this study, we show that myeloma cell lines and primary myeloma cells express BAFF, APRIL, and their receptors and that BAFF and APRIL are myeloma cell growth factors and rescue myeloma cells from apoptosis induced by dexamethasone. BAFF and APRIL activated nuclear factor (NF)-κB, phosphatidylinositol-3 (PI-3) kinase/AKT, and mitogen-activated protein kinase (MAPK) kinase pathways in myeloma cells and induced a strong up-regulation of the Mcl-1 and Bcl-2 antiapoptotic proteins. Finally, we demonstrated a 5-fold increase in the serum levels of BAFF or APRIL in patients with MM compared with age-related healthy individuals.

Materials and methods

Myeloma cell lines and primary samples

XG-1, XG-2, XG-5, XG-6, XG-7, XG-11, XG-13, XG-14, and XG-20 are IL-6-dependent human myeloma cell lines (HMCLs) obtained in our laboratory. Upon removal of IL-6, these cells progressively apoptosis within 10 to 14 days. These HMCLs were routinely maintained in RPMI 1640 and 10% fetal calf serum (FCS; Biowittaker, Walkersville, MD), except XG-14, which was maintained in X-VIVO 20 (Biowittaker) supplemented with 3 ng/mL IL-6 (Peprotech, Rocky Hill, NJ). The human myeloma cell lines RPMI8226, U266, LP1, and L363 (ATCC, Rockville, MD) grew autonomously in RPMI-10% FCS. All cell lines were free of Mycoplasma, as assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany). Peripheral blood B cells (PBBS) were purified using CD19 microbeads (Miltenyi Biotech, Paris, France), and dendritic cells (DCs) were generated from adherent monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-13.

Bone marrow or peripheral blood samples were collected from 5 patients with plasma cell leukemia (PCL) and 14 patients with intramedullary myeloma after informed consent was obtained. Mononuclear cells were obtained by centrifugation on Ficoll-hypaque medium. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, myeloma cells were purified (>95% purity) using CD138 microbeads (Miltenyi Biotech, Paris, France), whereas phenotype and apoptosis were analyzed on whole mononuclear cells. Polyclonal plasmablastic cells (PPCs) were generated by purified CD19+ PBBS in vitro. Briefly, PBBS were cultured in RPMI 1640 and 10% FCS in the presence of mitomycin-treated CD40L transfectant, IL-2 (20 U/mL), IL-4 (50 ng/mL), IL-10 (50 ng/mL), and IL-12 (2 ng/mL; R&D Systems, Abington, United Kingdom). After 4 days of culture, B cells were harvested and cultured without CD40 ligand (CD40L) transfectant and with IL-2, IL-10, IL-12, and IL-6 (5 ng/mL). On day 6 of culture, cells were stained with fluorescein isothiocyanate (FITC)–conjugated anti-CD20 (Beckman-Coulter, Marseilles, France) and phycoerythrin (PE)–conjugated anti-CD38 (Becton Dickinson, San Jose, CA) and CD20–CD38+ PPCs were sorted with a FACSVantage (Becton Dickinson).

Sera from 26 patients with myeloma at diagnosis, 10 patients with PCL, and 9 age-related healthy individuals were collected as described previously.

mRNA analysis

We generated cDNA with 2 μg total RNA using the Superscript II reverse transcriptase (Life Technologies) and oligo d(T) (Amersham Pharmacia Biotech, Orsay, France). Each 25-μL PCR reaction contained 1 μL of the first-strand cDNA, 1 μM of each primer (sense and antisense), 0.2 μM each of dNTP (2′-deoxynucleoside 5′-triphosphate), 1.5 mM MgCl2, 1 × polymerase buffer, and 2 units of Taq polymerase (Life Technologies). The following primers were used: BAFF, 5′-GGA GAA GAC GAC TCT CAG AAC (sense) and 5′-CAA TTC ATC CCC AAA CAT GAC ATG GAC (antisense); APRIL, 5′-CCT TGC TAC CCC ACT CCT G (sense) and 5′-ACA TCT AGA ATA TCC CTT TGG (antisense); BCMA, 5′-TTA CTT GTC CTT CCA GGC TGT TCT (sense) and 5′-CAT AGA AAG CAA GGA AGT TAC C (antisense); TACI, 5′-CAG CCT AAG CAA GAG TCT GC (sense) and 5′-TGG GAC TCA GAG TGC C (antisense); BAFF-R, 5′-GAA GGC AGC AACC CAC (sense) and 5′-AAG GAC GAC ACA CCA AA (antisense); bc20-globulin (b2M), 5′-CCA GCA GAT AGG AAG TC (sense) and 5′-GAT GCT GCT TAC ATG TCT CG (antisense). The sizes of the PCR products were as follows: BAFF, 311 bp; APRIL, 729 bp; BCMA, 806 bp; TACI, 931 bp; BAFF-R, 300 bp; and b2M, 269 bp. The amplification profile was 1 minute at 94°C, 1 minute at 62°C (BAFF), 67°C (APRIL), 58°C (BCMA), 60°C (TACI), 61°C (BAFF-R), and 60°C (b2M), 1 minute at 72°C, followed by a final extension of 10 minutes at 72°C. Reaction products were electrophoresed on a 1.5% agarose gel.

Flow cytometry analysis

The overall expression of receptors for BAFF on HMCLs was evaluated by incubating 5 × 10⁶ cells with 10 μg/mL of a human BAFF–murine CD8 (BAFF-muCD8) biotinylated fusion protein (Ancell, Bayport, MN) in phosphate-buffered saline (PBS) containing 30% human AB serum at 4°C for 30 minutes followed by incubation with PE-conjugated streptavidin (Beckman-Coulter). For primary samples, cells were double stained with BAFF-muCD8 fusion protein and FITC-conjugated anti-CD138 (Beckman-Coulter). The expression of BAFF was evaluated using an anti-BAFF antibody (Buffy-1; Alexis Biotechnology, Lausen, Switzerland). Flow cytometry analysis was done on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Study of apoptosis

IL-6-dependent HMCLs were starved of IL-6 for 3 hours and cultured in 24-well, flat-bottomed microtiter plates at 10⁵ cells per well in RPMI 1640–10% FCS or X-VIVO 20 culture medium with or without IL-6 (3 ng/mL), BAFF (200 ng/mL; Peprotech), or APRIL (200 ng/mL; R&D Systems). After 3 days of culture, cells were washed twice in PBS and apoptosis was assayed with FITC-conjugated annexin V labeling (Boehringer). Fluorescence was analyzed on a FACScan flow cytometer. In order to study the dexamethasone (DEX)–induced apoptosis, autonomously growing HMCLs were cultured for 3 days in 24-well, flat-bottomed microtiter plates at 10⁵ cells per well in RPMI 1640–10% FCS with or without DEX (10⁻⁶ M), IL-6 (3 ng/mL), BAFF (200 ng/mL), or APRIL (200 ng/mL) and apoptosis was assayed with annexin V labeling.

Proliferation assay

HMCLs were IL-6 starved for 3 hours and cultured for 5 days in 96-well, flat-bottomed microtiter plates at 10⁵ cells per well in RPMI 1640–10% FCS or X-VIVO 20 with or without IL-6 (3 ng/mL), BAFF (200 ng/mL),
APRIL (200 ng/mL), the B-E8 anti-IL-6 antibody (10 μg/mL) (Diaclone, Besancon, France), an inhibitor of PI-3K/AKT pathway (Ly 294002; 25 μM), an inhibitory peptide of NF-κB pathway (SN50), or the corresponding inactive peptide (100 μg/mL) (BIOMOL, Plymouth Meeting, PA), or a fusion protein of TACI and the human Fc fragment of immunoglobulin TACI-Fc; 10 μg/mL; R&D Systems). Cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech) for the last 12 hours of culture, harvested, and counted on a liquid scintillation analyzer.

Mononuclear cell culture

Mononuclear cells from tumor samples of 8 patients with MM were cultured for 4 days at 5 × 10^5 cells/mL in RPMI 1640 medium, 5% FCS, 1 ng/mL IL-6, with or without 10^{-6} M dexamethasone (DEX), BAFF (200 ng/mL), or APRIL (200 ng/mL). In each culture group, viability and cell counts were assayed and myeloma cells were stained with an anti-CD138-PE MoAb (ImmunoTech).

ELISA

ELISA microplates (Nunc MaxiSorp; Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 100 μL mouse anti-human BAFF antibody (RDI, Flanders, NJ) or mouse anti-human APRIL antibody (R&D Systems) (10 μg/mL in PBS). Plates were washed 5 times with PBS, 0.1% Tween 20, and blocked with PBS, 1% BSA for 2 hours at room temperature. Patients’ or healthy donors’ sera were added and plates were incubated for 2.5 hours at 37°C and washed. Rabbit anti-human BAFF antibody (Upstate, Lake Placid, NY) or goat anti-human APRIL antibody (R&D Systems) (2 μg/mL in PBS, 1% BSA, 0.05% Tween 20) were added for 2 hours at room temperature and the bound antibodies were detected with goat anti-rabbit (Sigma, 1:15,000) or rabbit anti-goat (Dako, Copenhagen, Denmark; 1:1,000) peroxidase-conjugated antibodies. The peroxidase reaction was developed with a tetramethylbenzidine (TMB) substrate kit (Sigma, St Louis, MO). Light absorbance was measured at 450 nm and standard curves were generated using known concentrations of recombinant human BAFF or APRIL. The sensitivity of the ELISA was 1.5 ng/mL for BAFF and 3 ng/mL for APRIL. The intra-assay variability of the ELISA was determined by measuring serum samples from 7 patients in 2 separate experiments and was less than 14% for BAFF and less than 17% for APRIL.

Western blot analysis

HMCLs were starved overnight in RPMI 1640–1% bovine serum albumin (BSA) without IL-6. Cells were lysed in 10 mM tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPP), 1% Triton X-100, 5 μM ZnCl2, 100 mM NaVO4, 1 mM dithiothreitol (DTT), 20 mM β-glycerophosphate, 20 mM P-nitrophenoxyphosphate (PNPP), 2.5 μg/mL aprotinin, 2.5 μg/mL leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM benzamidine, 5 μg/mL pepstatin, and 50 mM o릭akaid acid. Lysates were cleared by centrifugation at 10,000 g for 10 minutes and resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1 hour at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM tris-HCl (pH 7.4), 0.1% Tween 20 (TBS-T), 5% BSA, then incubated for 1 hour at room temperature with primary antibodies (phospho-specific antibodies anti-ERK1/2, anti–signal transducer and activator of transcription 3 (STAT3) and anti--AKT; New England Biolabs, Beverly, MA) at a 1:1000 dilution in 1% BSA TBS-T. The primary antibodies were visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Bio-Rad, Hercules, CA) peroxidase-conjugated antibodies using an enhanced chemiluminescence detection system. As a control for protein loading, we used anti-STAT3 (1:2000; Transduction Laboratories, Lexington, KY), anti-ERK1/2 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-AKT (New England Biolabs) antibodies. Rabbit polyclonal antibodies specific for Bcl-x and Mcl-1 were obtained from Santa Cruz Biotechnology and Bcl-2 antibody from Dako.

BLOTS were quantified by densitometry using acquisition into Adobe Photoshop (Apple, Cupertino, CA) and analyzing with the NIH Image software (National Institutes of Health, Bethesda, MD).

Nuclear transcription factor–κB assay

NF-κB activation was determined with a Trans-AM NF-κB p50 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. This ELISA used a 96-well plate coated with an oligonucleotide containing the NF-κB consensus binding site (5'-GGGACTTTCC-3'). Following overnight starvation, cells were seeded on a 24-well plate (10^5 cells/well) and were stimulated for 1 hour with IL-6 (3 ng/mL), BAFF (200 ng/mL), APRIL (200 ng/mL), or TNF-α (20 ng/mL). Cell lysates were diluted (1:10) and added to the ELISA plate. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p50, visualized by anti-IgG horseradish peroxidase conjugate, and quantified at 450 nm. Each condition was run in triplicate.

Statistical analysis

Statistical significance was tested using a nonparametric Wilcoxon test for pairs or a Student t test for pairs.

Results

BCMA, TACI, and BAFF-R expression in malignant and normal plasma cells

RT-PCR analysis indicated that 13 of 13 HMCLs expressed BCMA according to our microarray results (Figure 1A). The expression pattern of TACI and BAFF-R was more heterogeneous. As shown in Figure 1A, TACI and BAFF-R were expressed, respectively, by 8 of 13 and 9 of 13 HMCLs. Unlike DCs, purified B cells expressed BCMA, TACI, and BAFF-R as reported. We next looked for the expression in primary myeloma cells of 6 patients with intramedullary myeloma (patients 1-6) and of 5 patients with plasma cell leukemia (PCL) (patients 7-11). BCMA RNA was detected in 11 of 11 samples (Figure 1A). TACI and BAFF-R were simultaneously expressed by 8 of 11 primary myeloma samples.

Expression of BAFF and APRIL receptors was found in 5 of 5 in vitro–generated normal plasmablasts. In particular, BCMA and TACI were detected at a high level whereas BAFF-R was less expressed. These results are in agreement with our Affymetrix data and with the recent study of Avery et al (Figure 1A).

To confirm the membrane expression of receptors for BAFF, we used a biotinylated human BAFF–murine CD8 fusion protein, which binds to TACI, BCMA, and BAFF-R. In agreement with previous studies, this BAFF–murine CD8 did not label monocye-derived DCs but efficiently bound purified B cells (Figure 1B). BAFF–murine CD8 fusion protein bound to 8 of 13 HMCLs. All of them expressed high levels of TACI or BAFF-R (XG-2, XG-13, XG-14, XG-20, LP1, L363, U266, and RPMI 8226). The 5 HMCLs that were not labeled by BAFF–murine CD8 expressed BCMA alone or BCMA and a low level of BAFF-R (XG-1, XG-5, XG-6, XG-7, and XG-11; Figure 1A). In addition, we confirmed the presence of membrane receptors on primary myeloma cells and normal plasmablasts (Figure 1C).

BAFF and APRIL expression in malignant and normal plasma cells

Since an autocrine production of APRIL and BAFF was previously reported in several tumor models, we looked for their expression in HMCLs and in primary myeloma cells. BAFF RNA was detected in 12 of 13 HMCLs and in 11 of 13 primary myeloma samples. An anti-BAFF antibody stained 10 of 12 HMCLs that

From www.bloodjournal.org by guest on August 30, 2017. For personal use only.
expressed BAFF mRNA, showing the presence of the membrane-bound form of BAFF. Data for 4 cell lines are shown in Figure 2B. XG-6 showed no cell-surface expression of BAFF, in agreement with the absence of detectable BAFF RNA in these cells (Figure 2B). APRIL RNA was expressed in most primary samples (10 of 11) and in 6 of 13 HMCLs (Figure 2A). As APRIL is a secreted protein, we looked for APRIL protein in myeloma cell culture supernatants. Using ELISA, soluble APRIL levels were 30.9 ng/mL and 33.3 ng/mL in culture supernatants of RPMI8226 and XG-20, respectively, that expressed APRIL mRNA. APRIL was not detectable in culture supernatant of XG-6 which did not express the gene. Polyclonal plasmablasts expressed BAFF and APRIL RNA and were labeled by the anti-BAFF antibody (Figure 2A-B).

**BAFF and APRIL rescue IL-6–dependent HMCLs from apoptosis induced by IL-6 deprivation**

To investigate the effect of BAFF and APRIL on myeloma cell survival and proliferation, we first used 3 cell lines whose growth is dependent on addition of IL-6: XG-13 and XG-20 HMCLs that expressed TACI and BAFF-R and XG-14 that expressed mainly TACI. In the absence of exogenous cytokines, the 3 HMCLs did not proliferate and a strong proliferation was induced by recombinant IL-6 (Figure 3A).31 BAFF and APRIL were also potent proliferation factors for XG-13 and XG-20 cells, whereas XG-14 cells responded to APRIL only (Figure 3A). Using annexin V as an indicator of apoptosis, we looked for the effect of BAFF and APRIL on myeloma cell survival. BAFF
and APRIL efficiently protected XG-13 cells (respectively, $P = .01$ and $P = .001$; $n = 5$) and XG-20 cells (respectively, $P = .0003$ and $P = .0002$; $n = 5$) from IL-6 deprivation-induced apoptosis. Only APRIL protected XG-14 ($P = .003$; $n = 5$) cells from apoptosis, in agreement with the above-mentioned proliferation data (Figure 3B). A TACI-Fc fusion protein abrogated specifically the myeloma cell proliferation induced by BAFF or APRIL, whereas an anti–IL-6 MoAb did not affect it (Figure 3C). Conversely, TACI-Fc had no effect on IL-6–induced proliferation that was completely inhibited by an anti–IL-6 MoAb (Figure 3C).

Finally, we looked for the ability of BAFF and APRIL to support the long-term growth of XG-13 and XG-14 HMCLs. As shown in Figure 3D, XG-13 and XG-14 cells died within 17 to 18 days upon removal of IL-6. IL-6 induced an exponential growth of the 2 HMCLs, with a doubling time of 48 hours for XG-13 and 20 hours for XG-14. APRIL and BAFF were both able to support the long-term growth of XG-13 cells with a doubling time, respectively, 2.2-fold and 1.75-fold higher than that obtained with IL-6. In agreement with the survival and proliferation data shown above, only APRIL supported long-term growth of XG-14 cells with a doubling time 1.5-fold higher than that obtained with IL-6.

These data indicate that BAFF and APRIL myeloma cell growth factors are able to support the long-term growth of cytokine-dependent HMCLs.

### Autocrine BAFF and/or APRIL are involved in the autonomous growth of cytokine-independent HMCLs

As BAFF and/or APRIL are growth factors for IL-6–dependent HMCLs and are produced by some autonomously growing HMCLs, we investigated whether BAFF/APRIL could be autocrine myeloma growth factors. We used RPMI8226 and L363 HMCLs that expressed BAFF and/or APRIL together with their receptors (Figures 1 and 2). The autonomous proliferation of L363 and RPMI8226 cells was blocked by TACI-Fc, which neutralizes both BAFF and APRIL (Figure 4). Adding an excess of recombinant BAFF and APRIL abrogated the inhibitory effect of TACI-Fc. These data indicated that a BAFF/APRIL autocrine loop is involved in the autonomous growth of some HMCLs.
BAFF and APRIL inhibit apoptosis of primary myeloma cells

Our data showing that BAFF and APRIL are survival factors for malignant plasma cells suggest that new therapeutic agents inhibiting BAFF/APRIL may be promising for myeloma treatment. We thus investigated the effect of the TACI-Fc fusion protein, able to block BAFF and APRIL, on primary myeloma cell survival and on drug sensitization. Primary myeloma cells were cultured with their bone marrow environment and recombinant IL-6. Detailed results obtained with 6 patients are shown in Table 1. TACI-Fc significantly reduced the median number of viable myeloma cells by 48% \( (P = 0.028; n = 6) \). TACI-Fc also potentiated the inhibitory effect of DEX or B-E8 anti-IL-6 MoAb (respectively, \( P = 0.028; P = 0.046; n = 6 \)). When the 3 inhibitors were used together, a 90% reduction of viable myeloma cells was observed within 4 days of culture (Figure 6A). Of interest, the nonmalignant cells present in the culture wells were unaffected by these 3 inhibitors (results not shown).

Signal transduction and antiapoptotic protein regulation by BAFF or APRIL

As shown in Figure 7A, BAFF and APRIL induced a rapid phosphorylation of AKT and a late phosphorylation of MAPK in 3 myeloma cell lines (XG-13, XG-14, and RPMI8226), whereas no phosphorylation of STAT3 was detected. IL-6 induced the phosphorylation of STAT3, MAPK, and AKT, in agreement with previous data.36,37 We also looked for NF-κB signaling, as there is accumulating evidence that BAFF and APRIL activate NF-κB transcription factors in B cells.33,38-40 We found that BAFF and APRIL, like TNF-α, enhanced NF-κB binding activity in XG-13 and RPMI8226 cells, whereas IL-6 induced a weak and transient activation of NF-κB, in agreement with previous studies41 (Figure 7B). For XG-14 cells that biologically responded only to APRIL, we found that APRIL, unlike BAFF, enhanced NF-κB binding activity.

Table 1. TACI-Fc induces apoptosis of primary myeloma cells

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Control</th>
<th>TACI-Fc</th>
<th>DEX</th>
<th>DEX + TACI-Fc</th>
<th>Anti-IL-6 MoAb</th>
<th>Anti-IL-6 MoAb + TACI-Fc</th>
<th>Anti-IL-6 MoAb + DEX</th>
<th>Anti-IL-6 MoAb + DEX + TACI-Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>113 774</td>
<td>97 474</td>
<td>81 280</td>
<td>56 940</td>
<td>120 250</td>
<td>98 610</td>
<td>37 180</td>
<td>23 800</td>
</tr>
<tr>
<td>2</td>
<td>69 960</td>
<td>37 884</td>
<td>28 980</td>
<td>16 168</td>
<td>42 560</td>
<td>31 740</td>
<td>15 752</td>
<td>9 550</td>
</tr>
<tr>
<td>3</td>
<td>18 864</td>
<td>10 494</td>
<td>19 800</td>
<td>8 400</td>
<td>20 692</td>
<td>12 096</td>
<td>19 008</td>
<td>4 344</td>
</tr>
<tr>
<td>4</td>
<td>34 768</td>
<td>30 380</td>
<td>30 608</td>
<td>14 084</td>
<td>16 226</td>
<td>16 434</td>
<td>7 872</td>
<td>5 265</td>
</tr>
<tr>
<td>5</td>
<td>99 540</td>
<td>53 508</td>
<td>34 914</td>
<td>28 457</td>
<td>59 280</td>
<td>30 576</td>
<td>15 228</td>
<td>7 912</td>
</tr>
<tr>
<td>6</td>
<td>171 000</td>
<td>154 400</td>
<td>158 420</td>
<td>107 100</td>
<td>160 056</td>
<td>130 764</td>
<td>112 624</td>
<td>52 920</td>
</tr>
<tr>
<td>Median value</td>
<td>84 750</td>
<td>45 696</td>
<td>32 761</td>
<td>22 312</td>
<td>50 920</td>
<td>31 158</td>
<td>17 380</td>
<td>8 731</td>
</tr>
</tbody>
</table>

Mononuclear cells from tumor samples of 6 patients with MM were cultured for 4 days in the presence of IL-6 (1 ng/mL) with or without DEX (10^{-6} M), TACI-Fc (10 μg/mL), or B-E8 anti-IL-6 MoAb (10 μg/mL). At day 4 of culture, the cell count and viability were determined and the percentage of CD138^+ viable plasma cells was determined by flow cytometry.

BAFF/ APRIL inhibitor induces apoptosis of primary myeloma cells

We next sought to determine whether BAFF or APRIL could protect myeloma cells from the apoptosis induced by DEX, a potent drug for MM treatment. As indicated in Figure 5A, DEX induced apoptosis in RPMI 8226 and L363 HMCLs. Both BAFF and APRIL significantly protected the RPMI 8226 HMCL from DEX-induced apoptosis (respectively, \( P = 0.001 \) and \( P = 0.0002; n = 5 \)). The same results were obtained with L363 (respectively, \( P = 0.0007 \) and \( P = 0.001; n = 5 \)). In fact, both BAFF and APRIL were as potent as IL-6 in protecting myeloma cells from DEX-induced apoptosis (Figure 5A).

We next investigated whether BAFF and APRIL could protect primary myeloma cells from DEX-induced apoptosis. Since purified myeloma cells are highly susceptible to spontaneous apoptosis in vitro, myeloma cells were cultured in the presence of their bone marrow environment. In addition, recombinant IL-6 was added to reduce the variability resulting from the heterogeneous endogenous IL-6 production in cultured tumor samples.35 As shown in Figure 5B, DEX reduced the median number of viable myeloma cells of 8 patients by 58% \( (P = 0.03; n = 8) \). BAFF and APRIL enhanced survival of myeloma cells in the presence of DEX (respectively, \( P = 0.01 \) and \( P = 0.01; n = 8 \)) yielding a number of malignant plasma cells that was not statistically different between DEX and BAFF, DEX and APRIL, and the control group (Figure 5B).

BAFF/ APRIL inhibitor induces apoptosis of primary myeloma cells

Mononuclear cells from 8 patients with MM were cultured for 4 days in the presence of IL-6 (1 ng/mL) with or without DEX (10^{-6} M), BAFF (200 ng/mL), or APRIL (200 ng/mL). At day 4 of culture, the viability and total cell counts were assessed and the percentage of CD138^+ viable plasma cells was determined by flow cytometry. Results are median values of the numbers of myeloma cells in the culture wells. The values were compared with a Wilcoxon test for pairs.
Interestingly, an inhibitor of PI3K/AKT (Ly 294002) abrogated the proliferation of XG-13 cells induced by BAFF or APRIL. A peptide inhibitor of the NF-kB pathway (SN50) also inhibited BAFF- or APRIL-induced myeloma cell proliferation, unlike the corresponding inactive peptide (Figure 7C).

We then studied the regulation by BAFF or APRIL of 3 Bcl-2 family antiapoptotic members (Mcl-1, Bcl-2, and Bcl-xL) known to be involved in growth factor–mediated myeloma cell survival or in BAFF-mediated survival in B cells.42-47 BAFF and APRIL induced an up-regulation of Mcl-1 and Bcl-2 in XG-13 and RPMI8226 cells, whereas only APRIL increased Mcl-1 and Bcl-2 levels in XG-14 cells. In contrast, no change was noted in Bcl-xL protein expression (Figure 8A-B). IL-6 increased Mcl-1 but neither Bcl-2 nor Bcl-xL levels according to our previous studies.45

Levels of circulating BAFF and APRIL in sera of patients with MM

To further assess the biologic relevance of our data, we looked for levels of soluble BAFF and APRIL in the sera of 36 patients with MM and 9 age-related healthy individuals. Results shown in Figure 9 demonstrated that BAFF or APRIL median serum levels were increased, respectively, 4.2-fold (P = .02) and 5.9-fold in patients (P = 6.10^-9) compared with healthy individuals.

Discussion

Accumulating experimental evidence supports the notion that BAFF is essential for the survival of normal immature and mature B cells15 as well as normal plasmablastic cells.34 BAFF plays a key role in the survival of B-CLL tumor cells.28,29 In addition, APRIL stimulates the growth of some human and murine tumor cell lines in vitro and in vivo.17 As BAFF/APRIL receptor genes are overexpressed in malignant plasma cells,10,11 our aim was to look for a role played by BAFF and APRIL in MM.

We demonstrate here that BAFF and APRIL are growth factors for 2 myeloma cell lines that highly expressed TACI and BAFF-R and whose survival is completely dependent on addition of exogenous growth factors. APRIL is also a growth factor for a third cell line, XG-14, which expressed only TACI, unlike BAFF-R. We also show that an autocrine loop involving BAFF, APRIL, and their receptors is involved in the autonomous growth of 2 well-known HMCLs, L363 and RPMI8226. BAFF and APRIL contribute to the
XG-14 expresses a receptor specific for BAFF-R were sensitive to APRIL only. One hypothesis is that the expression of TACI and BAFF-R is tightly regulated during the B-cell maturation process, and one can hypothesize that these 2 molecules could have different biologic activities depending on the cell type. Erythematosus immune diseases such as Sjögren syndrome and systemic lupus erythematosus have been identified, as reported for adenocarcinoma cells. Novak et al reported that all myeloma cells they tested bound soluble BAFF.

In that study, BAFF-R was not detectable on the cell surface of HMCLs using an anti–BAFF-R antibody, whereas purified primary myeloma cells expressed BAFF-R. Thus, these data and our results indicate that we cannot yet draw firm conclusions on the respective role of TACI and BAFF-R in myeloma cells that express the 2 receptor genes, that is, the majority of purified primary myeloma cells.

In order to better understand the effect of BAFF and APRIL on myeloma cells, we examined intracellular signaling pathways. BAFF was reported to induce NF-κB activation in B cells and an overexpression of BCMA in human 293 cells activates the Rel/NF-κB, JNK, Elk-1, and p38 kinase transcription factors. Activation of TACI in Jurkat T cells also results in activation of AP-1, NF-κB, and nuclear factor of activated T cells (NF-AT).

In myeloma cells, we and others have shown that IL-6 activates 3 essential pathways: the JAK/STAT, MAPK, and PI3K/Akt cascades, whereas IGF-1 activates MAPK and PI3K/Akt. IGF-1 also activates NF-κB. We show here that neither BAFF nor APRIL was able to induce STAT3 phosphorylation but did activate ERK1/2 and PI3K/Akt pathways. An inhibitor of PI3K/AKT abrogated the growth of myeloma cells induced by BAFF and APRIL.

Figure 8. Regulation of Bcl-2 family antiapoptotic proteins by BAFF and APRIL.

Figure 9. Serum level of circulating BAFF and APRIL in myeloma patients. Serum levels of BAFF and APRIL were determined by ELISA in the sera from 36 patients with myeloma and 9 age-related healthy individuals.
the rescue from apoptosis induced by growth-factor removal or DEX treatment.

The data mentioned previously in this paper were that obtained with myeloma cell lines could be extended to primary myeloma cells that expressed BAFF-R and/or TACI. As primary myeloma cells rapidly apoptosed as soon as they were purified, they were cultured together with their bone marrow environment. The TACI-Fc fusion protein, able to block both BAFF and APRIL, reduced the survival of primary myeloma cells, and increased inhibition was obtained when TACI-Fc was used together with DEX or anti-IL-6 MoAb. Interestingly, when the 3 inhibitors were combined, virtually all primary myeloma cells died, whereas the cocultured nonmyeloma cells were unaffected. These in vitro data suggest that inhibitors of BAFF and TACI could be very useful to induce apoptosis of myeloma cells when used alone or in combination with DEX and or anti-IL-6 MoAb. The advantage of using BAFF/APRIL inhibitors in MM is emphasized by the current finding that serum levels of BAFF and APRIL were increased roughly 5-fold in patients with MM as compared with age-related healthy individuals. These serum concentrations were in the range of those able to promote myeloma cell growth in vitro. The presence of circulating APRIL has not been reported in humans. The circulating serum levels of BAFF reported here were close to those found in autoimmune diseases, where it was correlated with the autoantibody level. Further studies are necessary to determine whether BAFF or APRIL serum levels are prognostic factors in patients with multiple myeloma.

New therapeutic agents have now been developed to inhibit BAFF/APRIL in B-cell neoplasia and autoimmune diseases, such as anti-BAFF MoAb and the TACI-Fc, or the BAFF/APRIL signaling pathway, such as the PS-1145 1kB kinase inhibitor. Thus, the present report suggests that these novel inhibitors may be promising elements in the treatment of patients with MM, possibly in association with DEX and/or anti-IL-6 MoAb.

References

9. Klein B, Wijdenes J, Zhang XG, et al. Murine anti–IL-6 MoAb. Interestingly, when the 3 inhibitors were combined, virtually all primary myeloma cells died, whereas the cocultured nonmyeloma cells were unaffected. These in vitro data suggest that inhibitors of BAFF and TACI could be very useful to induce apoptosis of myeloma cells when used alone or in combination with DEX and or anti-IL-6 MoAb. The advantage of using BAFF/APRIL inhibitors in MM is emphasized by the current finding that serum levels of BAFF and APRIL were increased roughly 5-fold in patients with MM as compared with age-related healthy individuals. These serum concentrations were in the range of those able to promote myeloma cell growth in vitro. The presence of circulating APRIL has not been reported in humans. The circulating serum levels of BAFF reported here were close to those found in autoimmune diseases, where it was correlated with the autoantibody level. Further studies are necessary to determine whether BAFF or APRIL serum levels are prognostic factors in patients with multiple myeloma.

New therapeutic agents have now been developed to inhibit BAFF/APRIL in B-cell neoplasia and autoimmune diseases, such as anti-BAFF MoAb and the TACI-Fc, or the BAFF/APRIL signaling pathway, such as the PS-1145 1kB kinase inhibitor. Thus, the present report suggests that these novel inhibitors may be promising elements in the treatment of patients with MM, possibly in association with DEX and or anti-IL-6 MoAb.

References

8. Klein B, Wijdenes J, Zhang XG, et al. Murine anti–IL-6 MoAb. Interestingly, when the 3 inhibitors were combined, virtually all primary myeloma cells died, whereas the cocultured nonmyeloma cells were unaffected. These in vitro data suggest that inhibitors of BAFF and TACI could be very useful to induce apoptosis of myeloma cells when used alone or in combination with DEX and or anti-IL-6 MoAb. The advantage of using BAFF/APRIL inhibitors in MM is emphasized by the current finding that serum levels of BAFF and APRIL were increased roughly 5-fold in patients with MM as compared with age-related healthy individuals. These serum concentrations were in the range of those able to promote myeloma cell growth in vitro. The presence of circulating APRIL has not been reported in humans. The circulating serum levels of BAFF reported here were close to those found in autoimmune diseases, where it was correlated with the autoantibody level. Further studies are necessary to determine whether BAFF or APRIL serum levels are prognostic factors in patients with multiple myeloma.

New therapeutic agents have now been developed to inhibit BAFF/APRIL in B-cell neoplasia and autoimmune diseases, such as anti-BAFF MoAb and the TACI-Fc, or the BAFF/APRIL signaling pathway, such as the PS-1145 1kB kinase inhibitor. Thus, the present report suggests that these novel inhibitors may be promising elements in the treatment of patients with MM, possibly in association with DEX and or anti-IL-6 MoAb.

References


BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone

Jérôme Moreaux, Eric Legouffe, Eric Jourdan, Philippe Quittet, Thierry Rème, Cécile Lugagne, Philippe Moine, Jean-François Rossi, Bernard Klein and Karin Tarte

Updated information and services can be found at:
http://www.bloodjournal.org/content/103/8/3148.full.html

Articles on similar topics can be found in the following Blood collections
- Apoptosis (747 articles)
- Gene Expression (1086 articles)
- Genomics (149 articles)
- Neoplasia (4182 articles)
- Signal Transduction (1930 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml