Role of B-Cell–Activating Factor in Adhesion and Growth of Human Multiple Myeloma Cells in the Bone Marrow Microenvironment

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Abstract

Recent studies have underscored the role of B-cell-activating factor (BAFF), a member of the tumor necrosis factor superfamily, in promoting the survival of malignant B cells, including human multiple myeloma. We here characterized the functional significance of BAFF in the interaction between multiple myeloma and bone marrow stromal cells (BMSC) and further defined the molecular mechanisms regulating these processes. BAFF is detected on BMSCs derived from multiple myeloma patients as evidenced by flow cytometry. BAFF secretion is 3- to 10-fold higher in BMSCs than in multiple myeloma cells, and tumor cell adhesion to BMSCs augments BAFF secretion by 2- to 5-fold, confirmed by both ELISA and immunoblotting. Adhesion of MM1S and MCCAR multiple myeloma cell lines to KM104 BMSC line transfected with a luciferase reporter vector carrying the BAFF gene promoter (BAFF-LUC) significantly enhanced luciferase activity, suggesting that nuclear factor-κB (NF-κB) activation induced by multiple myeloma adhesion to BMSCs mediates BAFF up-regulation. Moreover, BAFF (0-100 ng/mL) increases adhesion of multiple myeloma lines to BMSCs in a dose-dependent manner; conversely, transmembrane activator and calcium modulator and cyclophylin ligand interactor-Ig or B-cell maturation antigen/Fc blocked BAFF stimulation. Using adenoviruses expressing dominant-negative and constitutively expressed AKT as well as NF-κB inhibitors, we further showed that BAFF-induced multiple myeloma cell adhesion is primarily mediated via activation of AKT and NF-κB signaling. Importantly, BAFF similarly increased adhesion of CD138-expressing patient multiple myeloma cells to BMSCs. These studies establish a role for BAFF in localization and survival of multiple myeloma cells in the bone marrow microenvironment and strongly support novel therapeutics, targeting the interaction between BAFF and its receptors in human multiple myeloma. (Cancer Res 2006; 66(13): 6675-82)

Introduction

Multiple myeloma is a clonal B-cell malignancy characterized by accumulation of malignant plasma cells within the bone marrow in close contact with stromal cells, which secrete factors/cytokines, promoting tumor cell growth and survival. Among the factors and cytokines produced by multiple myeloma microenvironment, paracrine secretion of interleukin-6 (IL-6) in bone marrow stromal cells (BMSC) promotes multiple myeloma cell proliferation and protects against drug-induced cytotoxicity (1, 2). Other cytokines (i.e., insulin-like growth factor-1, macrophage inflammatory protein-1α, and hepatocyte growth factor) similarly provide stimulatory signals for multiple myeloma growth and survival (2–6). Most recently, B-cell-activating factor of the tumor necrosis factor (TNF) family (BAFF; also known as B lymphocyte stimulator), a CD40L-related molecule produced by myeloid cells, was identified as a critical factor in normal B-cell development and homeostasis (7–10). BAFF is expressed predominantly by macrophages and dendritic cells and provides a key survival signal for the maturation of peripheral B cells (7, 10). It is produced as both a membrane-bound and a proteolytically cleaved soluble protein (7, 10). Subsequent studies found elevated BAFF secretion in autoimmune diseases (11) and many B-cell malignancies, including multiple myeloma (12–16). Importantly, dysfunctional BAFF signaling occurs in many B-cell neoplasias with an autocrine loop stimulating tumor cell growth and survival (14, 16). BAFF also attenuates tumor cell apoptosis because exposure of non–Hodgkin's lymphoma (NHL) B cells to recombinant BAFF increases nuclear factor-κB (NF-κB) activation, up-regulates Bcl-2 and Bcl-x(L), and down-regulates Bax (17). Therefore, NHL B cells deregulate an otherwise physiologic autocrine survival pathway to evade apoptosis. BAFF is present in multiple myeloma cells and in the serum derived from patients with multiple myeloma, suggesting an autocrine loop of stimulation from these tumor cells (13, 18). However, the biological role of BAFF in multiple myeloma pathogenesis within the bone marrow microenvironment is not fully characterized.

BAFF binds to three receptors specifically expressed on B cells, including transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI), B-cell maturation antigen (BCMA), and BAFF-R (also known as BR3; refs. 19–21). In a mouse model, BCMA plays an important role in long-term plasma cell survival (22). In addition, although BCMA is found in the intracellular Golgi storage (23), surface expression of BCMA has been reported in multiple myeloma (24, 25). The membrane expression of BCMA represents a target of donor B-cell immunity in patients with multiple myeloma responding to donor lymphocyte infusion (25). Novak et al. (13) reported that TACI and BCMA are expressed in a heterogenous pattern on patient multiple myeloma cells. Moreaux et al. (26) suggested that the biological function of TACI expression is linked to the multiple myeloma cell dependence on their bone marrow microenvironment. Furthermore, patients with lower TACI expression have clinical variables associated with bad prognosis (26). Although expression of BAFF-R in multiple myeloma cells is detected by reverse transcription-PCR (RT-PCR) and gene expression profiling (13, 18), to date the

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functional significance of this BAFF-binding receptor in multiple myeloma is undefined.

As with CD40, TACI, BCMA, and BAFF-R signal by recruiting TNF receptor-associated factors (TRAF) to their cytoplasmic tails (24, 27). By activating IκB kinase (IKK), TRAFs induce phosphorylation-dependent degradation of IκB, a cytoplasmic inhibitor of NF-κB. The subsequent nuclear translocation of NF-κB transcriptionally activates genes involved in B-cell proliferation, differentiation, and survival. In addition, BAFF also induces another pathway of NF-κB activation involving ubiquitin-mediated processing of the NF-κB2 inhibitory protein p100 to p52, which in turn translocates to the nucleus and activates NF-κB-dependent transcription of target genes. This cascade has an essential function in the survival and progression of maturing splenic B cells (28) as well as chronic lymphocytic leukemia (CLL) B cells (15), but its significance in multiple myeloma is unknown.

High levels of BAFF were detected in patients with multiple myeloma, suggesting that the primary site of production for this factor is the bone marrow microenvironment (18). Moreover, BMSCs and osteoclasts are two main components in the bone marrow milieu that secrete abundant BAFF (26). In the present study, we define the functional significance of BAFF in human multiple myeloma cells both alone and within their bone marrow milieu and delineate the molecular and signaling events regulating these sequelae.

Materials and Methods

Cell culture. The $CD138^+$ human multiple myeloma-derived cell lines were maintained as described (29). All multiple myeloma lines express CD138 and CD38 (>95% of cells) as evidenced by flow cytometric analysis. Dexamethasone-sensitive MM1S and dexamethasone-resistant MM1R cells were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). MCCAR, U266, and RPMI8226 cells were obtained from the American Type Culture Collection (Manassas, VA). Doxorubicin-resistant RPMI8226 line (DOX40) was provided by Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). 12BM, 12PE, and 28BM multiple myeloma lines were provided by Dr. Otsuki (Kawasaki Medical School, Okayama, Japan; ref. 30). Freshly isolated multiple myeloma cells ($CD138^+$) obtained after Institutional Review Board–approved (Dana-Farber Cancer Institute, Boston, MA) informed consent were prepared by positive selection using CD138 microbeads (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. More than 95% of the multiple myeloma cells of the selected cell population expressed CD138 and CD38 as confirmed by flow cytometry.

Reagents. BAFF was obtained from PeproTech, Inc. (Rocky Hill, NJ) or Research Diagnostics, Inc. (Flanders, NJ). Antibodies used for flow cytometry were phycoerythrin (PE) anti-human BAFF and PE anti-human BR3 (obtained from eBioscience, San Diego, CA) as well as anti-human BCMA and PE anti-human TACI (obtained from R&D Systems, Minneapolis, MN). TACI-Ig fusion protein [TACI (human):Fc (human)] and BCMA/Fc (blocking the binding of BAFF to its receptors TACI and BCMA, thus inhibiting BAFF-mediated signaling) were purchased from Alexis Biochemicals (San Diego, CA). Anti-BAFF antibody was obtained from ProSci, Inc. (Poway, CA) for immunoblotting. The other antibodies used for immunoblotting were purchased from Cell Signaling Technology (Beverly, MA). The IKK inhibitor PS1145 was obtained from Millennium Pharmaceuticals (Cambridge, MA; refs. 31, 32). All other reagents were purchased from Sigma Chemicals (St. Louis, MO).

Gene expression and microarray data analysis. More than 2 million CD138-expressing patient plasma cells, purified as described above, were lysed using Trizol; total RNA was isolated by Qiagen RNeasy kit (Qiagen, Valencia, CA). Gene expression was measured using human genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) scanned on a GeneArray scanner (Affymetrix). Normalization of arrays and calculation of expression values were done using the DNA-Chip Analyzer (dChip) program (33).

Cell proliferation assay and survival assay. Cell proliferation was measured by [^{3}H]thymidine (Perkin-Elmer, Boston, MA) incorporation. Multiple myeloma cells [1×10^4 (cell line) or 5×10^5 (patient multiple myeloma cells) per well, in triplicates] were incubated in 96-well culture plates at 37°C in the presence or absence of BMSCs with or without dexamethasone (Sigma) and lenalidomide (Celgene, Warren, NJ). Cells were pulsed with [^{3}H]thymidine (0.5 μCi/well for cell lines and 1 μCi/well for patient multiple myeloma cells) for 8 (cell lines) or 48 (patient multiple myeloma cells) hours, harvested, and counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). Cell survival was assessed by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colometric assays as described previously (29). Dye absorbance in viable cells was measured at 570 nm, with 630 nm as a reference wavelength. Data reported are average values plus SD of three representative experiments. Additional assays of apoptosis included Annexin V/propidium iodide staining for the percentage of sub-G_0-G_1 phase cells as in our prior studies (29, 31).

BMSC culture. Human BMSC lines KM101, KM103, KM104, and KM105 were kindly provided by Dr. Kenichi Harigaya (Chiba University Graduate School of Medicine, Chiba, Japan; ref. 34). Bone marrow mononuclear cells were first obtained after Ficoll-Hypaque density centrifugation of bone marrow aspirates from multiple myeloma patients, and BMSCs were obtained from CD138-nonexpressing fraction separated from CD138-positive patient multiple myeloma cells. BMSCs were cultured in Iscove's medium supplemented with 20% FCS at a concentration of 2×10^6/mL. The culture medium was replenished with fresh Iscove's medium/20% FCS once weekly. When a confluent layer of adherent cells was obtained, the cells were trypsinized and cultured in RPMI 1640/10% FCS for experiments.

ELISA. Multiple myeloma cells and BMSCs (3×10^5/mL) alone were seeded onto culture plates for 48 hours. For coculture experiments, BMSCs were seeded onto culture plates overnight, and multiple myeloma lines or patient multiple myeloma cells were added to these BMSC-coated plates the following day. Supernatants harvested from 48-hour cultures were tested for soluble BAFF by ELISA (R&D Systems) according to the manufacturer's instructions. The minimum detectable level of BAFF was 0.73 to 6.67 pg/mL.

Immunoblotting. Total cell lysates were subjected to 8% or 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes as reported previously (35).

Luciferase reporter assay. A luciferase reporter vector carrying the BAFF gene promoter (BAFF-LUC) contains at least six NF-κB-binding κB sites. The $\kappa B_{(2\times)}$-LUC reporter vector, as a positive control reporter, is driven by a NF-κB-responsive minimal promoter with two NF-κB-binding κB sites. To normalize firefly luciferase activities for transfection efficiency in KM104 BMSCs, we first cotransfected cells with either pCMX-lacZ or pRL-TK (Promega, Madison, WI), which encode β-galactosidase and *Renilla* luciferase, respectively. Relative luciferase activities were normalized to β-galactosidase activity (for pCMX-lacZ) or *Renilla* luciferase activity (for pRL-TK). We measured β-galactosidase activity using a β-galactosidase assay kit (Invitrogen, Carlsbad, CA), and luciferase activities using either single or dual luciferase reporter assay systems (Promega, Carlsbad, CA). The transfection efficiency in KM104 BMSCs is >85%. Next, KM104 BMSCs (20×10^6/mL) were cotransfected with plasmid DNA solution containing 20 μg BAFF-LUC or $\kappa B_{(2\times)}$-LUC reporter vectors with or without increasing amounts of pcDNA3.1 expression vector containing *IκBα* gene (IκBα-pcDNA3.1) that inhibits NF-κB activity. Transfection was done using Fugene 6 transfection reagent following manufacturer's instruction (Roche Diagnostics, Indianapolis, IN). Transfected cells (1×10^6/mL) were cultured for 48 hours. MM1S or MCCAR multiple myeloma cells were then added to transfected KM104 BMSCs for another 24 hours. The luciferase activity was measured with the Luciferase Assay System (Promega). BAFF-LUC, $\kappa B_{(2\times)}$-LUC, and IκBα-pcDNA3.1 expression vectors were kindly provided by Dr. Andrea Cerutti (Weill Medical College of Cornell University, New York, NY).

Cell adhesion assays. Cell adhesion assay was done as described previously (6). In brief, multiple myeloma cells and patient multiple myeloma cells (5×10^6/mL) were labeled with calcein AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C, washed, and resuspended in culture medium. Cells were stimulated with BAFF at 0 to 400 ng/mL

for 15 minutes and added to BMSC-coated 96-well plates at 37°C for 45 minutes (multiple myeloma cell lines) or 2 hours (multiple myeloma patient $CD138^+$ cells); unbound cells were removed by four washes with RPMI 1640. The absorbance of each well was measured using 492/520 nm filter set with a fluorescence plate reader (Wallac VICTOR2, Perkin-Elmer). In some experiments, labeled multiple myeloma cells were preincubated with or without TACI-Ig (1 μg/mL) or BCMA/Fc (μg/mL). To identify the involvement of BAFF signaling molecules in multiple myeloma adhesion, cells were pretreated with phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin (0.2 μmol/L) and Ly294002 (30 μmol/L) as well as NF-κB pathway inhibitors PS1145 and SN50 (0.5, 2.5 μmol/L) for 90 minutes. Treatments with these inhibitors alone produced no significant toxicity, evidenced by trypan blue exclusion at the end of experiments. To address AKT activation in BAFF-induced multiple myeloma cell adhesion, multiple myeloma cells were infected with adenoviruses expressing dominant-negative (dnAKT) and constitutively expressed AKT (myrAKT). Multiple myeloma cell adhesion to BMSCs was then done in the presence of BAFF (100 ng/mL). An adenovirus expressing β-galactosidase was used as a control.

Statistical analysis. Statistical significance of differences observed in experimental versus control cells was determined using the Student's *t* test. The minimal level of significance was $P < 0.05$.

Results

Expression of BAFF and BAFF-Rs in multiple myeloma patients. To define a role of BAFF signaling in multiple myeloma pathogenesis, we first analyzed the expression of BAFF and its three receptors, BAFF-R, TACI, and BCMA, on patient multiple myeloma cells ($n = 36$) using human genome U133 Plus 2.0 array data. As seen in Fig. 1, BAFF was heterogeneously expressed in CD138-expressing patient multiple myeloma cells, with 19 of 36 (>52.8%) above a cutoff arbitrary unit of expression of 400. Among its receptors, the expression of BCMA is much higher than TACI, with BAFF-R expression is the lowest: the maximum arbitrary units of expression for BCMA, TACI, and BAFF-R are 8,000, 2,500, and 300, respectively. These results confirm that BAFF and its receptors BCMA, TACI, and BAFF-R are heterogeneously expressed in multiple myeloma patient RNA: all patient multiple myeloma cells express low levels of BAFF-R, higher levels of TACI, and highest levels of BCMA.

BAFF rescues multiple myeloma cells from drug-induced apoptosis. Because BAFF can promote survival and proliferation of malignant B cells, including multiple myeloma (18, 13), we next determined the effect of BAFF on drug-induced multiple myeloma cell death triggered by dexamethasone and lenalidomide. Previously, Moreaux et al. (18) showed that BAFF rescues myeloma cells from dexamethasone-induced apoptosis in RPMI8226 and L363 multiple myeloma lines. As shown in Fig. 2*A* (*left*), BAFF triggered proliferation of 28PE multiple myeloma cells at levels similar to IL-6. In addition, BAFF protects 28PE multiple myeloma cells against dexamethasone-induced growth inhibition similar to IL-6. BAFF also overcomes lenalidomide-induced growth inhibition in MM1S multiple myeloma cells (Fig. 2*A, right*). Importantly, BAFF similarly rescues patient multiple myeloma cells from dexamethasone-induced growth inhibition and cell death as evidenced by MTT assay (Fig. 2*C*) and Annexin V staining (Fig. 2*D*). These results further confirmed a role of BAFF in growth and survival in multiple myeloma.

BMSCs are the main source of BAFF in multiple myeloma. We next measured surface BAFF expression by flow cytometric analysis using an anti-BAFF antibody. Cell membrane BAFF protein expression is barely detectable in all multiple myeloma cells (three multiple myeloma lines and two patient multiple myeloma cells; Fig. 3*A, top*). Using similar methods, we evaluated surface BAFF protein expression on BMSCs derived from CD138-negative mononuclear cells remaining after separation of CD138-expressing multiple myeloma cells. As shown in Fig. 2*A* (*bottom*), cell membrane BAFF expression was detected in all BMSCs, including one BMSC line (KM104) and four BMSCs from multiple myeloma patients. To further examine BAFF protein production, we measured BAFF secretion from multiple myeloma cells and BMSCs using BAFF ELISA. BAFF secretion is 2-fold greater in BMSCs than in multiple myeloma cells (Fig. 3*B*). The range of BAFF secretions in seven multiple myeloma lines (3×10^5) is from 16.22 plus 1.35 pg/mL to 84.38 plus 4.36 pg/mL, except for U266 cells that secrete >254.35 pg/mL. The mean BAFF secretion from three patient multiple myeloma cells is 62.4 pg/mL. In contrast, BAFF secretion from four BMSC lines ranges from 463 plus 14 pg/mL to 1,546 plus 23 pg/mL. Importantly, BMSCs derived from six multiple myeloma patients secrete even more BAFF than BMSC lines: from 973.27 plus 59 pg/mL to 3,409 plus 98 pg/mL. Therefore, the bone marrow environment is the main source of BAFF production in multiple myeloma.

Adhesion of multiple myeloma cells to BMSCs triggers BAFF secretion mediated by NF-κB activation in BMSCs. Because the bone marrow microenvironment is critical for multiple myeloma cell growth and survival, we next asked whether multiple myeloma adhesion to BMSCs alters BAFF production. BAFF secretion in

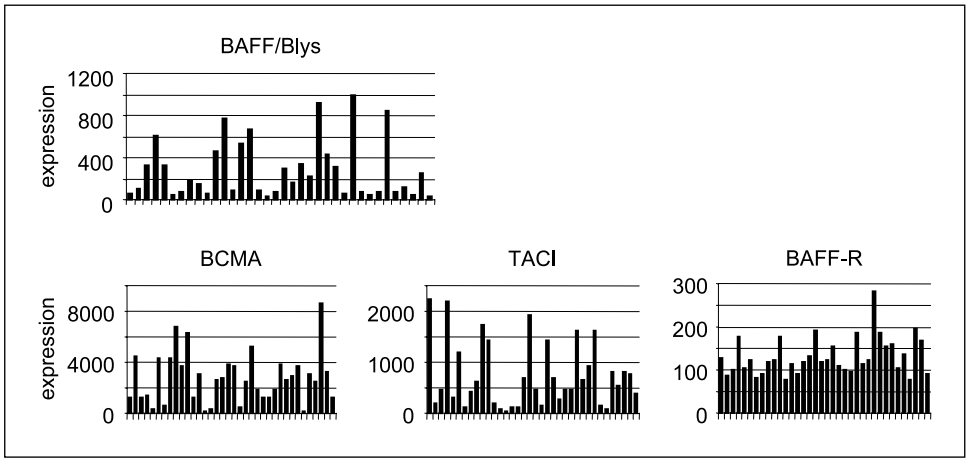

Figure 1. BAFF and BAFF-Rs are expressed heterogeneously in patient multiple myeloma cells. Expression profile of BAFF and BAFF-Rs (BCMA, TACI, and BAFF-R) in CD138-expressing (>98%) multiple myeloma cells using Affymetrix U133 Plus 2.0 array data. Total RNA isolated from CD138-purified multiple myeloma cells from each patient was subjected to microarray analysis.

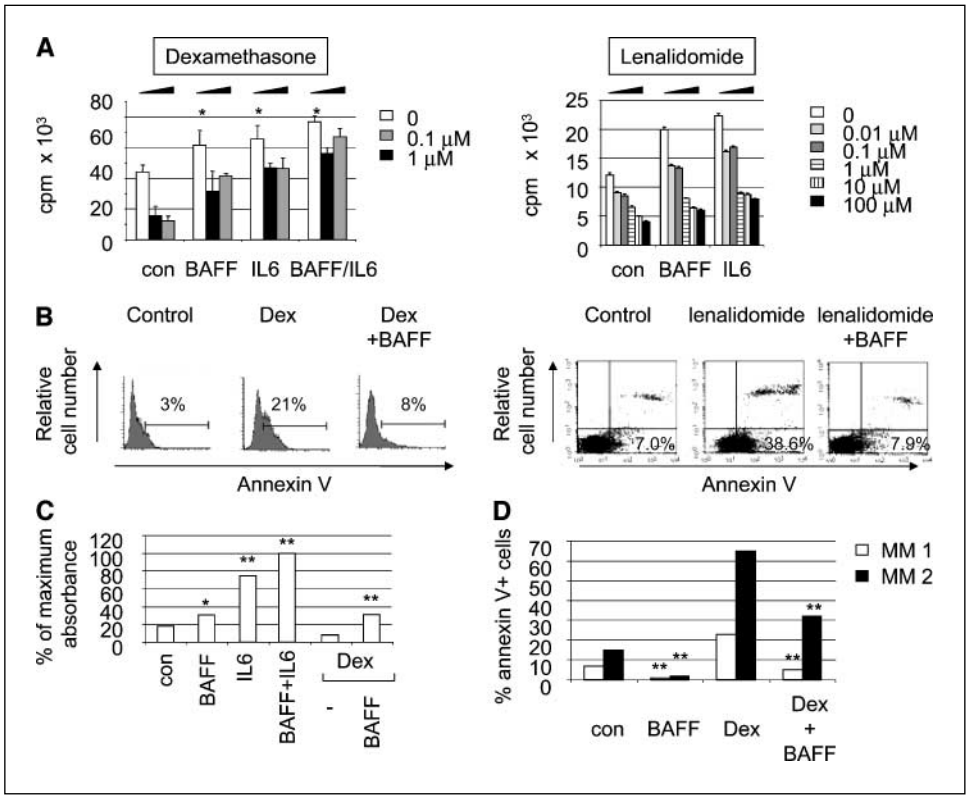

Figure 2. Effect of BAFF on multiple myeloma cell proliferation and survival. *A,* BAFF protects multiple myeloma cells from conventional and novel therapy. 28PE line was incubated with increasing doses of dexamethasone (*left*) and MM1S line was incubated with lenalidomide (*right*) in the presence or absence of exogenous BAFF (200 ng/mL) and proliferation assessed by [^{3}H]thymidine uptake. IL-6 (50 ng/mL) was included as a positive control for protection against these drugs. *B,* BAFF inhibits drug-induced apoptosis. *Left,* 28PE line was incubated with dexamethasone (*Dex*; 0.5 μmol/L) in the presence or absence of BAFF followed by Annexin V staining. *Right,* MM1S line was treated with lenalidomide (10 μmol/L) in the presence or absence of BAFF followed by Annexin V staining. *C,* BAFF protects patient multiple myeloma cells from dexamethasone-induced apoptosis. CD138$^+$ patient multiple myeloma cells were isolated from bone marrow aspirates and incubated with BAFF (200 ng/mL) alone or IL-6 (100 ng/mL) alone or both cytokines as well as with dexamethasone (1 μmol/L) in the presence of control medium (−) or BAFF (200 ng/mL) followed by MTT assay to determine cell survival. *, $P < 0.05$; **, $P < 0.01$. *Columns,* mean of duplicate wells; *bars,* SE. *D,* two patient multiple myeloma cells were incubated with control medium or BAFF (200 ng/mL) or treated with dexamethasone (1 μmol/L) in the presence or absence of BAFF (200 ng/mL) for 72 hours followed by Annexin V staining. *White columns,* patient multiple myeloma 1 (*MM 1*); *black columns,* patient multiple myeloma 2 (*MM 2*). **, $P < 0.01$.

supernatants from BMSCs from two multiple myeloma patients was measured before and after adhesion of multiple myeloma cell lines. As shown in Fig. 4*A*, BAFF secretion in cocultures of multiple myeloma cells and BMSCs is 1.8- to 4.2-fold higher than in BMSCs alone. We also did similar experiment using patient multiple myeloma cells with or without BMSCs. Similarly, BAFF in the supernatants of 8-hour cocultures of patient multiple myeloma cells with BMSCs was increased by ~3.5-fold relative to BMSCs alone, which persisted in 24-hour cocultures (Fig. 4*B*). BAFF secretion in multiple myeloma cells alone was significantly lower than in BMSCs alone. We next did immunoblotting of cell lysates prepared from these cultures using a specific anti-BAFF antibody. As shown in Fig. 4*C*, this significant BAFF protein up-regulation occurred as early as 8 hours after multiple myeloma adhesion to BMSCs, which was maintained at 24 hours. Thus, these results confirm that multiple myeloma adhesion to BMSCs significantly increased BAFF secretion.

Because NF-κB site is an essential regulatory element in multiple myeloma cell adhesion-induced IL-6 transcription in BMSCs (1) and the BAFF gene promoter contains at least six NF-κB-binding κB sites (36), we next determined whether multiple myeloma adhesion induces NF-κB transcriptional activity within the BAFF promoter. We transfected KM104 BMSCs with BAFF-LUC or a positive control $\kappa B_{(2\times)}$-LUC reporter in the presence or absence of NF-κB-inhibiting plasmid IκBα-pcDNA3.1. One day after transfection, multiple myeloma cells (MM1S and MCCAR) were added to these transfected KM104 BMSCs for additional 24 hours followed by luciferase assays. As expected, overexpression of IκBα by IκBα-pcDNA3.1 blocked baseline activity of BAFF-LUC and $\kappa B_{(2\times)}$-LUC in the KM104 BMSC line, indicating that the *BAFF* gene is transcriptionally activated in KM104 BMSC line and that NF-κB is critical to regulate BAFF transcription. When multiple myeloma cell lines adhered to KM104 cells, a significant increase in BAFF-LUC luciferase activity was detected: a 3- and 4-fold increase in cocultures of MM1S/KM104 and MCCAR/KM104, respectively (Fig. 4*D*). Conversely, inhibiting IκBα progressively inhibited multiple myeloma adhesion-induced activation of BAFF-LUC and $\kappa B_{(2\times)}$-LUC, further indicating that NF-κB mediates BAFF up-regulation triggered by tumor cell adhesion.

Effect of BAFF on multiple myeloma cell adhesion to BMSCs. We also studied whether BAFF increases multiple myeloma cell adhesion to BMSCs. As shown in Fig. 5*A*, BAFF induces dose-dependent multiple myeloma cell adhesion to BMSCs, with maximal adhesion of MM1S cells (3-fold increase) at 100 ng/mL of BAFF. Conversely, inhibition of BAFF binding to its receptors using TACI-Ig and BCMA/Fc significantly decreased adhesion. These data confirm that BAFF induces increased multiple myeloma cell adhesion to BMSCs via BAFF signaling. Similar experiments in

four additional multiple myeloma lines also indicated that BAFF (100 ng/mL) induces increased (1.6- to 4.4-fold) multiple myeloma cell adhesion to BMSCs (Fig. 5*B*). Importantly, we determined whether BAFF induces adhesion of $CD138^+$ patient multiple myeloma cells to BMSCs. $CD138^+$ multiple myeloma cells from two patients were incubated with increasing amounts of BAFF (0-1,000 ng/mL) and then added to BMSC-coated plates. As shown in Fig. 5*C*, BAFF induces a dose-dependent increase in adherence to BMSCs. Thus BAFF induces patient multiple myeloma cells to adhere to BMSCs, further supporting its important role of BAFF in multiple myeloma pathophysiology.

BAFF induces multiple myeloma cell adhesion to BMSCs via activation of NF-κB and AKT. We next characterized BAFF signaling in multiple myeloma cells and defined which BAFF pathway mediates BAFF-induced increased multiple myeloma adhesion. Specifically, PI3K/AKT, NF-κB, and mitogen-activated protein kinase pathways were evaluated by immunoblotting of cell lysates from BAFF-stimulated MM1S cells. Phosphorylation of AKT and IκBα was observed 5 minutes following BAFF stimulation and sustained until 40 minutes; in contrast, phosphorylation of p38 and extracellular signal-regulated kinase was undetectable (Fig. 6*A*). Phosphorylation of IκBα correlated with degradation of IκBα, suggesting activation of the classic NF-κB pathway (Fig. 6*A*). Because prior studies in normal B cells and CLL B cells indicated that BAFF could also induce activation of NF-κB2, a pathway critical for the growth and/or survival of these cells (15, 28), we next examined whether BAFF also induces activation of NF-κB2, which involves processing of p100 to p52 and subsequent translocation of p52 to the nucleus. We found that BAFF also induces activation of NF-κB2 in MM1S cells. In addition, BAFF induces translocation of p65 to the nuclear fraction (Fig. 6*B*), further confirming activation of the classic NF-κB pathway as shown in Fig. 6*A*.

We next defined BAFF-mediated signaling triggered by multiple myeloma adhesion using pharmacologic inhibitors against PI3K/AKT and NF-κB as well as adenoviruses expressing dominant-negative (dnAKT) and constitutively expressed AKT (myrAKT). Pretreatment with PI3K/AKT inhibitors wortmannin or Ly294002 significantly down-regulated BAFF-induced multiple myeloma adhesion (Fig. 6*C*). Constitutive activation of AKT by myrAKT further augmented BAFF-induced multiple myeloma cell adhesion in a dose-dependent manner, whereas inactivation of AKT by dnAKT completely inhibited BAFF-induced multiple myeloma cell adhesion (Fig. 6*D*). In addition, pretreatment with NF-κB inhibitors PS1145 or SN50 blocked BAFF-induced multiple myeloma cell adhesion in a dose-dependent fashion (Fig. 6*E*). These results indicate that BAFF-induced multiple myeloma cell adhesion to BMSCs is mediated via activation of AKT and NF-κB.

Discussion

The role of cells and factors within the bone marrow microenvironments promoting multiple myeloma cell growth and drug resistance is well defined. Although the mechanism of action of BAFF remains poorly understood, in part because of the complexity of its multiple receptors, accumulating data indicate that BAFF influences growth and survival of malignant B cells, including multiple myeloma. In the present study, we therefore characterized its role in regulating multiple myeloma cell localization, growth, and survival in their bone marrow microenvironment. To our knowledge, this is the first report to identify a role for BAFF in the interaction and adhesion of multiple myeloma cells with BMSCs. Significantly, we further defined the molecular mechanisms regulating these processes. We showed that BAFF functions in both an autocrine and paracrine

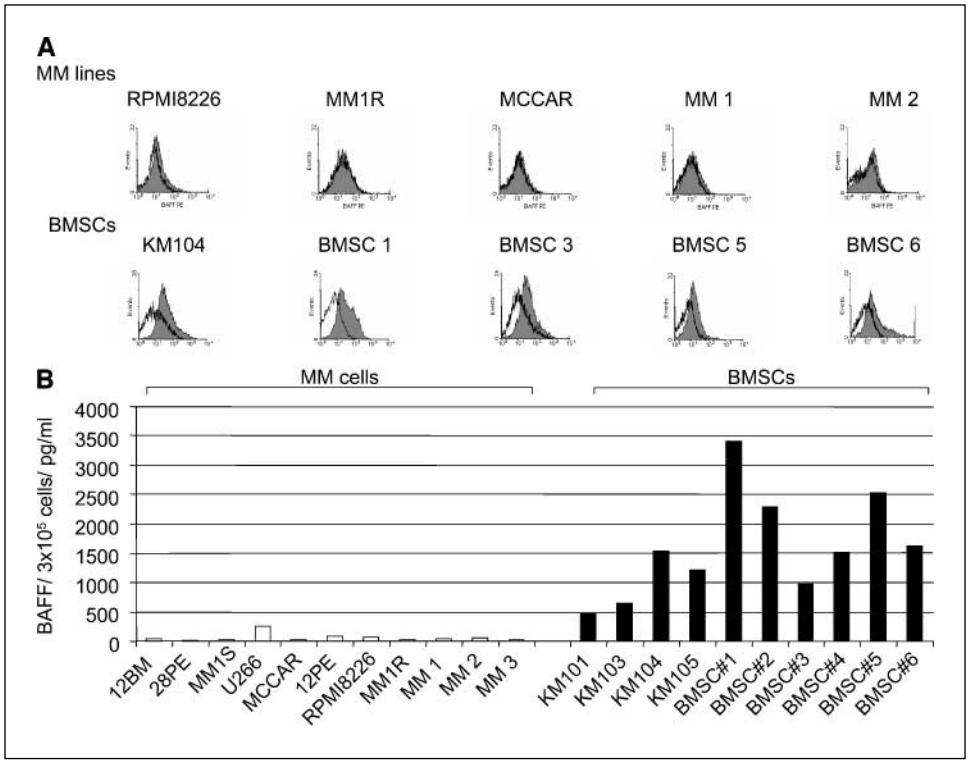

Figure 3. Expression of BAFF in multiple myeloma cells and BMSCs. *A,* expression of BAFF was determined by flow cytometric analysis using anti-BAFF antibody. *Filled histogram,* BAFF; *open histogram,* isotype control. *Top,* RPMI8226, MM1R, and MCCAR multiple myeloma cell lines as well as two patient multiple myeloma cells (multiple myeloma 1 and 2); *bottom,* KM104 BMSC line as well as BMSCs derived from four multiple myeloma patients. *B,* multiple myeloma cells and BMSCs at 5×10^5/mL were cultured at 37°C in triplicate for 3 days, and then supernatants were harvested. BAFF levels in the supernatants were determined by ELISA. *Columns,* mean of culture triplicates; *bars,* SE. *White columns,* multiple myeloma cell lines and patient multiple myeloma cells (multiple myeloma 1-3); *black columns,* BMSC lines and patient BMSCs (BMSC 1-6).

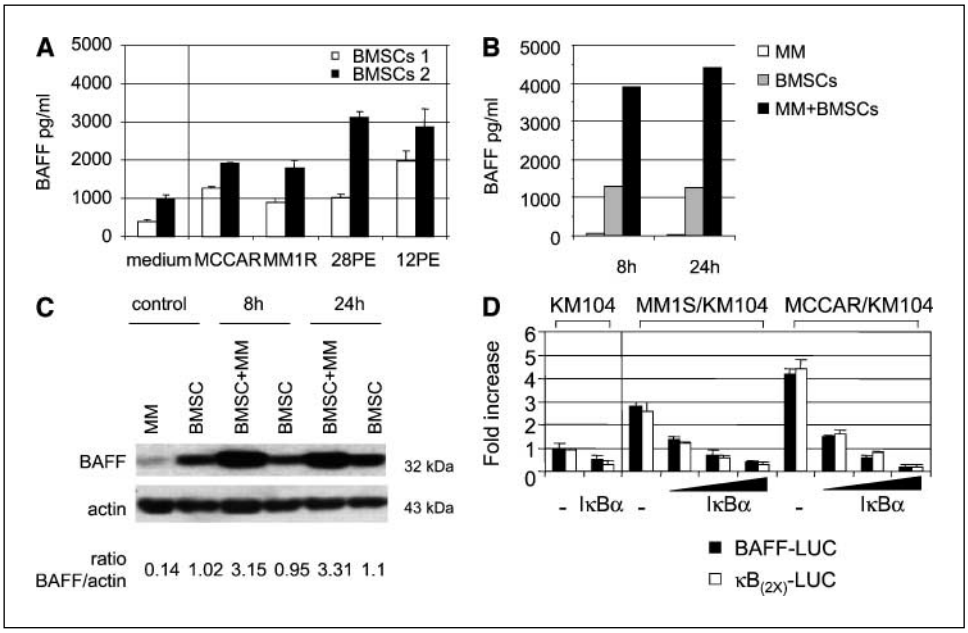

Figure 4. Adhesion of multiple myeloma cells augments BAFF secretion from BMSCs mediated by NF-κB activation in BMSCs. *A,* supernatants were collected after 24-hour culture of BMSCs derived from two multiple myeloma patients (BMSC 1 and 2) cocultured in medium alone or with indicated multiple myeloma lines. BAFF levels in the supernatants were quantitated by ELISA. *Columns,* mean from duplicate independent experiments; *bars,* SE. *B,* CD138-purified patient multiple myeloma cells were cultured alone or with BMSCs, and the supernatants were harvested after 8 and 24 hours followed by ELISA for BAFF. *White columns,* multiple myeloma cells alone; *gray columns,* BMSCs; *black columns,* multiple myeloma cocultured with BMSCs. *C,* cell lysates from patient multiple myeloma cells alone or cocultured with BMSCs [shown in (*B*)] at indicated time points were subjected to immunoblotting using anti-BAFF antibody. Anti-α-actin monoclonal antibody (mAb) was used as a loading control. *D,* adhesion-induced BAFF secretion is mediated by NF-κB activation in BMSCs. KM104 BMSCs were transfected with BAFF-LUC (*black columns*) or $I\kappa B_{(2x)}$-LUC (*white columns*) in the presence or absence (−) of increasing amounts of IκBα-pcDNA3.1 (*IκBα*) and maintained in medium for 24 hours. MM1S or MCCAR multiple myeloma cell lines were added to the confluent layer of transfected KM104 BMSCs. After 24 hours of incubation, cells were subjected to luciferase reporter assays. Relative increases in luciferase activities (fold induction) were calculated: activity in the extracts from cocultures of adherent multiple myeloma cells and KM104 BMSCs relative to that in transfected KM104 BMSCs alone (in the absence of IκBα; *column 1*). A value of 1 represents control baseline BAFF-LUC (or $I\kappa B_{(2x)}$-LUC) activity in KM104 BMSCs. *Columns,* mean of three independent experiments; *bars,* SE.

manner to support multiple myeloma growth and survival, suggesting that inhibiting this pathway may provide a novel therapeutic strategy.

The role of the three individual BAFF receptors in multiple myeloma biology remains unclear. In this study, we found that BAFF and its three receptors were heterogeneously expressed in both multiple myeloma cell lines and patient multiple myeloma cells, consistent with previous reports (13, 18). Although expression profiling and RT-PCR detect BAFF-R, flow cytometric analysis shows that multiple myeloma cells express little, if any, cell surface BAFF-R. This low-level expression of BAFF-R on multiple myeloma cell membrane is a reflection of its lower mRNA expression: RNA expression of BCMA and TACI among our patient cohort is approximately >30-fold and >10-fold higher, respectively, than that of BAFF-R. These results are in accord with increased reported levels of BCMA but not BAFF-R or TACI mRNA (22). Because BCMA is essential for the survival of long-lived bone marrow plasma cells (22) and high expression of BCMA in multiple myeloma cells was observed in the present study and by others (18, 25, 37), BCMA is a key receptor for BAFF in multiple myeloma cells. Our data showing that BAFF induces PI3K/AKT and NF-κB activation, which regulates multiple myeloma adhesion, further support this hypothesis. Therefore, both BCMA and TACI are the main receptors for BAFF function in multiple myeloma, in contrast to normal B cells where BAFF signaling/function is predominantly through high-affinity binding of BAFF to BAFF-R. Moreover, as shown here in multiple myeloma cells, BAFF also triggers signaling, mediating cell survival and proliferation in malignant Reed-Sternberg cells from Hodgkin's lymphoma cells that lack BAFF-R (38). These results further confirm the importance of BCMA and TACI in BAFF function and indicate that the expression and function of the three BAFF receptors are highly dependent on the differentiation stage of B cells. In addition, because both BCMA and TACI also bind to another CD40L-related factor (a proliferation-inducing ligand, APRIL) other than BAFF, our results suggest a potential role for APRIL in multiple myeloma pathogenesis.

Because BAFF originally was found to be expressed in myeloid lineage cells, such as monocytes, macrophages, or dendritic cells, high expression of BAFF in the multiple myeloma bone marrow microenvironment was anticipated. We found that BAFF secretion in BMSCs is >3-fold higher than in multiple myeloma cells. Using immunohistochemistry, Novak et al. (13) also showed that bone marrow sections from multiple myeloma patients stained brightly for BAFF. Although some multiple myeloma cells secrete BAFF, we found that the major source of BAFF in multiple myeloma is BMSCs. We therefore focused on determining the effect of tumor cell adhesion on BAFF secretion in BMSCs and related tumor cell survival. Importantly, significant increases in BAFF secretion were triggered by multiple myeloma cell binding to BMSCs; because paraformaldehyde fixation of BMSCs significantly inhibited BAFF secretion and adhesion-induced protection against dexamethasone-induced cell death, BAFF secretion was primarily in BMSCs rather than in multiple myeloma cells. These studies support the view that multiple myeloma cell adhesion to BMSCs may not only localize tumor cells in bone marrow but also permits cell-to-cell contact that is essential to trigger BAFF secretion by BMSCs.

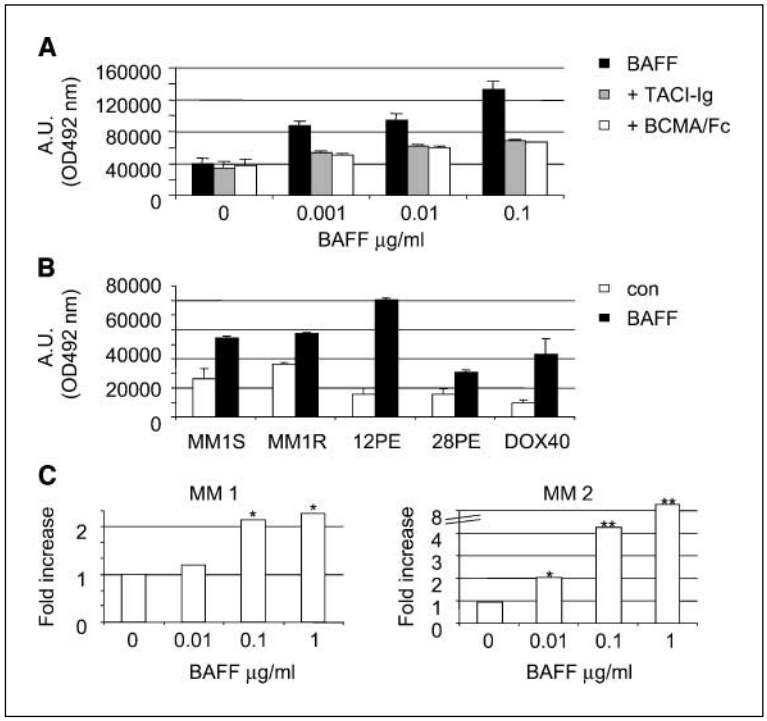

Figure 5. BAFF directly induces multiple myeloma cell adhesion to BMSCs. *A,* serum-starved MM1S cells were labeled, washed, incubated with increasing amounts of BAFF (*black columns*), and then added to BMSC-coated plates for 2 to 4 hours with medium control (*black columns*), TACI-Ig (*gray columns*), or BCMA/Fc (*white columns*); multiple myeloma cell lines were cultured in the presence (*black columns*) or absence (*white columns*) of 100 ng/mL BAFF. *Columns,* mean of triplicate samples from one representative of three independent experiments; *bars,* SE. *C,* BAFF enhances adhesion of CD138$^+$ patient multiple myeloma cells to BMSCs. CD138$^+$ multiple myeloma cells isolated from bone marrow aspirates from two patients (multiple myeloma 1 and 2) were subjected to adhesion assay as described above in the presence or absence of BAFF (0-1 μg/mL). *, $P < 0.05$; **, $P < 0.001$. *Columns,* mean of duplicate wells; *bars,* SE.

The current studies extend the recent observation that TACI plays a role in multiple myeloma adhesion (26, 39). Moreover, they also show that multiple myeloma cell adhesion to BMSCs stimulates transient induction of BAFF transcription dependent on NF-κB transactivation. Using BAFF promoter reporter constructs harboring at least six NF-κB-binding sites, we show that NF-κB is at least one crucial regulatory element for induction of the BAFF promoter triggered by multiple myeloma cell adherence to BMSCs. This correlates with an increase in BAFF promoter luciferase activity; conversely, a decrease in activity is observed in

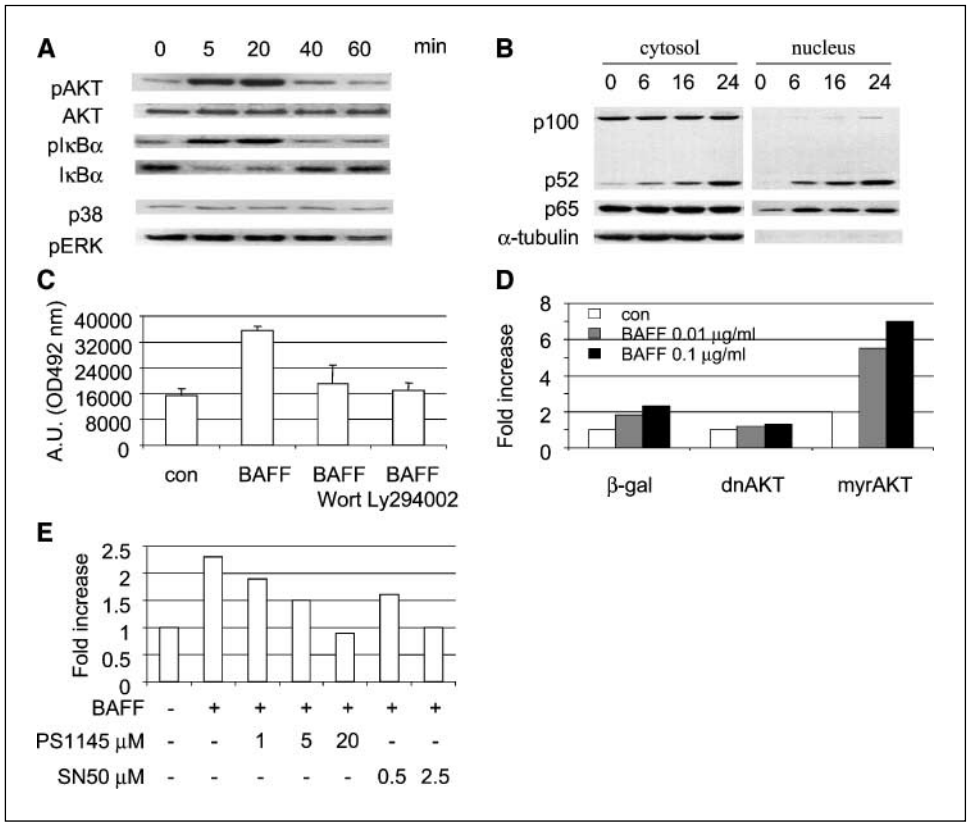

Figure 6. BAFF-induced multiple myeloma adhesion is mediated via activation of AKT and NF-κB. *A,* activation of AKT and NF-κB in multiple myeloma cells by BAFF. Serum-starved MM1S cells were incubated with BAFF (200 ng/mL) for the indicated times. Cell lysates were analyzed by immunoblotting with anti-phosphorylation-specific antibodies. Detection of total AKT and IκBα in the same lysates was used to show equal loading of samples and degradation of IκBα, respectively. *B,* processing of p100 and nuclear translocation of p52 or p65. MM1S cells were cultured with BAFF (200 ng/mL) for indicated times, and cytoplasmic and nuclear extracts were prepared for immunoblotting with anti-p100 or anti-p65 antibodies. Anti-α-tubulin mAb was used as a loading control and for confirmation of cytoplasmic versus nuclear fractions. *C,* MM1S cells were pretreated for 1 hour with medium control, wortmannin (*Wort*; 0.2 μmol/L), or Ly294002 (30 μmol/L), and adhesion assays were then done in the presence of BAFF (100 ng/mL). *D,* MM1S cells were transduced for 24 hours with adenoviruses expressing dnAKT, myrAKT, or control β-galactosidase (*β-gal*) followed by adhesion assays in the presence of increasing concentrations of BAFF. *E,* MM1S cells were preincubated with PS1145 (0, 1, 5, and 20 μmol/L) or SN50 (0, 0.5, and 2.5 μmol/L), and adhesion assays were done in the presence or absence of BAFF (100 ng/mL).

the presence of overexpressed NF-κB-inhibiting IκBα. Involvement of NF-κB motifs in BAFF regulation has recently been characterized in other cell systems (36). Our ongoing studies are directed at defining those signaling cascades acting through NF-κB to up-regulate BAFF in BMSCs to delineate and eventually interfere with BAFF-mediated paracrine growth mechanisms in multiple myeloma. In our severe combined immunodeficient-hu model, which permits characterization of gene expression profile in human multiple myeloma cells *in vivo* before and after binding to human bone marrow, preliminary data indicate that BAFF expression is increased (8-fold) following adhesion of INA-6 multiple myeloma cells to the bone marrow *in vivo* (40). Ongoing efforts are focused on the role of NF-κB activation in mediating BAFF up-regulation *in vivo*.

In summary, we here show that expression of BAFF and its three receptors (BCMA>TACI>>>BAFF-R) is heterogeneous in CD138-expressing multiple myeloma patient cells. BAFF rescues multiple myeloma cells from drug-induced apoptosis, confirming an autocrine pathway of BAFF signaling in multiple myeloma growth and survival. BMSCs secrete at least 3-fold higher levels of BAFF than multiple myeloma cells. Importantly, adhesion of tumor cells to BMSCs augments paracrine BAFF secretion in BMSCs, which promotes multiple myeloma cell growth and protects against dexamethasone-induced cell death. Adhesion-induced NF-κB activation in BMSCs in turn up-regulates BAFF gene expression. Finally, BAFF augments multiple myeloma adhesion to BMSCs via phosphorylation of AKT and NF-κB. These results establish a functional role of BAFF in localization and survival of multiple myeloma cells in the bone marrow microenvironment, providing the rationale to target BAFF pathway in novel therapeutics.

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