

Brief Definitive Report

BAFF Binds to the Tumor Necrosis Factor Receptor-like Molecule B Cell Maturation Antigen and Is Important for Maintaining the Peripheral B Cell Population

By Jeffrey S. Thompson,* Pascal Schneider,^{||} Susan L. Kalled,[‡] LiChun Wang,[‡] Eric A. Lefevre,[¶] Teresa G. Cachero,[§] Fabienne MacKay,** Sarah A. Bixler,* Mohammad Zafari,[‡] Zhong-Ying Liu,[‡] Stephen A. Woodcock,[‡] Fang Qian,[§] Marcel Batten,** Christine Madry,[¶] Yolande Richard,[¶] Christopher D. Benjamin,[‡] Jeffrey L. Browning,[‡] Andreas Tsapis,[¶] Jurg Tschopp,^{||} and Christine Ambrose*

From the *Department of Molecular Genetics, the [‡]Department of Immunology and Inflammation, and the [§]Department of Protein Engineering, Biogen, Incorporated, Cambridge, Massachusetts 02142; the ^{||}Institute of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland; [¶]Institut National de la Santé et de la Recherche Médicale U131, 92140 Clamart, France; and the **Garvan Institute of Medical Research, St. Vincent's Hospital, Darlinghurst NSW 2010, Australia

Abstract

The tumor necrosis factor (TNF) family member B cell activating factor (BAFF) binds B cells and enhances B cell receptor-triggered proliferation. We find that B cell maturation antigen (BCMA), a predicted member of the TNF receptor family expressed primarily in mature B cells, is a receptor for BAFF. Although BCMA was previously localized to the Golgi apparatus, BCMA was found to be expressed on the surface of transfected cells and tonsillar B cells. A soluble form of BCMA, which inhibited the binding of BAFF to a B cell line, induced a dramatic decrease in the number of peripheral B cells when administered in vivo. Moreover, culturing splenic cells in the presence of BAFF increased survival of a percentage of the B cells. These results are consistent with a role for BAFF in maintaining homeostasis of the B cell population.

Key words: tumor necrosis factor • B lymphocyte • receptor • cell survival • homeostasis

Introduction

Many members of the TNF family fulfill important roles related to the organization, function, and/or homeostasis of the immune system (1). The recently identified TNF family member B cell activating factor (BAFF) (TNF and apoptosis ligand-related leukocyte-expressed ligand 1 [TALL-1]/TNF homologue that activates apoptosis, nuclear factor κ B, and c-Jun NH₂-terminal kinase [THANK]/B lymphocyte stimulator [BlyS]; references 2–5) is expressed in monocytes, macrophages, and dendritic cells (2, 3, 5) and has been shown to bind to B cells and increase the proliferation of B cells in combination with an anti-B cell receptor antibody in vitro (2, 5). BAFF is a type II membrane pro-

tein and may act as either a membrane-bound or soluble form, the latter being generated by proteolytic cleavage at a furin consensus site (2, 5). Transgenic mice overexpressing BAFF have a greatly elevated number of mature B cells and an increased number of effector T cells in their spleen and mesenteric lymph nodes (6). These mice display autoimmune-like manifestations including high levels of rheumatoid factors, circulating immune complexes, anti-DNA autoantibodies, and Ig deposition in the kidney (6). However, splenic B cells from transgenic or control mice were found to proliferate at the same rate (6), suggesting that BAFF, in addition to its ability to costimulate B cell proliferation in vitro, may have alternative functions.

B cell maturation antigen (BCMA) was first identified as part of a translocation event in a malignant T cell lymphoma patient (7). Characterization of human BCMA identified it as a type I membrane protein primarily expressed in immune organs and mature B cell lines (7, 8).

J.S. Thompson and P. Schneider contributed equally to this work.

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Address correspondence to Christine M. Ambrose, Biogen, Inc., 12 Cambridge Center, Cambridge, MA 02142. Phone: 617-679-3340; Fax: 617-679-2304; E-mail: christine_ambrose@biogen.com

BCMA protein was localized to the Golgi apparatus in the U266 plasmacytoma cell line, which expresses high levels of BCMA (9). Subsequent identification of the mouse BCMA gene and further motif analysis led to the prediction that BCMA is a member of the TNF receptor superfamily (10).

Here, we show that BAFF is capable of interacting with the orphan TNF receptor, BCMA. We characterize the BAFF-BCMA interaction and show that injection of soluble BCMA-Ig fusion protein into mice leads to a dramatic reduction in the total number of B cells in the peripheral immune organs. This may be due to a B cell survival function of BAFF. Modulation of the peripheral B cell population using BCMA-Ig treatment may be efficacious in treating B cell-mediated disorders.

Materials and Methods

Cells and Constructs. Human embryonic 293 cells carrying the EBNA-1 gene were cultured in DMEM plus 10% FCS, glutamine, pen-strep, and 250 $\mu\text{g}/\text{ml}$ G418. Raji cells were obtained from American Type Culture Collection and were cultured as indicated. The human BCMA-hIgG1 (BCMA-Ig) expression vector was created using a murine κ light chain signal sequence, a PCR fragment generated to BCMA amino acids 1-48 (second Met), and a hIgG1 SalI + NotI fragment (11). The fragments were cloned into pCEP4 (Invitrogen) derivative. A COOH-terminal 6 \times His-tagged full-length human BCMA expression vector was generated by PCR. BCMA-Ig obtained by transient transfection of 293E cells using Lipofectamine (Life Technologies) was purified by protein A column chromatography, and contained <1 endotoxin U/mg of protein. The half-life of this protein in mice is ~ 3.5 d. The control Ig, nonbinding LFA3-Ig, was prepared from a Chinese hamster ovary (CHO) cell line as described (12). Murine lymphotoxin (LT) β R-Ig (13) and human LT β R-Ig (14) were made as described. Receptor-Ig fusion proteins and Flag-tagged soluble ligands were constructed and expressed as described using the following amino acid sequences: hTNF- α (85-263), mBAFF (127-309), hBAFF (136-285 or 83-285), human receptor activator of nuclear factor κ B ligand (hRANKL [151-316]), hCD30L (63-234), hCD40L (116-261), homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpesvirus entry mediator (HVEM), a receptor expressed by T lymphocytes (hLIGHT [89-240]), hTNFR2 (1-257), hTNF-related apoptosis-inducing ligand (hTRAIL)R2 (1-212), hCD30 (1-380), hCD40 (1-193), hHVEM (1-200), and mRANK (1-200) (2, 15). Unless indicated, BAFF in 15-fold concentrated serum-free Optimum medium (Life Technologies) was used (2).

Anti-BCMA Antibodies. Rabbit anti-hBCMA antibody, Rb 1593, was generated against purified hBCMA-Ig. Affinity-purified anti-BCMA antibodies and control affinity-purified anti-glutathione *S*-transferase (GST) antibodies originating from the same rabbit were used for the U266 cells and tonsillar B cell staining. These antibodies have been described (9).

BIAcore Analysis. All experiments were performed at 25°C with a 10 $\mu\text{l}/\text{min}$ flow rate using a BIAcore 2000™. HBS buffer (10 mM Hepes, 150 mM NaCl, 0.005% P20 surfactant, pH 7.4) was used both as the running buffer and as the sample diluent. The CM5 chip (BIAcore) surface was first activated with *N*-hydroxysuccinimide/*N*-ethyl-N9-(3-diethylaminopropyl)-carbo-

diimide hydrochloride. 25 μl of BCMA-Ig or hLT β R-Ig was diluted to 30 $\mu\text{g}/\text{ml}$ in 10 mM acetic acid (pH 5), then injected. The unreacted groups of the chip's dextran matrix were blocked twice with ethanolamine-HCl (pH 8.5). For the experiment, 100 μl of Flag-hBAFF, Flag-mBAFF, or hLT $\alpha_1\beta_2$ at 30 $\mu\text{g}/\text{ml}$ in diluent buffer were injected over the surface of the chip. The surface was regenerated between experiments by washing with 1 mM formic acid.

BCMA-Ig Blocking of BAFF Binding to Raji Cells. Flag-tagged human BAFF at 200 ng/ml was pre-incubated for 30 min on ice in FACS® buffer (PBS, 10% FCS, 0.05% azide) with either hBCMA-Ig or hLT β R-Ig at twofold dilutions ranging from 20 $\mu\text{g}/\text{ml}$ to 39 ng/ml, or with BAFF alone. The mixture was added to Raji cells for 30 min on ice. Bound BAFF was detected using 5 $\mu\text{g}/\text{ml}$ anti-Flag antibody M2 (Sigma-Aldrich) followed by PE-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). The samples were read using a FACSCalibur™ flow cytometer (Becton Dickinson).

Anti-BCMA Antibody and BAFF Binding to Full-length BCMA-transfected Cells. 293E cells were transfected with either vector alone or His-tagged full-length BCMA and a green fluorescent protein transfection control plasmid (gift of N. Horikoshi, Beth Israel Deaconess Medical Center, Boston, MA) using Lipofectamine 2000. After 48 h, the cells were detached in PBS containing 5 mM EDTA, washed, and stained for 30 min on ice with anti-hBCMA Rb 1593 (1:1,000) or preimmune sera (1:500) followed by PE-conjugated donkey anti-rabbit Ig (1:100; Jackson ImmunoResearch Laboratories). Alternatively, cells were stained plus or minus Flag-BAFF (2 $\mu\text{g}/\text{ml}$) and detected using M2 as described above.

BAFF and Anti-BCMA Antibody Binding to Human Tonsillar B Cells. Human mononuclear cells were obtained from palatine tonsils by gentle dissociation with forceps. T cells were removed by one cycle of rosette formation and further depleted with anti-CD2 magnetic beads (Dynabeads M-450; Dynal [16]). The remaining B cell populations were 93 \pm 4% CD19⁺, 59 \pm 6% IgD⁺, 56 \pm 5% IgM⁺, 81 \pm 16% CD44⁺, 21 \pm 6% CD38⁺, 42 \pm 1% CD95⁺, and \leq 1% CD14⁺, CD3⁺, and DRC1⁺ cells. Cells were preincubated with 10% human AB serum and stained plus or minus Flag-BAFF (amino acids 83-285) at 1 $\mu\text{g}/\text{ml}$ followed by M2 (7.5 $\mu\text{g}/\text{ml}$) and PE-conjugated goat anti-mouse IgG (Immunotech). Anti-BCMA staining was done using 20 $\mu\text{g}/\text{ml}$ antibody and FITC-conjugated goat anti-rabbit IgG (Immunotech).

BCMA-Ig Injection into Mice. 6-wk-old female BALB/c mice (four per group; The Jackson Laboratory) received intraperitoneal injections of either 400 μg of BCMA-Ig or 400 μg of LFA3-Ig (control Ig) on days 1, 4, 8, and 11. On day 19, the mice were killed. Blood was collected via cardiac puncture into tubes containing EDTA. Single cell suspensions were prepared from spleens and mesenteric lymph nodes, and RBCs were lysed in a hypotonic buffer. Flow cytometry was performed using PE-, FITC-, or CyChrome-conjugated anti-CD45R/B220 (B cells) and anti-CD4, anti-CD8 (T cells), or anti-Gr1/anti-Mac-1 (neutrophils and macrophages). All antibodies were purchased from BD PharMingen. In brief, Fc receptors were blocked with 10 $\mu\text{g}/\text{ml}$ FcBlock (BD PharMingen) for 15 min on ice followed by addition of the conjugated mAbs, and were incubated on ice for 30 min. Cells were washed twice, suspended in 0.5% paraformaldehyde, and analyzed by flow cytometry.

B Cell Survival Assay. Mouse spleens were collected under sterile conditions and homogenized in RPMI medium plus 10% FCS using SuperFrost glass slides (Menzel-Glaser). Cells were fil-

tered through a 70- μ m nylon cell strainer (Falcon) before centrifugation at 1,000 rpm (300 *g*) for 5 min. The pellet was resuspended in 2 ml/spleen RBC lysis solution and incubated on ice for 5 min. The remaining white blood cells were collected by centrifugation and resuspended in media. Splenocytes were incubated at a concentration of 3×10^6 cells/ml in media for 72 h. The splenocytes were either untreated or were in the presence of 2 μ g/ml human Flag-BAFF (amino acids 83–285) alone or with 20 μ g/ml BCMA-Ig or 20 μ g/ml murine LT β R-Ig. The percent live cells remaining was determined using the annexin V-FITC Apoptosis Detection Kit 1 (BD PharMingen). Cells were washed twice and then analyzed using a FACSCalibur™ machine (Becton Dickinson).

Results and Discussion

BAFF Interacts with Soluble BCMA. TNF receptor family members are generally type I proteins with a leader sequence and a cysteine-rich extracellular domain. The extracellular domains are organized as a series of alternating A and B modules, which are stabilized by internal disulfide bridges (17). A single C module, not involved in ligand binding, is found in the fourth cysteine repeat of TNFR1 (17). In a previous study, BAFF failed to bind to 16 mem-

bers of the TNF receptor family (2). Subsequently, an additional candidate receptor for BAFF was identified, termed B cell maturation antigen (10). BCMA appears to be distantly related to the TNF receptor family, because it is devoid of a signal sequence and contains a single A and one C module instead of multiple A and B modules (10). However, the expression pattern of this atypical receptor in mature B cells prompted us to examine its interaction with BAFF (8, 9). To this end, the extracellular domain of human BCMA was expressed as a human IgG1 fusion protein and was targeted to the secretory pathway with an added signal peptide. BCMA-hIgG1 (BCMA-Ig) fusion protein immunoprecipitated both recombinant human and murine BAFF very efficiently, but not five other TNF ligands (CD40L, RANKL, CD30L, LIGHT, and TNF- α) which, however, interacted with their respective receptors in control immunoprecipitations (Fig. 1 A). When the interaction was tested in another format using surface plasmon resonance (BIAcore) analysis, similar results were obtained, and both murine and human BAFF exhibited a significant affinity for human BCMA-Ig but not for LT β R-Ig (Fig. 1 B). In addition, BCMA-Ig interacted with membrane-bound BAFF expressed in 293 cells (Fig. 1 C), and in a dose-

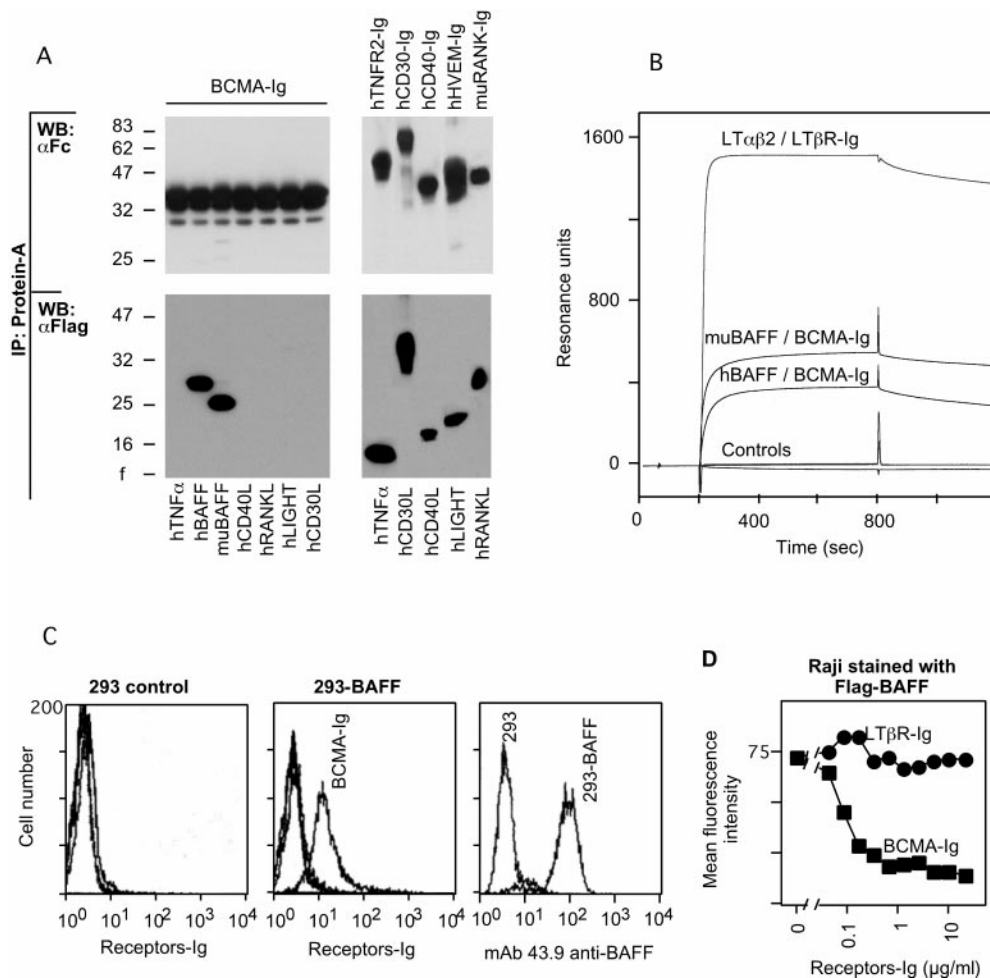


Figure 1. The extracellular domain of BCMA interacts with BAFF. (A) Human BCMA-Ig and various control TNFR-Ig molecules were mixed with Flag-tagged TNF ligands, immunoprecipitated (IP) with protein A Sepharose, and analyzed by Western blot (WB). Receptor-Ig is revealed with anti-human IgG (top), and coimmunoprecipitating ligands are detected using anti-Flag M2 antibody (bottom), α , anti-. (B) Interaction of BAFF with BCMA assayed by surface plasmon resonance (BIAcore). (C) BCMA-Ig binds stable 293 cell line expressing surface full-length BAFF. The wild-type 293 cells or a stable cell line expressing BAFF (reference 2) were stained with media containing similar amounts of BCMA-Ig, TNFR2-Ig, TRAILR2-Ig, or control media using PE-conjugated anti-hIgG1 (left and middle). The presence of surface BAFF was detected using an anti-hBAFF rat mAb, 43.9 (reference 2; right). (D) BCMA-Ig blocks BAFF binding to Raji cells. Various concentrations of either BCMA-Ig or LT β R-Ig were preincubated with Flag-BAFF, then added to Raji cells. BAFF binding was detected with the anti-Flag M2 antibody and analyzed by flow cytometry.

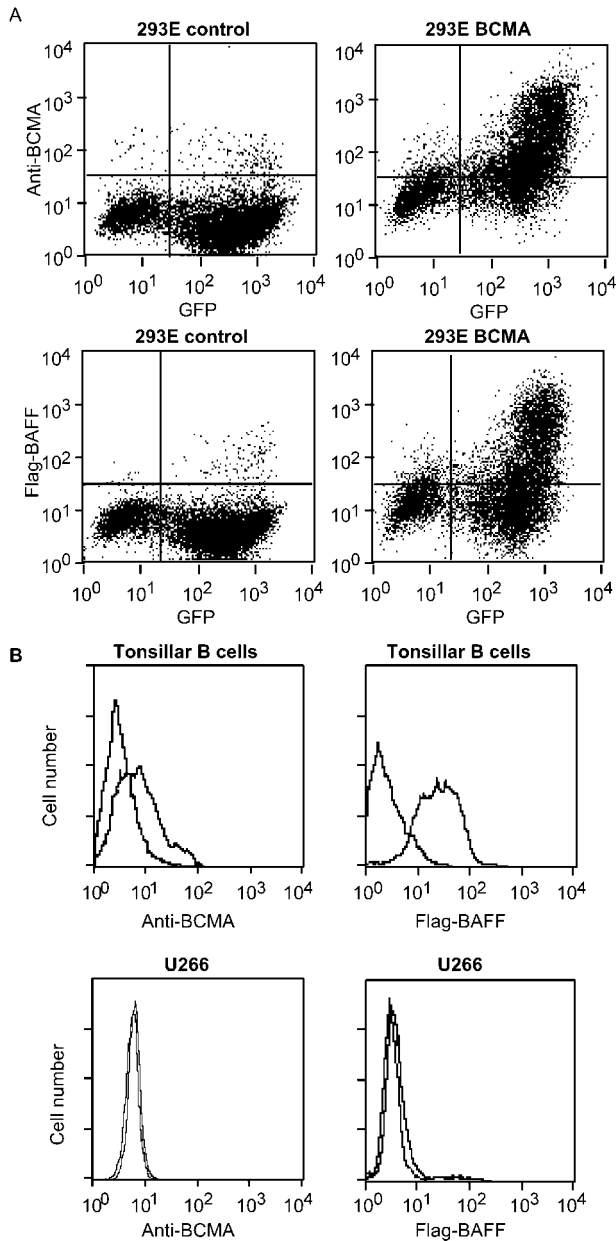


Figure 2. Cell surface expression of BCMA in transfected and primary cells. (A) 293E cells were cotransfected with expression vectors for green fluorescent protein (GFP) and full-length human BCMA or a control plasmid. Surface expression of BCMA was detected with either an anti-BCMA antibody (top) or Flag-BAFF (bottom). (B) Human tonsillar B cells and the U266 plasmocytic cell line were stained with either anti-BCMA, control rabbit antibody, or Flag-BAFF, and were analyzed by flow cytometry.

dependent manner specifically blocked the interaction of BAFF with Raji B cells (Fig. 1 D). Thus, the short extracellular domain of BCMA is sufficient for high-affinity binding to BAFF, and there is no species barrier for the murine BAFF-human BCMA interaction. The BAFF-BCMA interaction is presently unique in the family, as it involves only two modules, one predicted to be of the C type. In the two cases where the crystal structure of the tri-

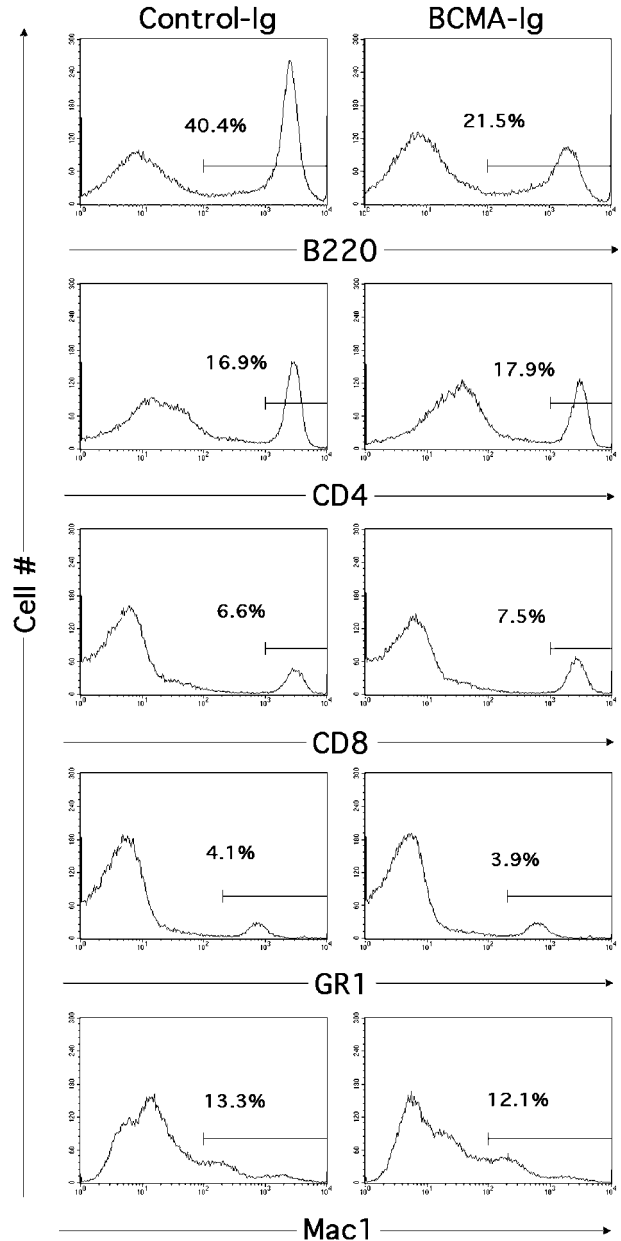


Figure 3. Administration of BCMA-Ig reduces the peripheral B cell population. Adult Balb/c mice were treated with four intraperitoneal injections of BCMA-Ig or control Ig over a period of 11 d. At day 19, splenic B220⁺ B cells, CD4⁺ and CD8⁺ T cells, and Gr1⁺ and Mac-1⁺ populations were analyzed by flow cytometry. A representative mouse from each group is shown.

meric ligand-receptor complex is known (LT α -TNFR1 and TRAIL-TRAILR2), the contacts involved in the interaction have been shown to involve one B and two A modules (18-20).

Cell Surface Expression of BCMA. Cell surface expression of BCMA is a likely prerequisite for BAFF binding. Indeed, most 293E cells transfected with a construct encoding full-length BCMA with no added signal peptide were positive for surface staining with anti-BCMA anti-

Table I. The Total Number of Splenocytes and Splenic Subpopulations Examined after Treatment with BCMA-Ig

Mouse	Total cells	B cells	CD4 ⁺ T cells	CD8 ⁺ T cells	Neutrophils	Mac-1 ⁺ cells
BCMA-Ig treated						
B1	58.5	21.5	16.9	7.5	4.6	12.1
B2	66.0	24.4	19.5	8.3	3.9	12.4
B3	46.5	11.7	14.9	8.3	3.4	10.0
B4	36.0	11.0	12.7	6.5	2.2	6.9
Mean ± SD	51.8 ± 11.4	17.2 ± 5.9	16.0 ± 2.5	7.7 ± 0.7	3.5 ± 0.9	10.4 ± 2.2
Controls						
A1	73.5	40.4	17.9	6.2	4.1	13.3
A2	72.8	39.1	16.1	6.6	4.5	13.7
A3	81.8	42.9	20.4	9.2	3.8	13.2
A4	43.5	21.6	10.4	4.2	3.2	9.0
Mean ± SD	67.9 ± 14.5	36.0 ± 8.4	16.2 ± 3.7	6.6 ± 1.8	3.9 ± 0.5	12.3 ± 1.9
<i>P</i> values	<i>P</i> > 0.1	<i>P</i> = 0.02	<i>P</i> > 0.1	<i>P</i> > 0.1	<i>P</i> > 0.1	<i>P</i> > 0.1

The number of cells ($\times 10^6$) from individual mice in each group is shown. *P* values were determined by Student's *t* test.

bodies and to a lesser extent with Flag-BAFF, whereas untransfected cells or cells transfected with a control plasmid were not (Fig. 2 A). We also found that tonsillar B cells were stained with both anti-BCMA and Flag-BAFF (Fig. 2 B), suggesting that surface expression of BCMA also occurs in primary cells and is not an artifact of overexpression. Therefore, in the absence of a signal sequence, the transmembrane domain of BCMA must dictate the topology of membrane insertion for correct expression at the cell surface. In a previous study, BCMA was located exclusively in the Golgi apparatus of the plasmocytic cell line U266, and not at the cell surface, despite the fact that these cells express high levels of the protein (9). Consistent with this observation, we find that U266 cells were not stained with either the anti-BCMA antibody or Flag-BAFF (Fig. 2 B). These results suggest that surface expression of BCMA may be a tightly controlled, cell type-dependent mechanism. Intracellular pools of the cytotoxic receptor Fas can be mobilized for surface expression in response to p53 activation, a mechanism that probably contributes to the elimination of damaged cells (21). It is an intriguing possibility that cells may release intracellular BCMA at the cell surface in response to specific stimuli, and therefore become sensitive to BAFF effects.

B Cell Reduction with BCMA-Ig Treatment. Because BIAcore studies revealed that murine BAFF was able to effectively bind human BCMA-Ig, we used the fusion protein to examine the effect of disrupting the BAFF signal in normal mice. Four administrations of BCMA-Ig at intervals of 3–4 d dramatically reduced the total number of B cells in the blood and peripheral lymphoid organs examined. The B220⁺ B cell population in the spleen of BCMA-Ig-treated animals was reduced by 50% compared with the control Ig-treated mice. In contrast, populations of neutro-

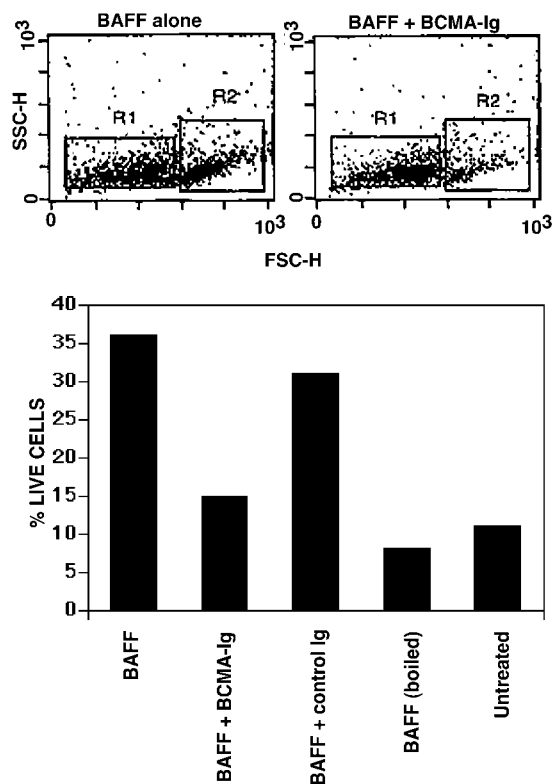


Figure 4. BAFF promotes the survival of splenocytes in vitro. (A) Forward (FSC) and side scatter (SSC) profiles of murine splenocytes in culture after 72 h in the presence or absence of BAFF. The R2 gate contains live cells (annexin V and propidium iodide negative). (B) Percentage of R2 cells surviving. BAFF concentration was 2 $\mu\text{g}/\text{ml}$, and fusion proteins were added at 10 $\mu\text{g}/\text{ml}$. This is a representative experiment of the assay done ~ 10 times.

phils, macrophages, CD4⁺ T cells, and CD8⁺ T cells were not significantly affected by BCMA-Ig (Fig. 3 and Table I). The decrease in absolute B cell number corresponded to the decrease in absolute total cell number (Table I), indicating that the reduction in the B cell population was not due to a concordant increase in other cell types. Therefore, the BCMA-Ig treatment specifically affected B cells. Similar results were observed in the blood and mesenteric lymph nodes, where approximately half of the B220⁺ B cells were lost (data not shown). As B cells do not express BAFF (2, 5), and because human BCMA-Ig does not bind to mouse splenic B cells (data not shown), we can exclude antibody-dependent cell-mediated cytotoxicity as the mechanism of action. Although BAFF is thought to be primarily secreted, staining of splenocytes using BCMA-Ig revealed that BAFF is found on the surface of Mac-1⁺ cells (data not shown).

BCMA-Ig treatment given over the course of 11 d may reduce the peripheral B cell pool by inhibiting production or release of immature B cells from the bone marrow, by blocking maturation of immature B cells, or by sequestering a factor required for B cell survival. At least 20×10^6 immature B cells are generated in the bone marrow each day, and from this population an estimated $2-3 \times 10^6$ B cells enter the periphery (22). It has been shown that maintenance of the peripheral B cell pool requires a minimum continuous input of newly formed cells (23), but that less than half of the B cells that reach the spleen progress to the mature phenotype (24). Although the percentage of long-lived and short-lived B cells in the periphery is controversial (22), the average half-life of the B220⁺ cells in the spleen is 4.3 wk, indicating that the majority of the B cells are long-lived mature cells (24). As we see a 50% decrease in the total B cell population in the periphery, we may be affecting the repopulation of the spleen with mature B cells or the survival of the existing mature B cells. One possible mechanism for sustaining B cell survival is to upregulate the antiapoptotic protein Bcl-2. Interestingly, elevated levels of Bcl-2 have been reported in the PBL B cells isolated from BAFF transgenic mice (6). To delineate the peripheral B cell subpopulations modulated by BCMA-Ig, the mechanism of action, as well as any potential effect on B cell development in the bone marrow, a more comprehensive study is in progress.

B Cell Survival with BAFF. The in vivo analysis, taken together with the finding that BAFF transgenic mice have an increased number of mature B cells and Bcl-2 expression, indicates that BAFF may promote B cell survival. We next examined the direct effect of BAFF on the survival of murine splenocytes in vitro. Splenocytes were cultured for 72 h in the presence or absence of BAFF and either BCMA-Ig or LT β R-Ig (Fig. 4). BAFF alone caused an increase in the percentage of cells that survived, and this effect was blocked with BCMA-Ig. The increase in cell numbers was not due to the increase in proliferation, as this has not been observed in the absence of B cell receptor stimulation (2, 3). The majority of the surviving cells, ~80%, were B220⁺ B cells, although a small percentage of

T cells also survived (data not shown). Thus, BAFF is capable of directly enhancing B cell survival.

In conclusion, we have identified BCMA as a receptor for BAFF. Soluble BCMA antagonizes BAFF action and leads to a rapid, significant B cell decline in vivo, suggesting that BAFF is necessary for homeostasis of the peripheral B cell pool. The increase in mature B cell numbers observed in the BAFF transgenic mice and the ability of BAFF to sustain B cells in vitro indicate that BAFF functions as a survival factor. Modulation of the peripheral B cell population using soluble BCMA may have applications in treating B cell-mediated autoimmune diseases, plasma cell disorders, and B cell cancers. A very recent report has demonstrated that blocking the BAFF pathway in two models of SLE using a soluble version of another receptor for BAFF, transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor (TACI)-Ig, eliminates proteinuria and prolongs the life span of the mice (25).

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