

# Soluble B-Cell Maturation Antigen Mediates Tumor-Induced Immune Deficiency in Multiple Myeloma

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## Abstract

**Purpose:** Reduced uninvolved immunoglobulin (Ig) levels are a hallmark of multiple myeloma. We previously showed that B-cell maturation antigen (BCMA) is solubilized and at high levels in multiple myeloma patient serum. We hypothesize that soluble BCMA binds B-cell-activating factor (BAFF) preventing its function to stimulate late B cells, and would result in lower polyclonal antibody levels in these patients.

**Experimental Design:** Mice were dosed with recombinant human BCMA (rhBCMA) and BCMA-BAFF complexes were analyzed in plasma, and its effects on antibody and Ig heavy chain mRNA levels determined. Using flow cytometry, BAFF binding to B cells was examined in the presence of rhBCMA and sera from multiple myeloma patients. In multiple myeloma sera, BCMA-BAFF complex formation and BCMA, IgA, IgG levels, and heavy-light chain isoform pair levels were determined.

**Results:** rhBCMA-BAFF complexes formed in immune-competent and deficient mice. Mice with human multiple myeloma xenografts, which contain plasma hBCMA and hBCMA-BAFF complexes, showed reduced plasma-free BAFF levels. rhBCMA administered to immune competent mice markedly reduced plasma IgA, IgG, and IgM levels and splenic Ig heavy chain mRNA levels. In serum from multiple myeloma patients, BCMA-BAFF complexes were detected and BAFF levels were reduced. Multiple myeloma patient sera containing BCMA prevented binding of BAFF to B cells. There is an inverse correlation between serum BCMA and uninvolved polyclonal Ig level in multiple myeloma patients.

**Conclusions:** Our results show that soluble BCMA sequesters circulating BAFF, thereby preventing it from performing its signaling to stimulate normal B-cell and plasma cell development, resulting in reduced polyclonal antibody levels in multiple myeloma patients. *Clin Cancer Res*; 22(13); 3383-97. ©2016 AACR.

## Introduction

Humans and mice bearing plasma cell tumors exhibit immune deficiency, specifically suppression of polyclonal antibody production (1-7). Thus, infections often occur in multiple myeloma patients (2). Active disease appears to be a factor that increases susceptibility to infections (2). Conversely, those responding to anti-multiple myeloma treatment show a decrease in infections (8). Normalization of uninvolved antibody levels occurs among multiple myeloma patients who achieve deeper responses and predicts improved overall survival (OS; ref.9). Assays that measure heavy chain-light chain (HLC) pairs have been introduced (10), and lower levels of the uninvolved HLC pair (isoform) have been shown to indicate a poor prognosis (11). This suggests that the lower risk of infection among multiple myeloma patients responding to

therapy likely results from their improved immune function with higher uninvolved antibody levels.

B-cell maturation antigen (BCMA also referred to as TNFRSF17 or CD269) is a cell surface receptor (12). It is expressed on normal B lymphocytes as they mature and on malignant cells from patients with terminally differentiated B lymphocytes, including tumor cells from multiple myeloma patients and cell lines (12-14). The ligands that bind BCMA are B-cell-activating factor (BAFF) and a proliferation inducing ligand (APRIL); these proteins stimulate B-cell differentiation and antibody production (15, 16).

We recently identified soluble BCMA in serum from multiple myeloma patients (17). We showed that levels were higher among patients with progressive disease (PD) than those with responsive disease and decreased with response to therapy (17). BAFF stimulates normal mouse and human B-cell proliferation, and addition of recombinant human BCMA-Fc (rhBCMA-Fc) abrogates this effect *in vitro* (18-20). On the basis of these studies, we hypothesized that soluble BCMA present in multiple myeloma patients' blood sequesters its ligand BAFF, thereby preventing it from binding to membrane-bound BCMA and BAFF-receptor (R) on the surface of B cells. This prevents BAFF from stimulating normal plasma cell development and production of polyclonal antibodies. Thus, the formation of circulating BCMA-BAFF complexes could account for the reduced levels of polyclonal antibodies in multiple myeloma patients.

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### Translational Relevance

We have previously shown that B-cell maturation antigen (BCMA) is solubilized and found at high levels in multiple myeloma patient serum. Normally, binding of the ligand B-cell-activating factor (BAFF) to BCMA present on late-stage B cells drives their development and antibody production. We show that solubilized BCMA from multiple myeloma cells sequesters BAFF preventing it from signaling B cells. This results in a lack of polyclonal antibody production, which is a hallmark of multiple myeloma. These studies provide the basis for evaluating this decoy soluble receptor in other B-cell malignancies associated with humoral deficiency, including chronic lymphocytic leukemia and Waldenstrom's macroglobulinemia. Higher circulating BCMA levels have been recently identified in these two diseases. It is possible that solubilized BCMA contributes to their humoral immune-deficient state. Identification of the mechanism(s) leading to the presence of circulating BCMA may lead to treatments that prevent this event. This should help reverse these patients' humoral immune deficiency.

## Materials and Methods

### Serum collection

Peripheral blood was drawn into red top vacutainers (Becton Dickinson). Serum was stored at  $-80^{\circ}\text{C}$  until analyzed. All studies involving human tissue were approved by the Western Institutional Review Board (WIRB BIO 001) and informed consent was obtained in accordance with the Declaration of Helsinki. Patients were diagnosed with multiple myeloma and their clinical status determined according to the International Myeloma Working Group criteria (21, 22).

### Cell line and cell culture conditions

The Raji and TB94 B-cell lines were obtained from the ATCC. Raji and TB94 cells were maintained in RPMI-1640 (Omega Scientific) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 i/u per mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and essential amino acids in an atmosphere of 5% carbon dioxide ( $\text{CO}_2$ ) at  $37^{\circ}\text{C}$ .

### Assessment of BCMA and BAFF levels

BCMA-Fc, Ig-Fc, and anti-BCMA monoclonal antibodies and ELISA kits were obtained from R&D Systems. Serum or plasma levels of human BCMA and human BAFF were determined using an ELISA (catalog # DY193E and catalog # DBLYSOB, respectively). Mouse plasma BAFF levels were assessed with an ELISA (catalog # DY2106-05). Samples were analyzed using a  $\mu\text{Quant}$  (Biotek Industries) plate reader set to 450 nm with KC Junior software.

### Identification of recombinant human (rh) BCMA-mouse BAFF complexes *in vitro*

We incubated recombinant mouse (rm) BAFF obtained from an mBAFF kit (catalog # AB-108-C) with recombinant human (rh) BCMA (catalog # DY193) overnight. A 96-well plate was coated overnight with an anti-mouse BAFF antibody (catalog # AB-108-C). Next, the samples were added and incubated for 2 hours. An

anti-BCMA detection antibody was then added (catalog # AB-108-C) and subsequent steps followed per the manufacturer's protocol.

### Identification of rhBCMA-mouse BAFF complexes in immune-competent and SCID mice

Four-week-old immune competent C57 Bl/6 and Balb/c and immune-deficient CB17 SCID mice were ordered from Charles River Laboratories. Animal studies were approved by the Institutional Animal Care and Use Committee. Mice were anesthetized using 3% isoflurane and blood was obtained retro-orbitally. Samples were collected using heparinized capillary tubes and spun, and plasma collected. ELISA plates were coated with a capture anti-mouse BAFF ELISA kit (catalog # MBLYS0) and plasma allowed to incubate for 2 hours. The anti-human BCMA detection antibody (catalog # DY193) was added for 2 hours. Following the incubation, the manufacturer's protocol was followed. Optical density of each well was determined using a  $\mu\text{Quant}$  microplate spectrophotometer with KC Junior software (Bio-Tek Instruments), absorbance at 450 nm with a reference wavelength of 550 nm.

### Identification of human BCMA-mouse BAFF complexes in multiple myeloma xenografts

We obtained 100  $\mu\text{L}$  of the anti-mouse BAFF capture antibody (catalog # DY2106-05) and added 25  $\mu\text{L}$  of undiluted plasma from mice with and without multiple myeloma xenografts, and vortexed it with mBAFF capture antibody overnight at room temperature. One hundred  $\mu\text{L}$  per well of this solution was added to a 96-well microplate, which was rotated on an orbital rotator overnight at room temperature. The plates were decanted and washed three times with 200  $\mu\text{L}$  wash solution, patted dry and incubated with 200  $\mu\text{L}$  blocking solution for 1 hour at room temperature. The plates were decanted again and washed X 3 with 200  $\mu\text{L}$  wash solution and patted dry. The anti-human BCMA detection antibody (catalog # DY193) was diluted in the working concentration using PBS and 100  $\mu\text{L}$  of this solution added. The plate was rotated overnight at room temperature, decanted and washed X3 with 200  $\mu\text{L}$  wash solution and patted dry. Next, the streptavidin was prepared using the anti-human BCMA ELISA Kit (catalog # DY193) to the working concentration in PBS, and 100  $\mu\text{L}$  added to the wells for 20 minutes at room temperature in the dark and decanted, and the plate washed X3 with 200  $\mu\text{L}$  of wash solution, patted dry, and substrate added (catalog # DY999) with 100  $\mu\text{L}$  per well in the dark. After 20 minutes, 50  $\mu\text{L}$  of stop solution was added (2N  $\text{H}_2\text{SO}_4$ , catalog # DY994).

### Flow cytometric analysis of the effects of rhBCMA and human multiple myeloma serum on BAFF binding to human Raji and TB94 B cells

Five hundred ng/mL BAFF-his-Flag-Tag (AB Bioscience) was preincubated with 3.0  $\mu\text{g}/\text{mL}$  rhBCMA or serum from a healthy subject (BCMA level 0.02  $\mu\text{g}/\text{mL}$ ) or a multiple myeloma patient in complete remission (CR) with a similarly low serum BCMA level (0.02  $\mu\text{g}/\text{mL}$ ) or with PD with a high serum level (0.75  $\mu\text{g}/\text{mL}$ ) or control mouse IgG in 1 mL 1X PBS at  $4^{\circ}\text{C}$  overnight. Six million Raji or TB94 B cells were incubated with or without BAFF with or without healthy subject or multiple myeloma patient serum or rhBCMA for 45 minutes at  $37^{\circ}\text{C}$ . For the blocking experiment, the anti-human BCMA antibody (3.0  $\mu\text{g}/\text{mL}$ ) obtained from the BCMA Kit (R&D Systems; catalog # DY193,

detection antibody), was added to rhBCMA or multiple myeloma serum overnight at 4°C, and this was then cultured with the Raji or TB94 cells. After washing three times, the cells were incubated with either anti-Flag antibody conjugated with FITC (Genscript) or isotype control IgG-FITC (Genscript) for 1 hour at 37°C away from light and then washed three times with PBS. Flow cytometric analysis was performed using a Beckman-Coulter FC500 cytometer with Cytomics CXP software (Beckman Coulter).

#### Multiple myeloma xenograft studies

Four-week-old male CB17 SCID mice were implanted with human multiple myeloma tumors (LAGκ-2, LAGκ-1A, or LAGλ-1) developed from multiple myeloma patients as previously described (17). Tumors were measured and the formula for an ellipsoid volume was applied [ $4/3 \pi \times (\text{width}/2)^2 \times (\text{length}/2)$ ]. Mouse plasma IgA, IgG, and IgM levels were determined with an ELISA according to the manufacturer's specifications (Bethyl Laboratories). Tumor-bearing mice were bled via retro-orbital bleeding and plasma collected. Absorbance at 450 nm with a reference wavelength of 550 nm was determined on a  $\mu$ Quant microplate spectrophotometer with KC Junior software (Bio-Tek Instruments).

#### Pokeweed antigen stimulation in C57 Bl/6 mice with and without injection of rhBCMA-Fc

C57 Bl/6 mice were administered 100  $\mu$ g of rhBCMA-Fc i.p. on days -4, -2, 0, 2, and 4 and also injected with 40  $\mu$ g/mouse of pokeweed Mitogen (PWM, Sigma, catalog # L8777 5MG) via intraperitoneal injection on day 0 (23). On days -4, 0 and 7, mice were bled and plasma IgM levels were determined.

#### qPCR methods

Quantitative PCR (qPCR) was applied to measure the relative abundance of mouse Ig heavy chain C $\alpha$  and C $\mu$  and BAFF transcripts, and compared with the mRNA levels of the housekeeping gene, mouse beta-actin (Actb). The qPCR was applied using TaqMan technology on an OneStepPlus instrument (Life Technology). First, total RNA was extracted using the RNeasy Kit (Qiagen). To eliminate DNA contamination, DNase I was used as recommended in the protocol's optional step. RNA was then converted to cDNA with the SuperScript III reverse transcriptase Kit (Life Technology). For mouse C $\mu$  qPCR, the mouse C $\mu$  probe was fluorescence labeled as 6FAM-CCTCTTCTACAGCACCAC-MGBNFQ and amplified with PCR primers C $\mu$  qPF1, ACTGCCTC-CACCTTCATCGT and C $\mu$  qPR2, CTGAGAGTCATTTACCTTGAACAG. For mouse C $\alpha$  qPCR, the mouse C $\alpha$  probe was fluorescence labeled as 6FAM-ACCTAGTGTGAGCCCG-MGBNFQ and amplified with PCR primers C $\alpha$  qPF1, GGAGCTGTCCCCA-GAAAGC and C $\alpha$  qPR2, GCTCCCTCGCCTGGCT. Mouse Actb was selected as the housekeeping control gene. Actb probe was labeled with another fluorescence probe VIC-ATCATTGCT-CCTCCTGAG-MGBNFQ and amplified with PCR primers mouse Actb qPF1, TGGTCCCTAGCACCATGAAGA and mouse Actb qPR2, CACCGATCCACACAGACTTTC. All amplicons were designed to span introns to eliminate possible false signals from DNA contamination. The relative abundance of C $\mu$  or C $\alpha$  mRNA was compared as folds of transcripts of C $\mu$  or C $\alpha$  versus that of Actb mRNA in the same wells that started with the same cDNA amount. The levels represented the average of triplicates. Similarly, mBAFF probe was fluorescence labeled as 6FAM-AGTCTA-GAAGACACACATA-MGBNFQ and amplified with PCR pri-

mers BAFF qPF1, TGACAAAGCATCATCTTCCCC and BAFF qPR2, CTGAGAGTCATTTACCTTGAACAG. The same mouse Actb gene was used as the housekeeping control.

#### Uninvolved Ig levels versus BCMA

Serum IgA and IgG levels were determined among multiple myeloma patients using nephelometry (Image 800, Beckman-Coulter). Hevylite Assays (Binding Site) were used to quantify HLC isoform pair levels in multiple myeloma patients.

#### Statistical analyses

When comparing two groups, significance was determined using a Student *t* test. The minimal level of significance was  $P < 0.05$ . Statistical significance of differences observed between two or more groups was determined using ANOVA (two-way). Analysis was determined using GraphPad Prism version 4.03 for Windows, GraphPad Software. All *in vivo* experiments contained 4 or 5 mice per treatment group. The Mann-Whitney test, a non-parametric test that compares the distributions of two unmatched groups, was used to analyze both uninvolved HLC and Ig levels in multiple myeloma patients as plotted against serum BCMA levels.

## Results

#### Mouse BAFF and recombinant human BCMA form complexes *in vitro*

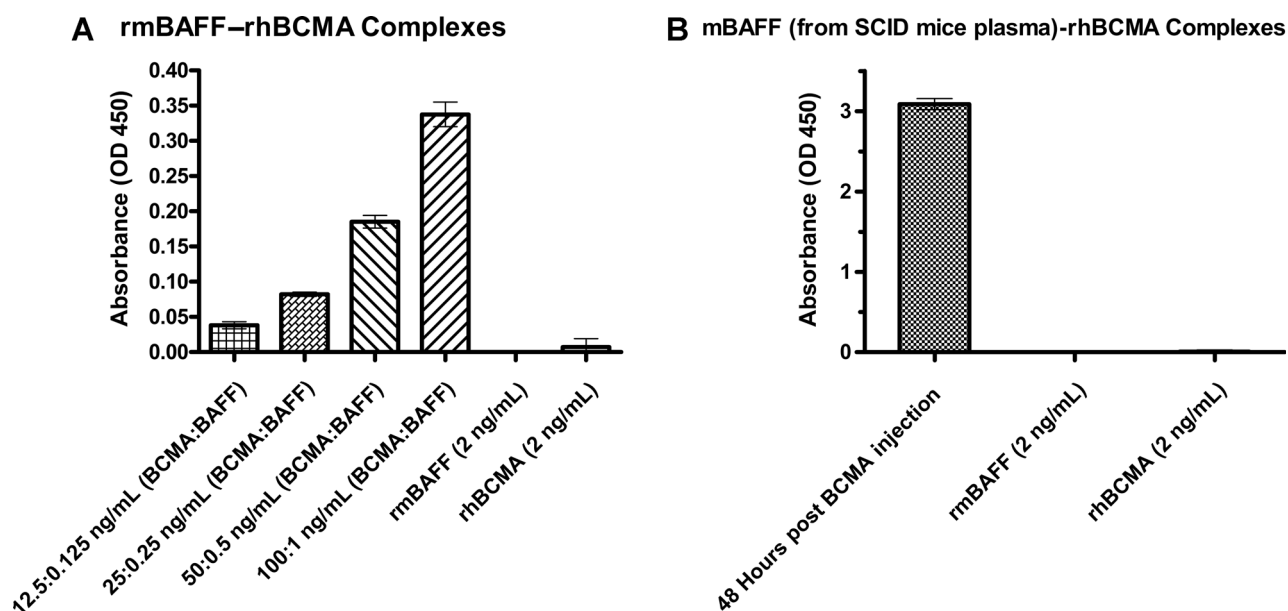
BAFF is a survival factor for normal B lymphocytes and plasma cells and has been shown to be critical for normal B-cell development (24–27). *In vitro* studies have shown that BCMA-Fc (or BCMA-Ig or TACI-Ig) prevents the proliferative effects of BAFF on normal B cells (18–20). We proposed that BCMA-Fc may neutralize the effects of BAFF via direct binding of this ligand. To this end, we incubated increasing concentrations of both rhBCMA and rmBAFF and measured complex formation using an anti-mBAFF capture antibody and an anti-rhBCMA detection antibody. The highest absorbance occurred at concentrations of 100 ng/mL BCMA and 1 ng/mL BAFF (Fig. 1A), which are similar to those found in plasma from multiple myeloma patients (28, 29).

Given that rhBCMA forms complexes with rmBAFF, we determined whether mBAFF present in plasma from SCID mice is also bound to rhBCMA. Plasma obtained from mice was incubated overnight with rhBCMA. Recombinant human BCMA formed complexes with mBAFF (Fig. 1B). The anti-mouse BAFF capture and anti-human BCMA detection antibodies did not cross react with rhBCMA and mBAFF, respectively.

#### The plasma of immune-competent and deficient mice dosed with rhBCMA-Fc reveals rhBCMA-mBAFF complexes

We investigated whether BCMA-BAFF complexes form *in vivo*. Plasma samples from C57 Bl/6 mice injected with rhBCMA-Fc were incubated in wells pre-coated with anti-mBAFF capture antibodies. A polyclonal anti-hBCMA detection antibody was then added. A strong absorbance indicating the presence of rhBCMA-mBAFF complexes was observed in samples collected on days 4 and 6 post-rhBCMA injection (Fig. 2A). Only minimal background absorbance was observed in samples from mice injected with the control protein Ig-Fc or from untreated control mice, and antibody cross-reactivity was negative.

Similarly, SCID mice were dosed with rhBCMA-Fc at day 0; animals were bled just before its administration, and on post-injection days 1, 2, and 7. Plasma from immune-deficient SCID



**Figure 1.**

rhBCMA and mBAFF form complexes *in vitro*. A, detecting rmBAFF–rhBCMA complexes *in vitro*. Varying concentrations of 100:1 ratio of rhBCMA:rmBAFF were incubated overnight in a 35°C water bath, and their levels were determined using an ELISA against negative controls of rmBAFF and rhBCMA at high standard concentrations (2 ng/mL) using anti-mBAFF capture and anti-hBCMA detection Abs. The experiment shows that complexes are detected at ratios and concentrations of BCMA to BAFF that typically are present in multiple myeloma patients. B, detecting mBAFF (derived from SCID plasma)–rhBCMA complexes. Samples that included CB17 SCID plasma and recombinant human BCMA, and IgG were incubated overnight in a 35°C water bath. Negative controls were determined using an ELISA against rmBAFF and rhBCMA at high standard concentrations (2 ng/mL) using anti-mBAFF capture and anti-hBCMA detection Abs. All samples were run in triplicates and the average  $\pm$  SEM was graphed. Absorbance was determined by a  $\mu$ Quant microplate spectrophotometer with KC Junior software.

mice contained rhBCMA–mBAFF complexes (Fig. 2B; top), which could still be detected on days 2 and 7 (data not shown). The corresponding mBAFF level following rhBCMA-Fc treatment is shown in Fig. 2B (bottom). rhBCMA–mBAFF complexes were also observed on day 1 in plasma samples from immune competent C57 Bl/6 mice following rhBCMA injection (Fig. 2C).

#### Plasma hBCMA levels correlate with tumor volume in mice bearing human multiple myeloma xenografts, and hBCMA–mBAFF complexes are detected in these animals

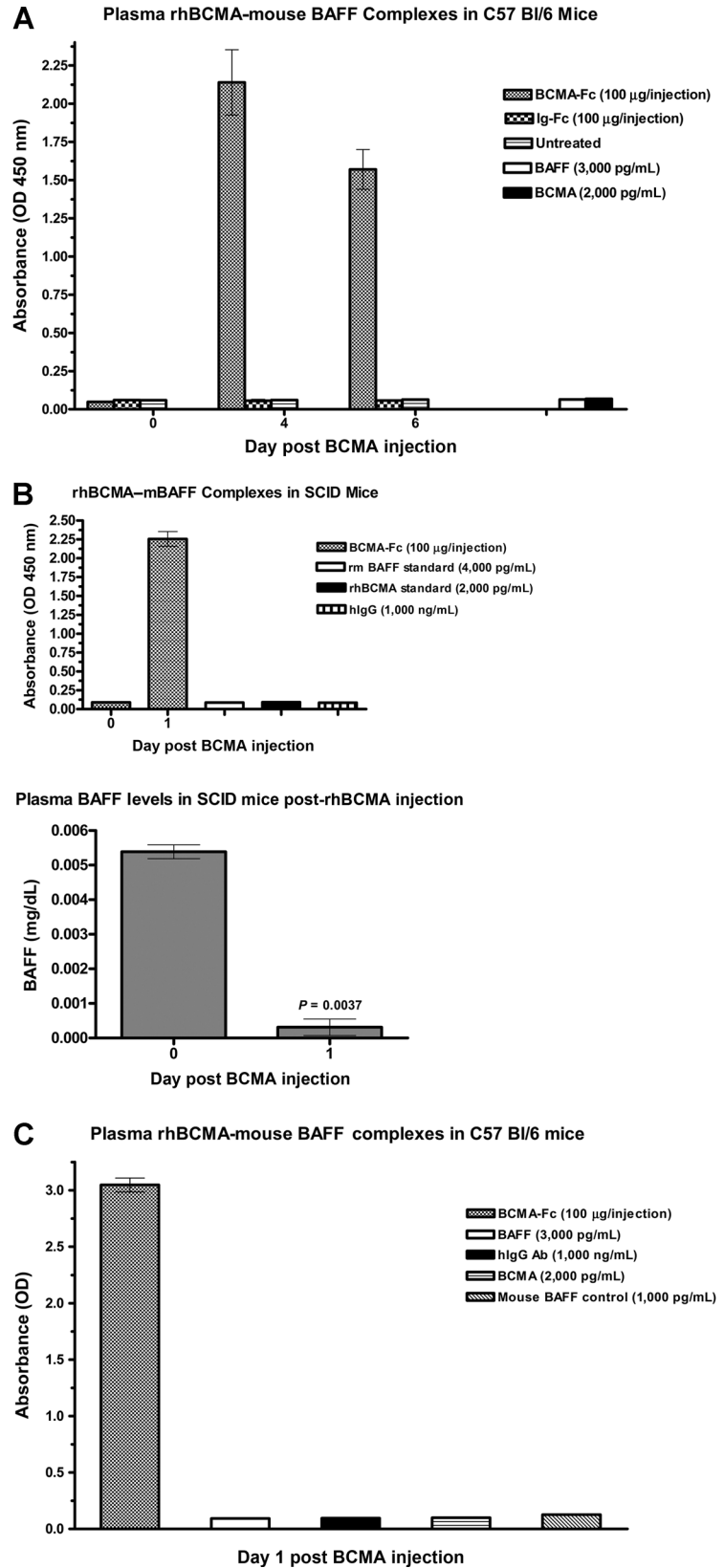
Human BCMA (hBCMA) levels correlated with increases in tumor volume in human multiple myeloma xenograft models (Fig. 3A–C). Notably, we detected mBAFF–hBCMA complexes in plasma samples from mice (measured on day 28 post tumor implantation) containing the three human multiple myeloma xenografts (Fig. 3D). The hBCMA–mBAFF complex levels in the plasma correlate with the amount of BCMA in the plasma in these 3 different human multiple myeloma xenograft models (Fig. 3A–C). Specifically, LAG $\kappa$ -2 (Fig. 3B) had the highest level of plasma BCMA, followed by LAG $\lambda$ -1 (Fig. 3A), and LAG $\kappa$ -1A (Fig. 3C) had the least amount of plasma BCMA; the hBCMA–mBAFF complexes correlated with the amount of BCMA in the plasma of these mice (Fig. 3D).

#### BAFF levels are significantly lower in SCID mice bearing human multiple myeloma

We next evaluated whether human BCMA produced by multiple myeloma xenografts affects mouse BAFF plasma levels.

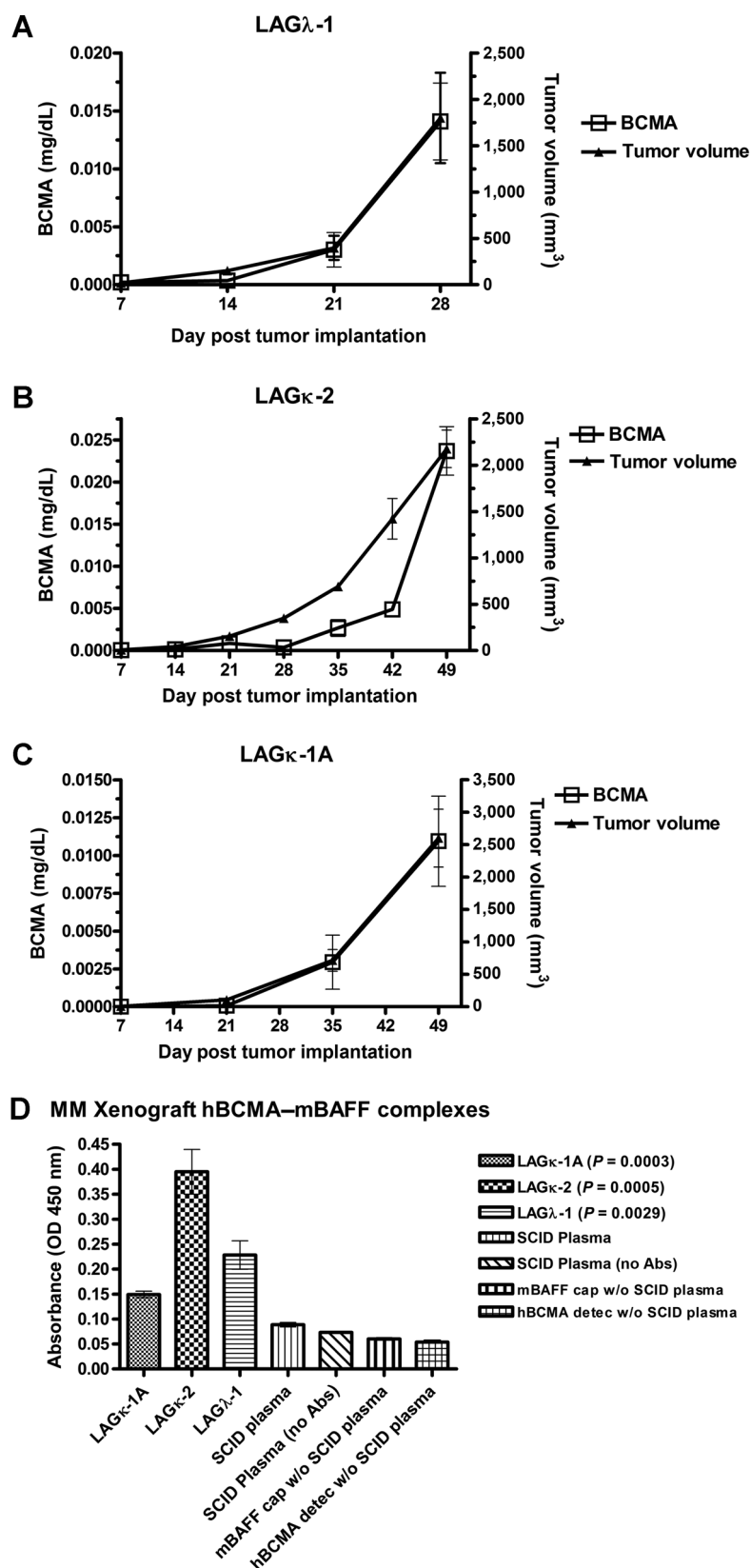
Results showed that SCID mice implanted with the human multiple myeloma tumor LAG $\lambda$ -1 had lower mBAFF levels (day 14,  $P = 0.0143$ ; day 21,  $P = 0.0002$ ; and day 28,  $P = 0.0008$ ) when compared with age and sex-matched SCID mice without tumors (Fig. 4A). mBAFF was also decreased in plasma collected on days 14, 21, 28, and 35 from mice implanted with LAG $\kappa$ -2 tumors, compared with mice without tumors ( $P = 0.0015$ ,  $P < 0.0001$ ,  $P < 0.0001$ , and  $P < 0.0001$ , respectively; Fig. 4B). Similarly, mice bearing LAG $\kappa$ -1A xenografts had significantly lower mBAFF levels when compared with mice without tumors (data not shown).

Because we had established that rhBCMA-Fc injected into mice formed rhBCMA–mBAFF complexes, the decrease in mBAFF levels in the multiple myeloma xenografts was likely due to the formation of rhBCMA–mBAFF complexes and the inability of the anti-mBAFF antibody from the ELISA BAFF Kit to detect this protein when it was bound to rhBCMA. To confirm this, we incubated mBAFF with rhBCMA overnight and performed an mBAFF ELISA. At the higher and clinically relevant concentration of BCMA found in multiple myeloma patients (100 ng/mL), rhBCMA inhibited the ability of the anti-mBAFF antibody to detect mBAFF (Fig. 4C; top). In contrast, human IgG at an identical concentration to that of rhBCMA did not interfere with mBAFF detection (Fig. 4C; top). Similarly, we incubated rhBAFF with rhBCMA overnight and performed an rhBAFF ELISA. At the clinically relevant concentration of BCMA found in multiple myeloma patients (100 ng/mL), rhBCMA inhibited the ability of the anti-BAFF antibody to detect rhBAFF (Fig. 4C; bottom).

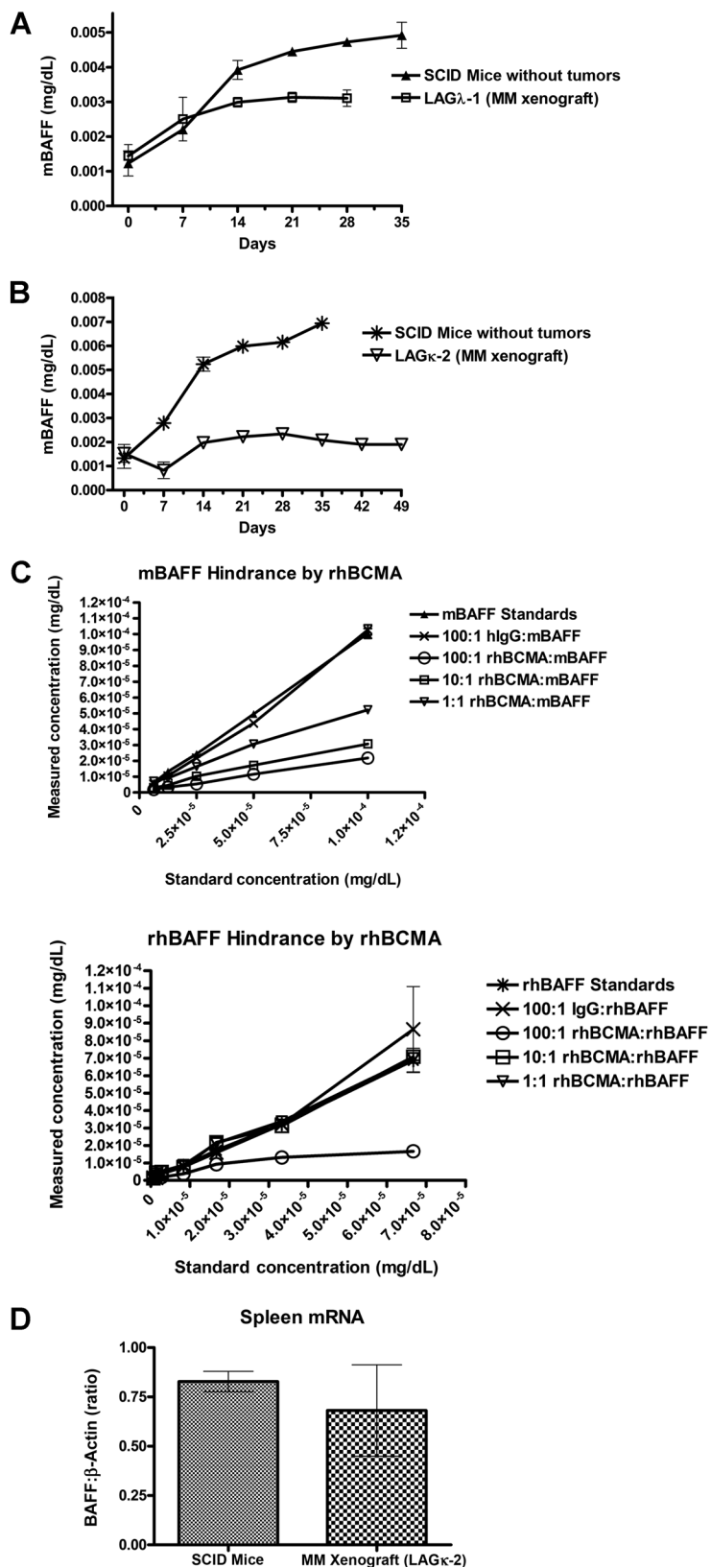


**Figure 2.** rhBCMA-BAFF complexes form *in vivo*. A, rhBCMA-Fc injected into C57 Bl/6 mice binds mouse BAFF, forming hBCMA-mBAFF complexes. B, hBCMA-mBAFF complexes were detected in SCID mice dosed with hBCMA-Fc (top) and BAFF levels in these mice following formation of hBCMA-mBAFF complexes (bottom). C, hBCMA-mBAFF complexes were detected as early as day 1 in C57 Bl/6 mice dosed with rhBCMA-Fc. Negative controls include samples of mBAFF, hlgG, and rhBCMA. Absorbance was determined by a µQuant microplate spectrophotometer with KC Junior software. Data are presented as means ± SEM, obtained from two independent experiments and samples were analyzed twice.

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**Figure 3.** Plasma human BCMA levels correlate with human multiple myeloma (MM) tumor volume and BAFF-BCMA complexes are found in SCID mice bearing these tumors: In SCID mice bearing the A, LAG $\lambda$ -1; B, LAG $\kappa$ -2; and C, LAG $\kappa$ -1A human multiple myeloma xenografts. D, human BCMA (from human multiple myeloma tumors growing in SCID mice) binds mouse BAFF in mouse plasma and forms hBCMA-mBAFF complexes. D, in the plasma from mice bearing these three human multiple myeloma xenografts, the levels of BCMA in the plasma correlate with the amount of BAFF-BCMA complex formation. Abs, antibodies.



**Figure 4.** Plasma BAFF levels are lower in SCID mice bearing human multiple myeloma (MM) xenografts. A, decrease in plasma BAFF levels in mice implanted with the human multiple myeloma tumor LAGλ-1 when compared with SCID mice without multiple myeloma tumors (day 14,  $P = 0.0143$ ; day 21,  $P = 0.0002$ ; and day 28,  $P = 0.0008$ ). B, decrease in plasma BAFF levels in mice implanted with the human multiple myeloma tumor LAGκ-2 when compared with SCID mice without multiple myeloma tumor (day 14,  $P = 0.0015$ ; day 21,  $P < 0.0001$ ; day 28,  $P < 0.0001$ ; and day 35,  $P < 0.0001$ ). Results presented are group means  $\pm$  SEM ( $n = 4-5$  mice/group) from two independent experiments. C, increasing concentrations of rhBCMA interfere with mouse BAFF detection (top) and human BAFF detection (bottom), with the highest concentration of BCMA used being similar to concentrations found in multiple myeloma patients. D, using RT-PCR, no differences in BAFF mRNA levels were detected in SCID mice with or without human multiple myeloma xenografts.

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Human IgG at an identical concentration to that of rhBCMA did not interfere with rhBAFF detection (Fig. 4C; bottom).

The spleens and lymph nodes of human multiple myeloma-bearing and non-bearing SCID mice were analyzed for mBAFF mRNA levels using RT-PCR. No differences in these levels were detected in either the spleen (Fig. 4D) or lymph nodes (data not shown) of mice with or without human multiple myeloma. These results indicate that the lower mBAFF levels in mice containing human multiple myeloma xenografts were not from changes in the expression of this gene.

#### Administering rhBCMA-Fc to immune-competent mice reduces antibody levels

Decreases in serum levels of non-paraprotein immunoglobulins observed in multiple myeloma patients have been primarily attributed to a reduction in protein synthesis (30). Given our previous finding that BCMA is soluble and found at high levels in multiple myeloma patients (17), we determined the effect of soluble human BCMA on antibody levels in mice. First, we determined the concentrations of rhBCMA-Fc in C57 Bl/6 mice for 48-hours following its injection. Peak plasma concentrations occurred at 4 hours post-injection, with a half-life of approximately 36 hours (Fig. 5A). On the basis of these observations, 100 µg of rhBCMA-Fc was injected into immune competent C57 Bl/6 mice on days 0, 2, and 4, and plasma IgA, IgG, and IgM levels determined before and up to 6 days following its administration. A decrease in IgA levels was observed on days 4 and 6 following rhBCMA-Fc administration when compared with its baseline levels ( $P = 0.0031$  and  $P = 0.0064$ , respectively), mice injected with Ig-Fc control protein ( $P = 0.0087$ ) and untreated mice ( $P = 0.0221$ ; Fig. 5B). IgM levels were also determined (Fig. 5C), and were lower in rhBCMA-Fc-treated animals when compared with the untreated group ( $P = 0.0001$ ) and the Ig-Fc-treated mice ( $P = 0.0088$ ). At study termination, no differences in spleen size were observed between BCMA-Fc, Ig-Fc, or untreated controls (data not shown). Splenic cell lysates were analyzed for Ig transcript levels using RT-PCR, and heavy chain C $\mu$  and C $\alpha$  mRNA levels were significantly lower than among vehicle-treated mice (Fig. 5D and E).

For IgG levels, mice were dosed daily with rhBCMA-Fc, and a marked decrease was observed on day 6 when compared to baseline levels ( $P = 0.0023$ ), the Ig-Fc-injected mice ( $P = 0.0014$ ), and the untreated animals ( $P = 0.0129$ ; Fig. 5F). We observed a slower decrease in plasma IgG levels, when compared to IgA and IgM, following rhBCMA-Fc treatment, as expected, given the half-life of IgG in the blood is approximately three weeks compared to the shorter half-lives of IgA and IgM (31, 32).

An additional study in Balb/c mice also showed significant decreases in both IgA and IgM levels following administration of BCMA-Fc (data not shown). Because the median age at diagnosis of multiple myeloma is 66 years (33), older (one-year-old) C57 Bl/6 mice were dosed once (on day 0) with BCMA-Fc and mouse IgA levels were measured. Plasma IgA levels were similarly reduced ( $P = 0.0028$ ), when compared with baseline (Fig. 5G).

It is known that PWM stimulates B lymphocytes to selectively synthesize and secrete IgM, and plasma IgM levels in immune-competent C57 Bl/6 mice peak at 1 week following PWM immunization (34). To determine whether treatment with BCMA-Fc would prevent the stimulatory effect of PWM on normal B cells, C57 Bl/6 mice were pretreated with BCMA-Fc (100 µg/injection) on days -4 and -2 before injection of PWM and also on days 0, 2,

and 4 following PWM simulation. On day 0, mice were immunized with PWM (40 µg). The inhibitory effect on plasma IgM levels was observed in mice pre-treated with BCMA-Fc alone, as on day 0 these mice had significantly lower IgM levels when compared with the vehicle control group ( $P = 0.0035$ ). Interestingly, pre-treatment with BCMA-Fc completely blocked IgM production following PWM injection (Fig. 5H). On day 7, mice dosed with BCMA-Fc alone and with the combination of BCMA-Fc and PWM had reduced levels of plasma IgM when compared with mice treated with PWM alone (BCMA-Fc:  $P = 0.0375$ ; BCMA-Fc + PWM:  $P = 0.0490$ ) and also when compared with vehicle control mice (BCMA Fc:  $P = 0.0031$ ; BCMA-Fc + PWM:  $P = 0.0225$ ).

#### In multiple myeloma patient serum, BCMA-hBAFF complexes are present and BAFF levels are reduced

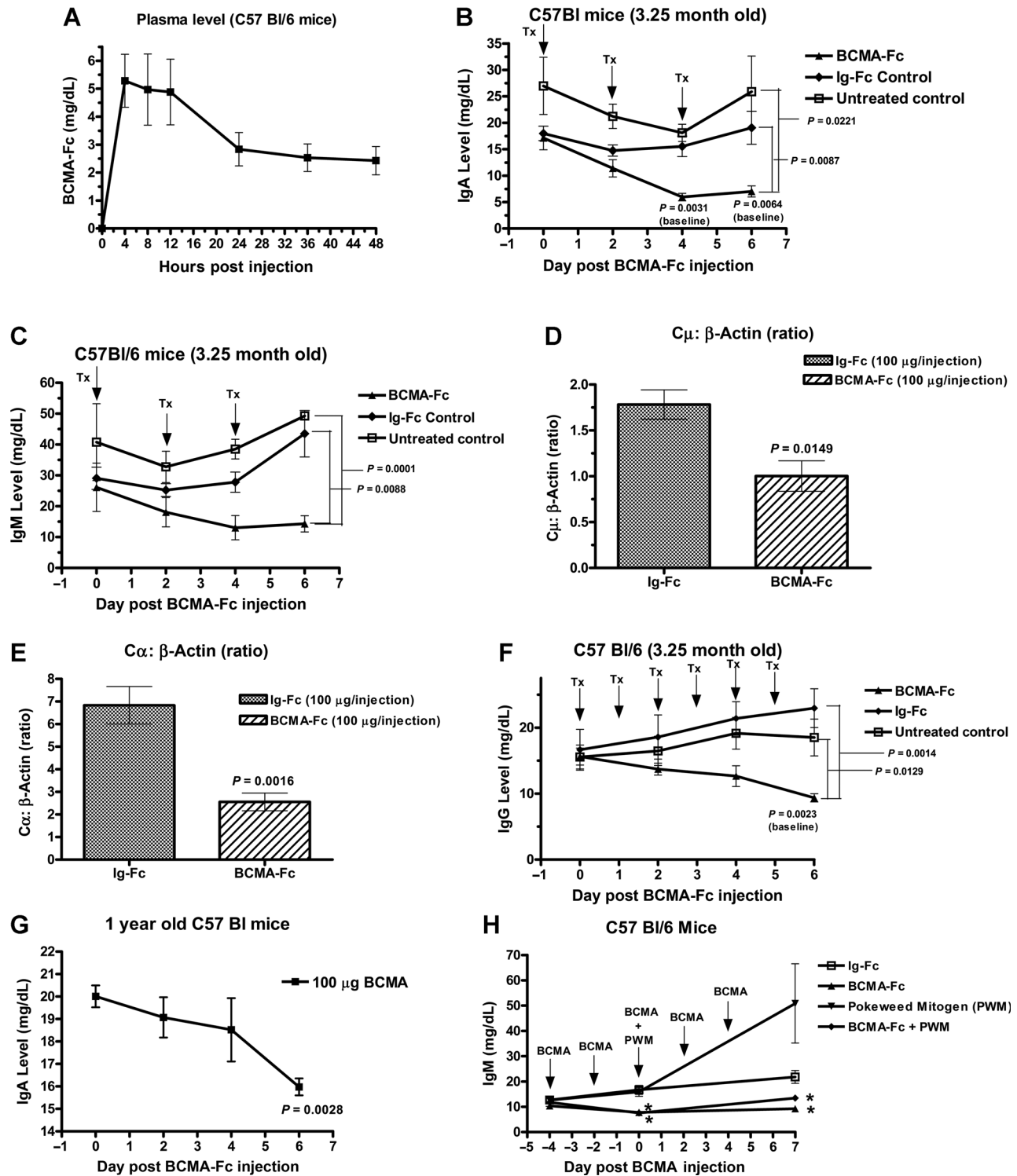
First, we determined whether sera from multiple myeloma patients contained BCMA-BAFF complexes with a polyclonal anti-human BCMA antibody, using ELISA plates coated with an anti-human BAFF antibody. As shown in Fig. 6A (2 representative samples from 12 multiple myeloma patients), a strong absorbance indicating the presence of BCMA-BAFF complexes was also detected in multiple myeloma patients.

Next, we determined the level of BAFF in the serum of active multiple myeloma patients compared with healthy donors (Fig. 6B). The level was lower in multiple myeloma patients (median 436.0 pg/mL) compared with healthy donors (median, 837.5;  $P = 0.0004$ ). These results are consistent with our findings in SCID mice, in which animals bearing human multiple myeloma showed reduced plasma free BAFF levels (Fig. 4A and B) from the sequestering of BAFF by BCMA (Fig. 3D).

#### Multiple myeloma patient serum and rhBCMA prevent BAFF from binding to Raji or TB94 B cells

To determine whether hBCMA present in multiple myeloma patient serum or rhBCMA-Fc would block BAFF from binding to the surface of B cells, Raji B cells were incubated with serum from a healthy subject (0.02 µg/mL), or a multiple myeloma patient with PD and serum containing high levels of BCMA (0.75 µg/mL) or in CR with low levels of serum BCMA (0.02 µg/mL) or rhBCMA-Fc (3.0 µg/mL) in the presence of rhBAFF (500 ng/mL). Serum from the multiple myeloma patient with high levels of serum BCMA and rhBCMA-Fc decreased rhBAFF detection on Raji cells from 98.6% to 53.9% and from 98.6% to 44.2%, respectively (Fig. 6C, top). An anti-hBCMA-blocking antibody added to Raji B cells incubated with BAFF and either rhBCMA or serum from this multiple myeloma patient increased BAFF binding back up to 87.1% and 77.0%, respectively. In contrast, serum from a healthy subject or multiple myeloma patient in CR with low levels of serum BCMA (both at 0.02 µg/mL) only minimally decreased BAFF binding, which is consistent with the low levels of serum BCMA present in healthy subjects and multiple myeloma patients in CR (17, 29). To confirm that BCMA (from multiple myeloma serum or rhBCMA) blocks BAFF from binding to the B-cell surface, and that an anti-BCMA antibody restores this binding, the above experiment was performed using an additional human B-cell line, TB94. Similarly, serum from the multiple myeloma patient with high levels of serum BCMA and rhBCMA-Fc decreased rhBAFF detection on these cells from 94% to 62.1% and from 98.6% to 48.7%, respectively (Fig. 6C, bottom). An anti-





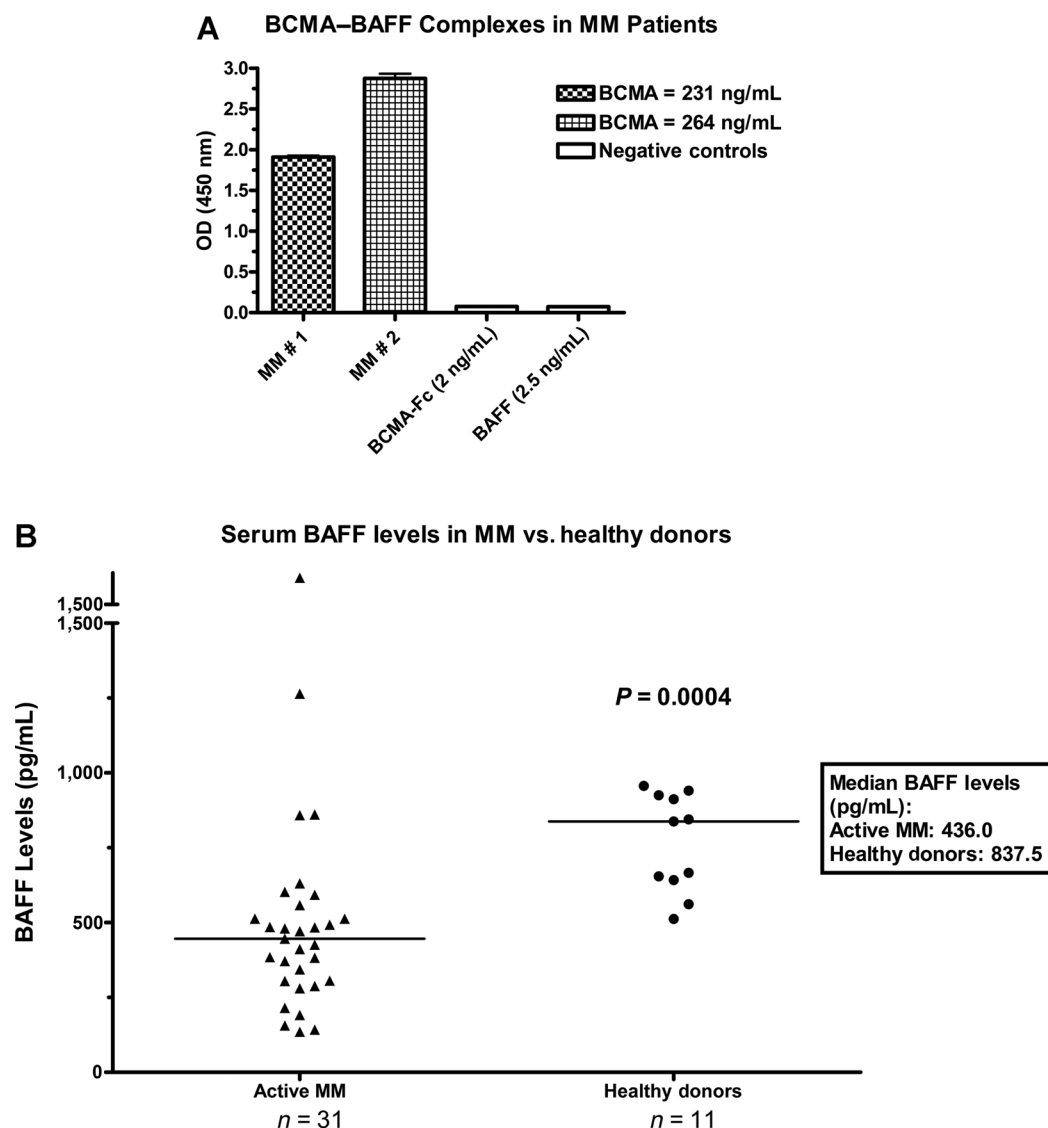
**Figure 5.** Administration of BCMA-Fc decreases antibody levels. A, 100 μg of rhBCMA was injected into mice to determine the half-life of this protein and plasma collected for 48 hours following its administration. B, reduction in plasma IgA levels in C57 Bl/6 mice following rhBCMA treatment (day 4:  $P = 0.0031$ ; day 6:  $P = 0.0064$ ). Tx, day of administration of BCMA-Fc or Ig-Fc. C, decline of plasma IgM levels in C57 Bl/6 mice following rhBCMA treatment (day 4,  $P = 0.0001$ ; day 6,  $P = 0.0088$ ). D, C<sub>μ</sub> mRNA levels were lower ( $P = 0.0149$ ) following BCMA treatment. E, C<sub>α</sub> mRNA levels were also lower ( $P = 0.0016$ ) following BCMA treatment. F, a significant reduction in plasma IgG levels occurred in C57 Bl/6 mice following daily rhBCMA treatment when compared with baseline, Ig-Fc, and untreated controls (day 6,  $P = 0.0023$ ;  $P = 0.0014$  and  $P = 0.0129$ , respectively). G, one-year-old mice dosed with BCMA on day 0 showed a reduction in their IgA levels on day 6 ( $P = 0.0028$ ). H, pretreatment of BCMA-Fc reduced IgM levels in C57 Bl/6 mice stimulated with the B-cell mitogen pokeweed mitogen (PWM), when compared with mice treated with PWM alone (day 7,  $P = 0.049$ ) and also when compared with vehicle control mice (day 7,  $P = 0.0225$ ). Arrows indicate injections of BCMA and PWM. Statistical significance of differences observed between two or more groups was determined using two-way ANOVA. Absorbance was determined by a μQuant microplate spectrophotometer with KC Junior software.

hBCMA-blocking antibody added to TB94 cells incubated with BAFF and either rhBCMA or serum from this multiple myeloma patient increased BAFF binding back up to 84.8.1% and 79.6%, respectively.

**Human BCMA levels are inversely correlated to uninvolved polyclonal antibody levels**

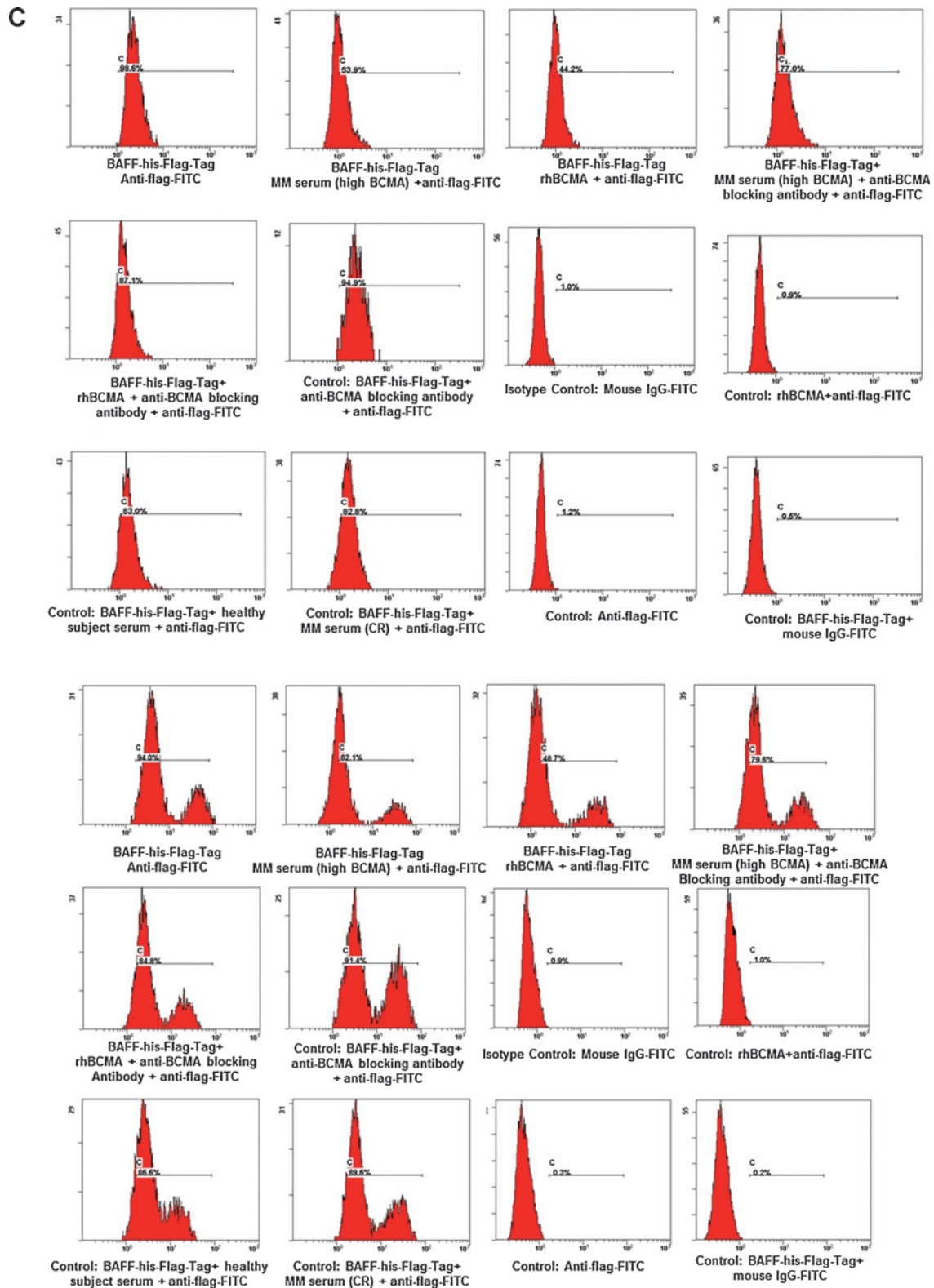
We then determined whether serum BCMA levels were also inversely related with uninvolved Ig and uninvolved HLC levels in multiple myeloma patients. Using assays for Hevylite, uninvolved

IgG $\lambda$  and BCMA levels were determined in the sera from samples of patients with IgG $\kappa$  MM ( $n = 117$ ). An inverse correlation between BCMA and IgG $\lambda$  levels was observed ( $P < 0.0001$ ; Fig. 6D). A similar inverse correlation was found when serum BCMA and IgG $\lambda$  levels were compared from samples of patients with IgG $\lambda$  multiple myeloma ( $n = 62$ ;  $P = 0.0006$ ; Fig. 6E). Samples from samples of patients with IgG multiple myeloma ( $n = 313$ ) also demonstrated an inverse correlation between serum levels of BCMA and uninvolved IgA ( $P < 0.0001$ ; Fig. 6F). Serum from samples of patients with IgA multiple myeloma ( $n = 134$ )



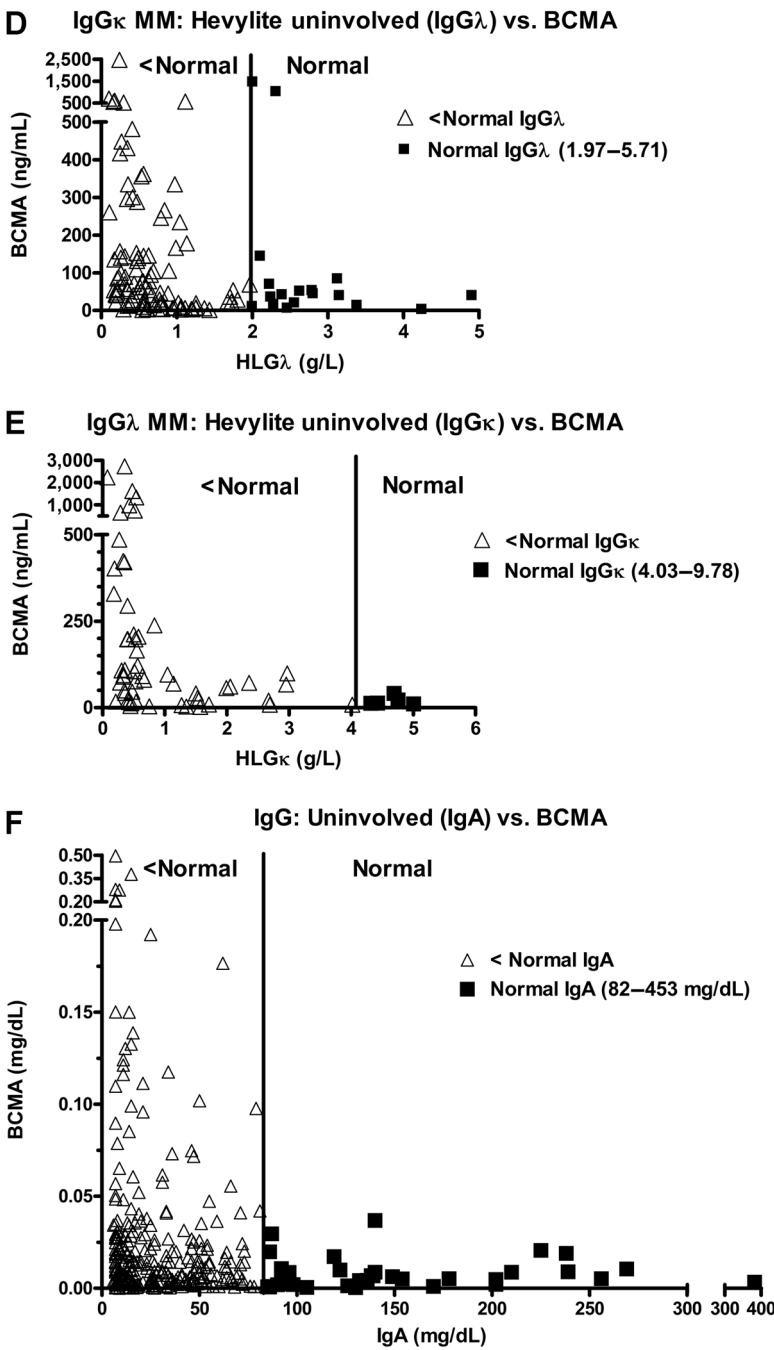
**Figure 6.** Sera of multiple myeloma (MM) patients contain hBCMA-hBAFF complexes and hBCMA is inversely related to uninvolved polyclonal antibody levels. A, detection of BCMA-BAFF complexes in multiple myeloma patients using anti-human BAFF capture Ab and anti-hBCMA detection Ab. B, determination of serum BAFF levels from 31 patients with active multiple myeloma (13 with PD and 18 untreated) versus healthy donors ( $n = 11$ ) shows reduced BAFF levels in the multiple myeloma patients. C (top), flow cytometric analysis of sBAFF (500 ng/mL) binding to Raji cells (top figures) in the presence of rhBCMA (3.0  $\mu$ g/mL, top row, third column) and serum from multiple myeloma patients with PD with a high level of serum BCMA (0.75  $\mu$ g/mL, top row, second column) or CR with a low level (0.02  $\mu$ g/mL, bottom row, second column) or a healthy subject (also 0.02  $\mu$ g/mL, bottom row, first column). Both rhBCMA and serum from the multiple myeloma patient with PD with a high level of serum BCMA markedly reduced BAFF binding to Raji B cells, and addition of anti-BCMA-blocking antibody restored BAFF binding. In contrast, minimal reduction in BAFF binding occurred in the presence of serum from the healthy subject (bottom row, first column) or multiple myeloma patient in CR (bottom row, second column) both with low serum BCMA levels. (Continued on the following page.)

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**Figure 6.** (Continued.) C (bottom), this same experiment was duplicated using an additional human B-cell line, TB94, and similar results were obtained. (Continued on the following page.)

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**Figure 6.** (Continued.) D, using assays for Hevylite and determining serum BCMA levels, uninvolved IgG $\lambda$  levels were determined in the sera from multiple myeloma patients with IgG $\kappa$  disease, and showed an inverse correlation between the levels of BCMA and IgG $\lambda$  ( $P < 0.0001$ ; open triangles,  $n = 99$ , solid squares,  $n = 18$ ). E, similarly, when BCMA and IgG $\kappa$  serum levels were determined among patients with IgG $\lambda$  multiple myeloma, an inverse relationship was observed ( $P = 0.0006$ ; open triangles,  $n = 57$ , solid squares,  $n = 5$ ). F, next, among patients with IgG multiple myeloma, serum levels of IgA and BCMA were found to inversely correlate ( $P < 0.0001$ ; open triangles,  $n = 275$ , solid squares,  $n = 38$ ). Flow cytometric analysis was performed using a Beckman-Coulter FC500 cytometer with Cytomics CXP software. Each data point represents one individual multiple myeloma patient's serum sample. Open triangles show serum samples from multiple myeloma patients with below normal uninvolved Ig levels and filled squares show serum samples from patients with normal levels of uninvolved Ig. Samples were run in duplicates with data presented as means  $\pm$  SEM.

similarly showed an inverse correlation between BCMA and uninvolved IgG levels ( $P < 0.0001$ ; data not shown).

**Discussion**

*In vitro* studies have demonstrated the importance of BAFF in B-cell proliferation (24–27), and the prevention of its function impairs B-cell formation (18–20). A reduction in splenic B cells occurred following administration of BCMA-Fc to normal mice (20). Administration of soluble BCMA-Ig to normal mice resulted

in reductions in B cells in the blood and peripheral lymphoid organs (15). When mice were dosed with TACI-Fc, which binds BAFF, were immunized with hapten-conjugated chicken gamma globulin (NP23-CgG), the production of NP-specific IgM was inhibited (35). Data from our *in vivo* studies are consistent with these findings. Specifically, we have shown that immune-competent C57 Bl/6 mice dosed with BCMA-Fc, which binds mBAFF, show reduced plasma IgM, IgA, and IgG levels.

We have previously shown that BCMA is present in the serum of multiple myeloma patients; its levels decreased with responses to

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anti-multiple myeloma therapy and inversely correlated with OS (17, 29). Recent preclinical studies demonstrate that antibody conjugates targeting BCMA may have anti-multiple myeloma effects (36). Therapies targeting its ligands, BAFF and APRIL, have been of minimal benefit for the treatment of multiple myeloma patients. Atacicept (TACI.Fc) is a fusion protein that binds soluble BAFF and APRIL; it reduces levels of these free ligands that prevent them from interacting with their corresponding receptors (37). A clinical study evaluating atacicept for treating multiple myeloma and Waldenstrom's macroglobulinemia patients showed little clinical benefit (37). Notably, a significant decrease in polyclonal antibodies was observed in 54% of multiple myeloma patients in this trial, and the authors stressed the need for infectious disease surveillance due to a possible association between suppression of normal antibody production and risk of infection. Our studies show that in multiple myeloma patients, BAFF is rendered inactive and unable to bind to B cells as it is sequestered through its binding to soluble BCMA, resulting in loss of polyclonal antibody production, similar to what was observed with the TACI.Fc treatment in the clinical trial.

High levels of soluble BCMA are found in the blood of multiple myeloma patients especially those with more advanced and PD (17, 29). Thus, it is likely that antibodies directed against BCMA that are currently in clinical development may primarily bind the circulating form of this molecule, which may severely limit their efficacy for treating multiple myeloma patients.

Several groups have investigated the basis for the immune deficiency in multiple myeloma patients. Studies have shown that host suppressor cells decreased the capacity of B lymphocytes to secrete Ig (1, 4). Negative feedback from M-proteins was also proposed as a mechanism, but was dismissed because hypogammaglobulinemia occurs in the absence of circulating M-component in non-secretory multiple myeloma (38). Our results are in agreement with observations from two groups (39, 40), who postulated that myeloma cells secreted specific inhibitors, which block normal B cells in response to antigenic challenge. Normally, the ligand BAFF binds membrane-bound BCMA and other receptors on the surface of B cells, stimulating B-cell growth with production of antibodies (25, 41–43). Our findings suggest that the immune deficiency manifest as a lack of polyclonal antibody production in multiple myeloma patients is due, at least in part, to the presence of circulating BCMA from tumor cells. This solubilized receptor sequesters BAFF, preventing this ligand from binding to its membrane B-cell receptors such as BCMA and BAFF-R, and blocking late B-cell development and antibody production. We have previously reported that BCMA is in the plasma of both secretory and non-secretory multiple myeloma xenografts and in the serum of patients with secretory and non-secretory multiple myeloma (17). Soluble BCMA is, therefore, capable of binding B-cell ligands irrespective of the presence or absence of serum M-protein. We have shown the presence of BCMA–BAFF complexes in plasma from mice containing human multiple myeloma xenografts and serum from multiple myeloma patients. We have shown that this results in reduced free BAFF levels in the plasma of SCID mice bearing human multiple myeloma xenografts and serum from patients with active multiple myeloma. In addition, both recombinant hBCMA and serum from multiple myeloma patients with high levels of serum BCMA prevent BAFF from binding to human B-cell lines (Raji and TB94 cells), and anti-BCMA antibody restores BAFF binding to these cells. Soluble BCMA administration

to immune-competent mice significantly reduces normal antibody levels, and we have shown an inverse relationship between serum BCMA levels and polyclonal antibody levels in multiple myeloma patients. Our current findings may explain why therapeutic agents that have been developed to inhibit the function of BAFF, have yielded disappointing results in the clinic while also resulting in marked reduction in polyclonal antibody levels (37).

This is the first study identifying a specific solubilized protein, BCMA, from multiple myeloma cells that directly contributes to the humoral immune-deficient state that so often occurs in multiple myeloma patients. These experiments help explain one of the important mysteries and hallmarks of multiple myeloma, the presence of hypogammaglobulinemia. Results from these studies indicate that plasma cell-induced immunosuppression in multiple myeloma patients is mediated, at least in part, through BCMA-binding BAFF, providing the rationale and basis for additional experiments evaluating this decoy soluble receptor in normal B-cell homeostasis, autoimmune diseases, transplantation, and other related B-cell malignancies known to express and solubilize this receptor. Higher BCMA expression on tumor cells was recently associated with a shorter progression-free survival in chronic lymphocytic leukemia (CLL) patients (44). Moreover, circulating levels of this protein have been recently found to be markedly elevated in Waldenstrom's macroglobulinemia and CLL patients, and higher levels were associated with worsening disease and shortened OS (45, 46). These soluble receptors may both prevent the efficacy of anti-BCMA antibody-based therapies and contribute to the humoral immune deficiencies that also similarly characterize these B-cell tumors. Identification of the mechanism (s) leading to the presence of circulating BCMA may lead to treatments that could prevent this event. This should both improve the efficacy of therapies currently in development to target BCMA and reverse the immune deficiency that is a hallmark of patients with these lymphoid malignancies.

#### Disclosure of Potential Conflicts of Interest

E. Sanchez, M. Li, H. Chen, and J.R. Berenson hold ownership interest (including patents) in OncoTracker, Inc. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

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**Writing and editing of the manuscript:** E. Sanchez, J.R. Berenson

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**Study supervision:** E. Sanchez, J.R. Berenson

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