

# Serum B-cell maturation antigen is elevated in multiple myeloma and correlates with disease status and survival

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Tumour necrosis factor receptor superfamily, member 17 (TNFRSF17, also designated as B-cell maturation antigen (BCMA) or CD269) is a receptor that was first identified in a T-cell tumour line (Laabi *et al*, 1992) and subsequently shown to be expressed in B lymphocytes as they mature (Laabi *et al*, 1994). Its ligands are BAFF (B cell-activating factor; TNFSF13B) and APRIL (a proliferation-inducing ligand; TNFSF13) (Rennert *et al*, 2000; Thompson *et al*, 2000). In multiple myeloma (MM) cell lines, these ligands activate cell proliferation pathways and upregulate antiapoptotic proteins (Moreaux *et al*, 2004). Both ligands also bind the receptor TACI (transmembrane activator and CAML interactor; TNFRSF13B) (Gross *et al*, 2000; Wu *et al*, 2000; Yu *et al*, 2000). Additionally, BAFF binds to a third receptor, called

## Summary

Although TNFRSF17 (also designated as B-cell maturation antigen (BCMA)) is expressed on tumour cells in B-cell malignancies, it has not been found in serum. The present study found that BCMA concentrations were higher in the supernatants of cultured bone marrow mononuclear cells from multiple myeloma (MM) patients than in healthy subjects. Serum BCMA levels were measured in samples from MM patients ( $n = 209$ ), monoclonal gammopathy of undetermined significance (MGUS) individuals ( $n = 23$ ) and age-matched controls ( $n = 40$ ). BCMA was detected in the serum of untreated MM patients ( $n = 50$ ) and levels were higher than in MGUS patients ( $P = 0.0157$ ) and healthy subjects ( $P < 0.0001$ ). Serum BCMA levels were higher among patients with progressive disease ( $n = 80$ ) compared to those with responsive disease ( $n = 79$ ;  $P = 0.0038$ ). Among all MM patients, overall survival was shorter among patients whose serum BCMA levels were above the median ( $P = 0.001$ ). We also demonstrated that sera from mice with human MM xenografts contained human BCMA, and levels correlated with the change in tumour volume in response to melphalan or cyclophosphamide with bortezomib. These results suggest that serum BCMA levels may be a new biomarker for monitoring disease status and overall survival of MM patients.

**Keywords:** multiple myeloma, B-cell maturation antigen, serum, xenografts, *in vivo*.

BAFF-receptor (BAFFR; TNFRSF13C), whereas APRIL does not (Thompson *et al*, 2001; Day *et al*, 2005). The ligands BAFF and APRIL are members of the tumour necrosis family (TNF) and binding of TNF members to their receptors can lead to apoptosis, differentiation or proliferation (Smith *et al*, 1994). TNF family members act through autocrine, paracrine and endocrine mechanisms (Smith *et al*, 1994).

Transgenic mice that overexpress BAFF or are TACI-deficient display symptoms of systemic lupus erythematosus (SLE) and show B cell hyperplasia and increased levels of serum immunoglobulin (Ig) (McKay *et al*, 1999; Yan *et al*, 2001; Groom *et al*, 2002; Seshasayee *et al*, 2003). APRIL-deficient mice do not show B- or T-cell abnormalities (Varfolomeev *et al*, 2004). BCMA-deficient mice have normal

peripheral B lymphocyte development, and their immune responses remain intact (Xu & Lam, 2001). However, others have shown that bone marrow (BM) plasma cells were functionally impaired in BCMA-deficient mice (O'Connor *et al*, 2004). These reports demonstrate the critical roles that these proteins play in B-cell homeostasis.

Serum BAFF levels are elevated in patients with autoimmune diseases and lymphoma (Cheema *et al*, 2001; Zhang *et al*, 2001; Oki *et al*, 2005). BCMA has been shown to be located intracellularly in plasma cell lines (Laabi *et al*, 1992, 1994). Surface expression of BCMA was found on human tonsillar B-cells (Thompson *et al*, 2000), and on human CD138-expressing MM cells (Novak *et al*, 2004). Malignant cells from Hodgkin lymphoma and Waldenstrom macroglobulinemia (WM) patients also express this protein (Elsawa *et al*, 2006; Chiu *et al*, 2007). However, serum levels of BCMA have not been previously reported in any disease.

Although the response to treatment of MM patients is traditionally followed with measurement of their monoclonal Ig levels, some patients do not produce this marker, and in others it is lost during the course of their disease. Although other serum markers, including albumin and beta-2 microglobulin ( $\beta$ 2M), are prognostic markers, their reliability regarding determining the response to therapy has not been shown and  $\beta$ 2M levels are also affected by renal function.

B-cell maturation antigen concentrations were measured in supernatants from cultures of BM and peripheral blood (PB) mononuclear cells (MCs) and sera from patients with MM, monoclonal gammopathy of undetermined significance (MGUS) individuals and healthy subjects. We also determined the relationship between serum levels and disease status and overall survival (OS) among MM patients, and during the disease course of individual patients. The sera of severe combined immunodeficiency (SCID) mice bearing human MM xenografts were measured for BCMA to determine whether levels changed during anti-MM treatment.

## Materials and methods

### *Serum collection and PB and BMMC cultures*

PB and BM aspirates were obtained from patients with MM, MGUS and age and gender-matched healthy control subjects. The study was approved by the Institutional Review Board (Western IRB BIO 001) and informed consent was obtained in accordance with the Declaration of Helsinki.

Patients were defined as having MGUS, MM patients as indolent MM or symptomatic disease, and treated patients as showing progressive or responsive disease [partial response (PR), very good (VG) PR, or complete response (CR)] according to the International Myeloma Working Group (IMWG) criteria (The International Myeloma Working Group, 2003; Rajkumar *et al*, 2011). Kaplan–Meier survival

of MM patients was determined from the time of initial serum BCMA measurement to death or the date of last follow-up. Individual patients with multiple BCMA samples were analysed from the time of their first assessment.

Serum from a Corvac™ serum separator tube (Becton Dickinson, Franklin Lakes, NJ, USA) was isolated by centrifugation and stored at  $-80^{\circ}\text{C}$ . PB and BM aspirates were collected in heparinized tubes and MCs were isolated using density-gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in RPMI 1640 medium (Omega Scientific, Tarzana, CA, USA) supplemented with 10% foetal bovine serum, non-essential amino acids, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 25 mmol/l HEPES, 200 units/ml penicillin, and streptomycin at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ .

### *Enzyme-linked immunosorbent assay for determination of BCMA concentrations in serum and supernatant from BMMC cultures*

Serum and supernatant samples were analysed by BCMA enzyme-linked immunosorbent assay (ELISA) obtained from R&D Systems, Minneapolis, MN, USA (catalogue # DY193E). Serum samples were diluted 1:50 and the BCMA ELISA assay carried out according to the manufacturer's protocol. The ELISA plates were analysed using a  $\mu$ Quant (Biotek Industries, Winooski, VT, USA) plate reader set to 450 nm with KC Junior software. Values represent the mean of triplicate samples on each specimen. Notably, this BCMA ELISA kit does not cross react with recombinant human APRIL or BAFF, recombinant human TACI/Fc or recombinant mouse BCMA/Fc or mouse BCMA.

### *Polyclonal anti-BCMA antibody (Ab) blocking experiment*

B-cell maturation antigen standards were incubated with another polyclonal goat anti-human BCMA Ab (catalogue # AF193; R&D Systems) or control Ab at a high (400 ng/ml) or low (40 ng/ml) concentration overnight at  $4^{\circ}\text{C}$ . Polyclonal goat IgG Ab was used as an isotype control (catalogue # AB-108-C; R&D Systems). We also tested the ability of this polyclonal anti-BCMA Ab to block detection of BCMA from the serum of MM Patient 1056 following an overnight incubation and BCMA levels were assessed using the BCMA ELISA protocol described above.

### *Detection of BCMA with a monoclonal anti-BCMA Ab*

B-cell maturation antigen standards or serum (diluted 1:50) from MM patients were incubated using a murine monoclonal anti-human BCMA Ab (catalogue # WH0000608M1; Sigma-Aldrich), instead of the polyclonal 'capture Ab' used in the BCMA ELISA. The samples were then assayed according to the BCMA ELISA protocol.

### MM xenograft studies

Six-week old CB17 SCID mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Animal studies were conducted according to protocols approved by the Institutional Animal Care and Use Committee. To establish the CD38 and CD138-expressing LAGκ-2 tumour, a BM biopsy from a MM patient showing IgGκ paraprotein was implanted into the hind limb of a SCID mouse (Campbell & Berenson, 2008). Sera from mice containing the xenograft did not show human IgG or free κ light chains; and, thus, we defined this xenograft as non-secretory. However, κ chains were observed in the cytosol of tumour cells using immunohistochemical (IHC) staining. The LAGκ-1A tumour was developed from a patient with an IgGκ-producing MM resistant to lenalidomide (Campbell & Berenson, 2008). The LAGλ-1 tumour was developed from a MM patient who showed IgGλ paraprotein (Campbell & Berenson, 2008). The xenografts were excised, sectioned into 20–40 mm<sup>3</sup> pieces, and implanted into the muscle. Seven days post-tumour implantation, mice were randomised into treatment groups. Animals were euthanized when the tumours reached 2.5 cm in diameter.

The proteasome inhibitor (PI) bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) was used as a 1 mg/ml stock solution and diluted using 0.9% sodium chloride (NaCl). Bortezomib was administered i.v. at 0.75 mg/kg twice weekly. Cyclophosphamide (Florida Infusion, Palm Harbor, FL, USA) was dissolved from a stock solution of 20 mg/ml with NaCl and administered at 10 mg/kg via oral gavage once weekly. Melphalan (Sigma-Aldrich) at 3 mg was dissolved in 100 μl Acid-EtOH (47 μl concentrated HCl and 1 ml 100% EtOH) and diluted to 1 ml with phosphate-buffered saline (PBS) to generate a 3 mg/ml stock solution. The drug was administered via intraperitoneal (i.p.) injection twice weekly at a dose of 3 mg/kg.

Tumours were measured using standard callipers and the formula for an ellipsoid volume was applied ( $4/3 \pi \times [\text{width}/2]^2 \times [\text{length}/2]$ ). Tumour growth and IgG curves were analysed in terms of treatment group means and standard error.

Mice were bled weekly via retro-orbital sinus to determine human IgG and BCMA levels. Samples were spun at 10 000 rpm for 5 min and serum was collected. The human IgG ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) was used according to the manufacturer's specifications. Absorbance at 450 nm with a reference wavelength of 550 nm was determined on a μQuant microplate spectrophotometer with KC Junior software (Bio-Tek Instruments, Winooski, VT, USA). The human BCMA ELISA kit (R&D Systems) was used to determine serum protein levels.

### Immunohistochemical analysis

B-cell maturation antigen protein expression was determined in MM and normal BMMCs and in our human MM xenografts. For the xenografts, 5 μm sections were cut after fixa-

tion in 4% paraformaldehyde. For the BMMCs, the cells were fixed with 1% paraformaldehyde and  $1 \times 10^5$  cells/slide were cytopsun. The slides were blocked with 0.05% Tween-20 PBS (PBST) and 3% bovine serum albumin (BSA) for 1 h at room temperature (RT). The samples were exposed to the anti-human BCMA Ab (5 μg/ml) at 4°C overnight. The slides were washed three times with TBST and treated with horseradish peroxidase conjugated with either anti-mouse, anti-rabbit or anti-goat antibodies (KPL, Gaithersburg, MD, USA) diluted 1:500 in TBST at RT for 2 h. The slides were washed three times in TBST and placed in 3-amino-9-ethylcarbazole (AEC) buffer for 5 min, and colour was detected using an AEC kit (Vector Laboratories, Burlingame, CA, USA). For light chain staining, BMMCs were resuspended in 100 μl PBS and cytopsun on slides. The samples were blocked with 3% BSA before the Ab was added to prevent non-specific binding. Goat anti-human λ light chain Ab (Sigma-Aldrich), anti-human κ light chain Ab (Sigma-Aldrich) or isotype control Ab (R&D System) was added to the corresponding samples. These antibodies were incubated overnight at 4°C. On the following day, the antibodies were washed with 0.05 mol/l TBST buffer. The samples were then treated with 10% H<sub>2</sub>O<sub>2</sub> methanol before the secondary Ab. The samples were then incubated with peroxidase-labelled rabbit anti-goat Ab (KPL) for 2 h at RT and then washed. Peroxidase substrate (Vector Laboratories) was added to the samples for 30 min. The cells were stained with haematoxylin for 1 min, and the samples were mounted. BCMA and λ and κ light chain expression was determined using a light microscope (Olympus BX51; Olympus, San Diego, CA, USA). Haematoxylin and eosin (H&E) staining was performed on BMMCs using standard staining procedures.

### Statistical analyses

Statistical significance of differences observed in supernatant, serum and xenograft studies was determined using a Student's *t*-test. The minimal level of significance was  $P < 0.05$ . Statistical analysis was determined using GRAPHPAD PRISM version 4.03 for Windows (GraphPad Software, San Diego, CA, USA).

## Results

### *BMMCs from MM patients showed BCMA and culture medium from these cells showed high concentrations of this protein*

First, we examined membrane-bound BCMA expression on BMMCs from MM patients and healthy subjects ( $1 \times 10^4$  cells) using flow cytometric analysis. The protein was detected on a very small proportion of BMMCs from healthy subjects whereas most BMMCs from MM patients showed strong BCMA staining (Fig S1). The expression of BCMA was also marked in PBMCs from a patient with plasma cell

leukaemia (Fig S1). As has previously been demonstrated (Novak *et al*, 2004), we also confirmed the presence of BCMA in CD138-expressing and light chain-restricted BMMCs from MM patients (data not shown). Next, to determine whether MM cells from BM are capable of releasing BCMA, BMMCs ( $1.6 \times 10^6$  cells/well) from MM patients with active disease ( $n = 12$  samples) or indolent disease ( $n = 2$ ), two MGUS individuals, and five healthy subjects were cultured for 72 h. Supernatants from MM patients showed high BCMA concentrations, whereas those from healthy subjects showed negligible amounts (Fig 1A). These levels were markedly higher in MM patients with active disease than among those with indolent MM or MGUS (Fig 1A). BCMA was not detectable in the supernatants from PBMCs from MM patients, except for a very high concentration (1589 pg/ml) in a patient with plasma cell leukaemia who lost the presence of measurable monoclonal Ig (data not shown). These findings demonstrate that high amounts of BCMA are released from MM-containing PB or BM MCs.

#### *Supernatant BCMA levels correlated with the percentage of MM plasma cells*

We correlated the concentrations of BCMA in the culture medium with the percentage of MM plasma cells in the BMMCs. To accomplish this, we performed both H&E and  $\kappa$  and  $\lambda$  light chain staining to identify malignant plasma cells. Total numbers of nucleated cells and plasma cells were then counted and the proportions of malignant cells were calculated. Patients with a high percentage of malignant cells, as determined by the proportion of cells showing light chain staining, demonstrated high concentrations of BCMA in their culture medium whereas those with few light-chain restricted cells had low BCMA levels in their culture medium (Fig 1B).

#### *Serum BCMA levels were elevated in MM patients*

We measured BCMA in sera from patients with newly diagnosed MM ( $n = 50$ ), MGUS individuals ( $n = 23$ ) and healthy control subjects ( $n = 40$ ) that were age- and gender-matched. The MM patients included those with IgG $\kappa$  ( $n = 24$ ), IgG $\lambda$  ( $n = 9$ ), IgA $\kappa$  ( $n = 6$ ), IgA $\lambda$  ( $n = 3$ ), IgM $\kappa$  ( $n = 1$ ),  $\kappa$  light chain only ( $n = 4$ ), and  $\lambda$  light chain only ( $n = 3$ ) disease. Using the International Staging System (Greipp *et al*, 2005); 30, 12 and seven patients were stages 1, 2 and 3 respectively, and one was unknown. The serum levels of BCMA in MM patients were elevated when compared to healthy controls ( $P < 0.0001$ ) and MGUS individuals ( $P = 0.0157$ ; Fig 2A). Specifically, the median serum BCMA concentrations in MM, MGUS and controls were 13.87, 5.30 and 2.57 ng/ml, respectively. Indolent MM patients ( $n = 16$ ) had lower levels (median 11.60 ng/ml) than among those with active MM ( $n = 34$ , median 17.79 ng/ml). Interestingly, the one MGUS patient who eventually progressed to MM had the highest serum BCMA level (12.62 ng/ml) of MGUS

patients, which had more than doubled to 25.68 ng/ml at the time she developed MM. Notably, BCMA levels did not correlate with serum creatinine levels (data not shown). Cross-reactivity of the ELISA with a high concentration of human IgG (1000 ng/ml) was found to be minimal, with a BCMA readout of only 0.015 ng/ml (data not shown).

#### *Serum BCMA levels in MM patients correlated with supernatant from cultured MM BMMCs, disease status and overall survival*

B-cell maturation antigen levels from serum and supernatants, of cultured BMMCs, of MM patients ( $n = 14$  samples) showed a strong correlation ( $r = 0.82$ ; Fig S2).

Next, we determined if serum BCMA levels correlated with the MM patient's response to therapy. Responsive ( $\geq$  PR) patients ( $n = 80$  samples) had lower serum BCMA levels (median 4.06 ng/ml) than individuals ( $n = 79$  samples) showing progressive disease (median 19.76 ng/ml;  $P = 0.0038$ ; Fig 2B). In addition, patients that achieved CR ( $n = 26$ ) had lower BCMA levels (median, 2.09 ng/ml) than patients with VGPR ( $n = 16$ , median 3.33 ng/ml) or PR ( $n = 38$ , median 5.44 ng/ml). Notably, BCMA levels did not correlate with serum creatinine levels (data not shown).

Changes in serum BCMA levels were found to correlate with changes in an individual patient's clinical status in response to anti-MM treatment. Except in one case, those patients responding to treatment showed decreases in these levels whereas those with disease progression showed increases in BCMA levels (Fig S3). Regardless of the patient's clinical status, serum BCMA levels did not correlate with the use of specific anti-MM agents that were being administered (Table S1).

With a median follow-up of 11 months (range, <1–83 months), MM patients ( $n = 162$ ) with BCMA levels above the median (10.85 ng/ml) showed a shortened survival compared to those with amounts below the median concentration ( $P = 0.0014$ ; Fig 2C). In addition, patients in the highest quartile ( $\geq 24.56$  ng/ml) showed a markedly shortened survival compared to the remainder of the patients ( $P = 0.0003$ ; Fig S4).

#### *Another polyclonal anti-BCMA Ab blocked detection of BCMA in standards and serum from MM patients*

Sera from MM patients was incubated overnight at 4°C with a different polyclonal anti-BCMA Ab (catalogue # AF193; R&D Systems) and the same ELISA was performed. This anti-BCMA Ab (400 ng/ml) blocked the detection of BCMA in the standards whereas an isotype-matched polyclonal goat control Ab at the same high concentration did not have any impact on detection of BCMA (Fig 3A, left panel). We then used a 10-fold lower concentration of this blocking polyclonal anti-BCMA Ab (40 ng/ml) and BCMA became detectable but only when high concentrations of the standards

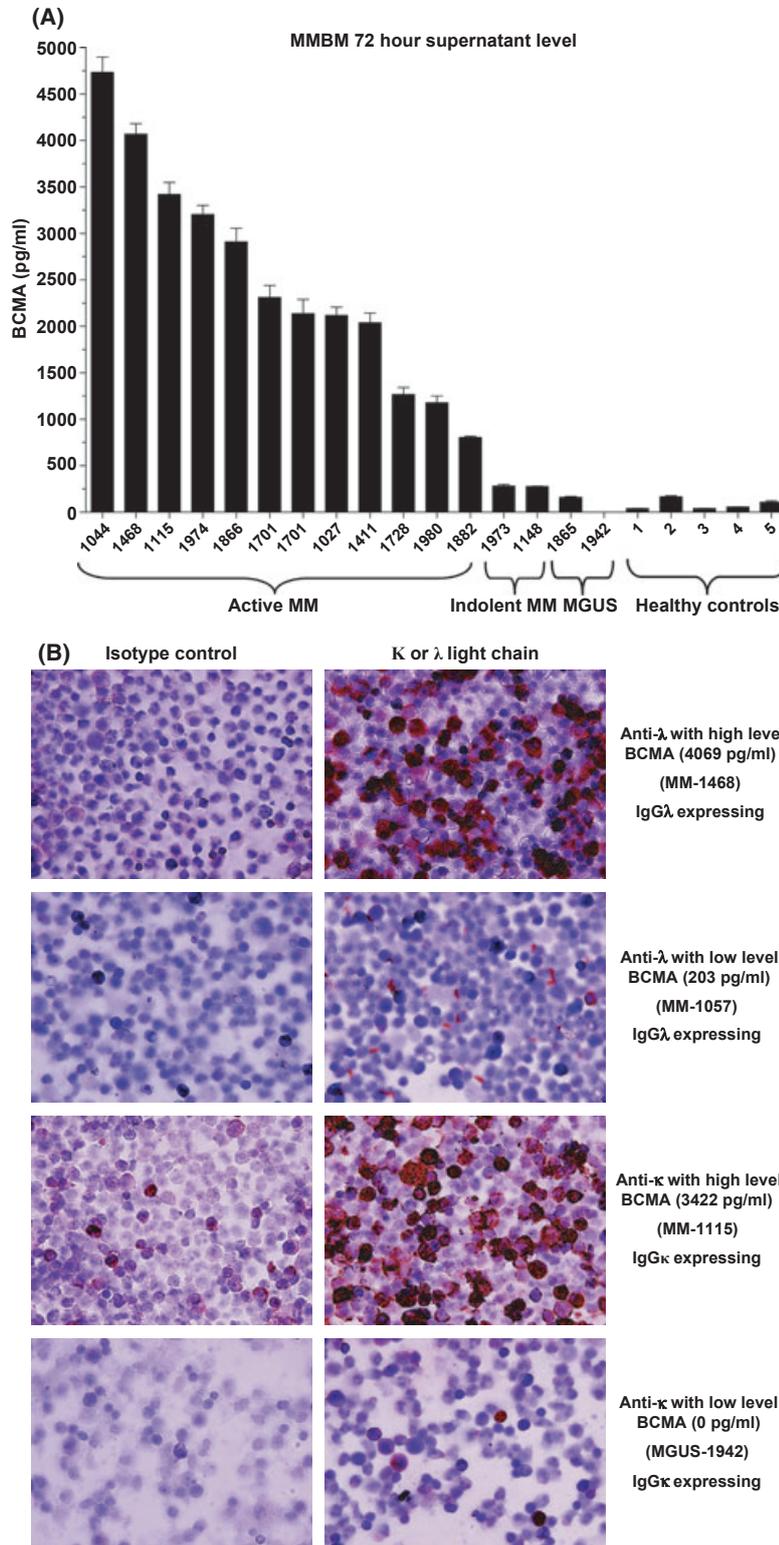
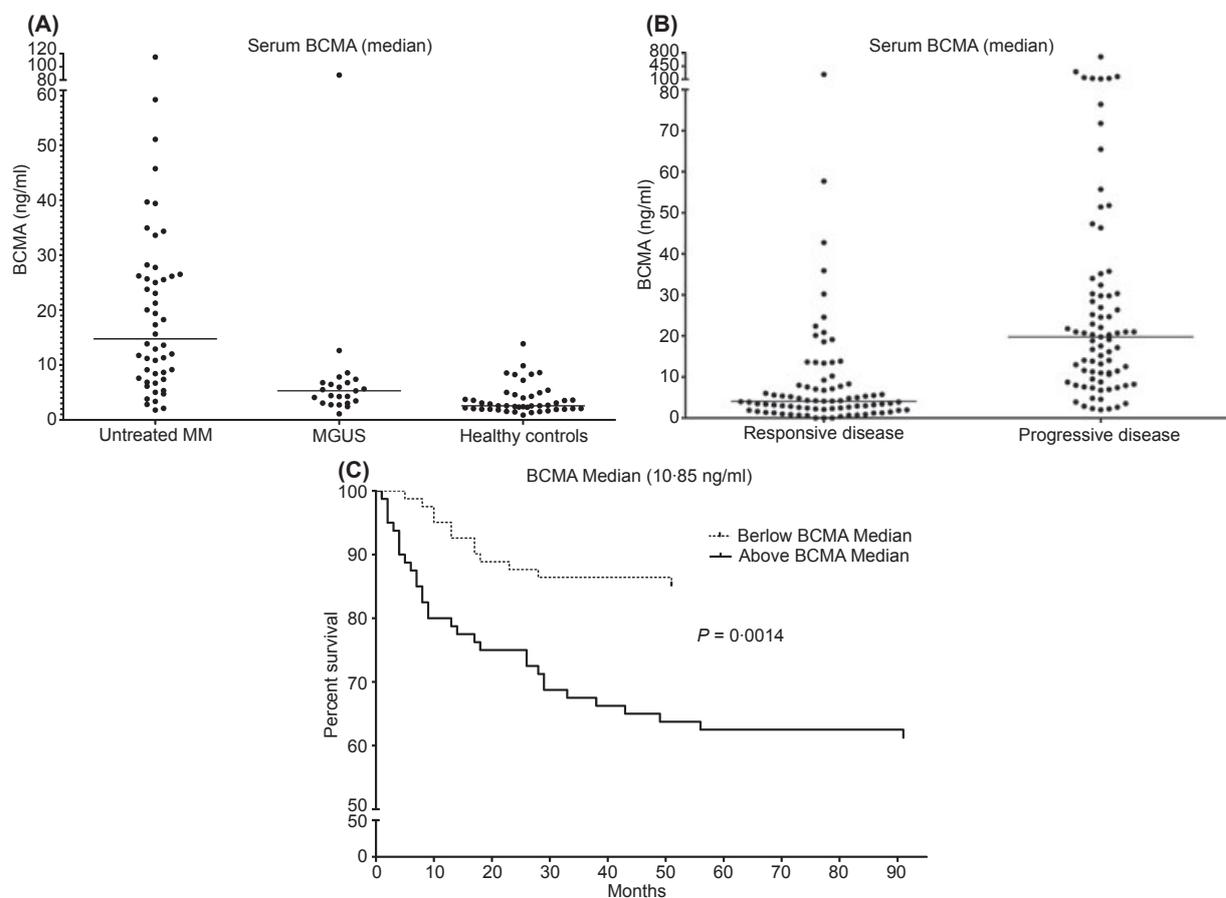


Fig 1. BCMA is found in supernatants from cultured BMMCs from MM patients. (A) Bone marrow mononuclear cells were cultured for 72 h, supernatants collected and a BCMA ELISA performed. Patients with active MM had markedly higher BCMA levels than those with indolent multiple myeloma (MM) or monoclonal gammopathy of undetermined significance (MGUS) or healthy controls (one patient was studied from bone marrow samples obtained following disease progression from two different regimens). (B) Patients with a high percentage of malignant cells, determined by light chain staining, demonstrated high concentrations of BCMA in their culture medium whereas those with few light-chain restricted cells had low BCMA levels in their culture medium (original magnification  $\times 100$ ). Data graphed are the mean  $\pm$  standard error of the mean using three replicates.



**Fig 2.** Soluble BCMA is found in the serum of MM patients and correlates with disease status and overall survival. (A) Untreated multiple myeloma (MM) patients had significantly higher serum BCMA levels compared to monoclonal gammopathy of undetermined significance (MGUS) patients and normal controls ( $P = 0.0157$ ,  $P < 0.0001$ , respectively). (B) Serum BCMA levels from responding patients were lower compared to patients with progressive disease ( $P = 0.0038$ ). (C) Kaplan–Meier survival of MM patients with BCMA levels above the median (10.85 ng/ml) showed shortened survival compared to those with amounts below the median. Individual patients with multiple BCMA levels were analysed from the time of their first assessment. Data graphed are of samples run in triplicates and are presented as medians.

were present (Fig 3A, right panel). Similarly, BCMA was not detected in diluted and undiluted MM serum in the presence of a high concentration (100 ng/ml) of the blocking anti-BCMA Ab (Fig 3B) whereas BCMA levels were high in undiluted MM serum and decreased as the sample alone was diluted (Fig 3B). We then used a lower concentration of this blocking polyclonal anti-BCMA Ab (10 ng/ml) and BCMA became detectable but at lower levels than in serum lacking the blocking Ab (data not shown). Thus, the exogenously added anti-BCMA Ab blocks the detection of BCMA identified with the standard anti-BCMA Ab used in the ELISA, indicating the specificity of this assay for detecting BCMA.

#### *Monoclonal anti-BCMA Ab showed similar serum BCMA levels as detected with the polyclonal Ab-based ELISA*

Plates were coated with a monoclonal anti-BCMA Ab (Sigma-Aldrich) as a capture Ab. BCMA standard antigens

from the R&D Systems kit or patient serum was incubated with monoclonal Ab (mAb)-coated plates and then the standard protocol described in the R&D Systems kit was used. BCMA was detectable with the mAb and the pattern of levels matched results obtained with the polyclonal Ab, when assessing the BCMA standards (Fig 3C) and serum samples from MM and MGUS patients (Fig 3D).

#### *SCID mice containing human MM xenografts contained human BCMA in their serum and these levels correlated with tumour volumes and response to anti-MM therapy*

As it is possible that BCMA detected in human MM serum and supernatants from cultured MM BMMCs may be derived from non-malignant cells, we evaluated human BCMA levels in our human MM xenografts. MM tumours (LAG $\kappa$ -2, LAG $\lambda$ -1 and LAG $\kappa$ -1A) were analysed for human BCMA expression using IHC. The protein was detected in the three xenografts with the anti-BCMA Ab, which does not

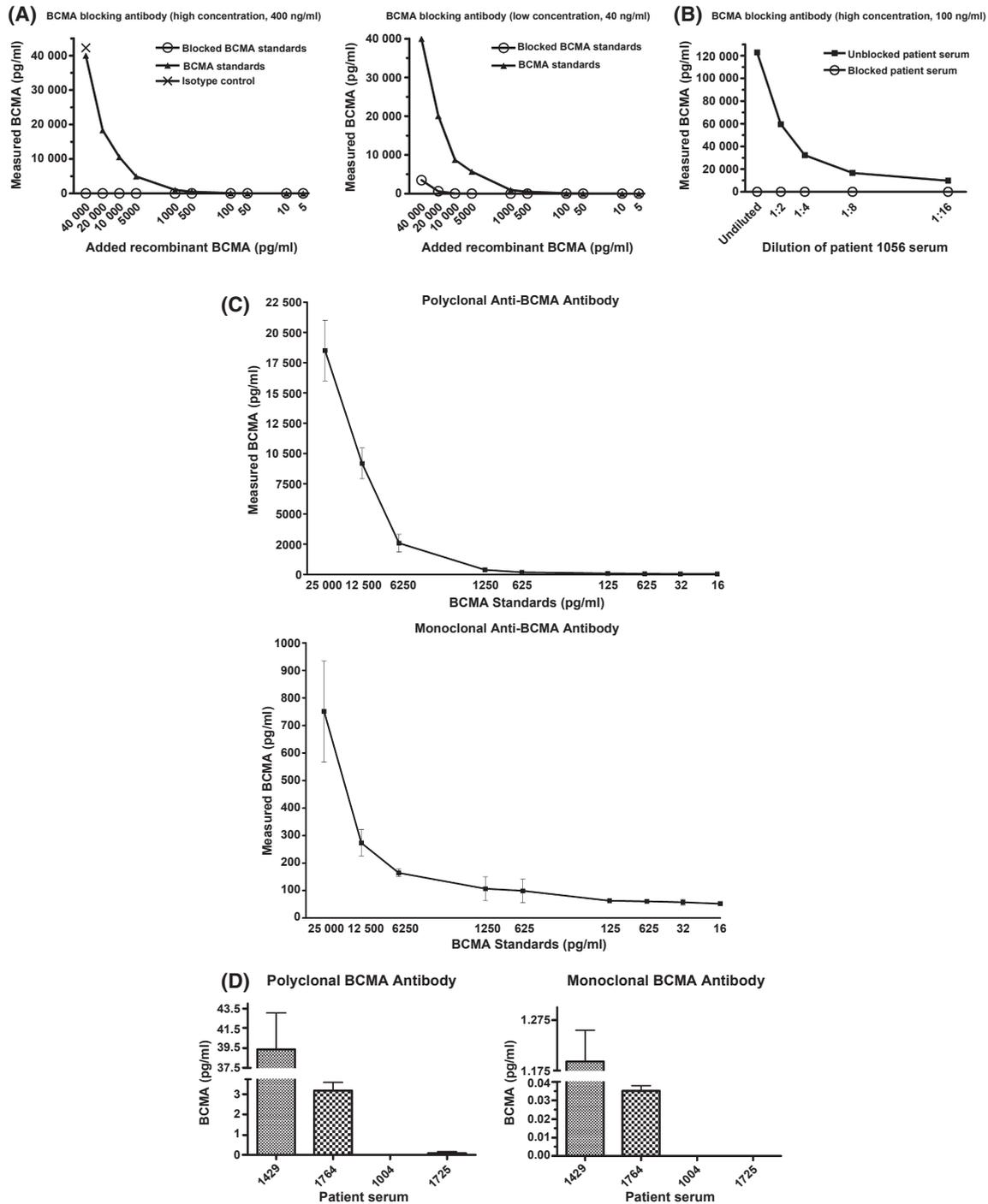


Fig 3. Polyclonal blocking and monoclonal anti-BCMA antibodies confirm the presence of BCMA in MM serum. (A) BCMA standards were undetectable when incubated with a polyclonal anti-BCMA Ab (left panel). An isotype-matched control goat Ab (at the same high concentration) or BCMA standards incubated without the blocking polyclonal anti-BCMA Ab, did not have any impact on detection of BCMA (left panel). When the blocking polyclonal anti-BCMA Ab was used at a 10-fold lower concentration, only the high-concentration BCMA standards were detectable (right panel). BCMA was detected when BCMA standards were not incubated with the polyclonal anti-BCMA Ab (right panel). (B) Polyclonal anti-BCMA Ab (100 ng/ml) blocked BCMA from undiluted and diluted (up to 1:16) serum of MM Patient 1056. In contrast, serum from the same patient in the absence of the blocking antibody detected BCMA, which decreased with serial dilution. (C) A monoclonal anti-BCMA Ab detected the BCMA standards (bottom panel), and the pattern of levels was similar to those obtained when using the polyclonal anti-BCMA Ab (top panel). (D) The pattern of BCMA levels from MM patients who were untreated (1429), in VGPR (1764) or CR (1004) or an MGUS individual (1725) using the polyclonal Ab (left panel) showed similar patterns of the results obtained with the monoclonal Ab (right panel).

bind to mouse BCMA (Fig 4). No staining of other murine tissue was observed (data not shown).

To exclude the possibility that the serum BCMA detected was not the result of dead cells, either from natural tumour cell turnover or anti-MM treatment, mice were allowed to grow non-paraprotein producing LAG $\kappa$ -2 tumours to approximately 1000 mm<sup>3</sup> at which time the mice were treated with bortezomib and cyclophosphamide. Mice were bled 8–12 h following this combination treatment ( $n = 7$ ) or untreated controls ( $n = 4$ ). A decrease in both tumour volume ( $P = 0.0006$ ) and serum human BCMA levels ( $P < 0.0001$ ) were observed among drug-treated mice compared to untreated animals (Fig 5A, B); a rise in serum BCMA levels from a possible release of membrane bound protein from dead cells was not observed following drug treatment. Thus, it could be concluded that the BCMA in the sera of the mice were from live plasma cells, and not the result of its release from dying MM cells.

Serum human BCMA levels also correlated with tumour growth, IgG levels and response to treatment in mice bearing another human MM xenograft, LAG $\lambda$ -1. Untreated mice ( $n = 4$ ) had large tumour volumes and high serum IgG and BCMA levels. Mice receiving melphalan (3 mg/kg,  $n = 4$ ) twice weekly via i.p. injection showed smaller tumour volumes, IgG and BCMA levels compared to untreated mice (tumour volumes:  $P < 0.0001$ ; hIgG:  $P = 0.0033$ ; BCMA:  $P = 0.0055$ ; Fig 6A–C). Human BCMA was not detected in

the serum of non-tumour bearing SCID mice (data not shown).

## Discussion

B-cell maturation antigen is expressed on the surface of normal and malignant B-cells (Laabi *et al*, 1992, 1994; Thompson *et al*, 2000; Novak *et al*, 2004). Another surface receptor present on normal and malignant B-cells, the interleukin (IL)-6 receptor, has been shown to be elevated in the serum of MM patients (Jones *et al*, 2001) but only in approximately 15% of patients (Stephens *et al*, 2012). We have now shown that BCMA is present in the serum of MM patients; and, moreover, its levels correlate with the patient's response to therapy and overall survival. We used a blocking anti-BCMA Ab to show the specificity of the ELISA to detect BCMA and a mAb to confirm the presence of BCMA in these samples. Supernatants from cultured BMMCs from MM patients with active disease contain much higher levels in their culture medium than among healthy subjects and those with MGUS or indolent MM. SCID mice bearing human MM xenografts also showed high serum BCMA levels, which decreased in response to anti-MM therapy.

Due to the difficulty in assessing the location of this BM-based malignancy and the heterogeneous involvement of malignant plasma cells within different BM sites, measurement of MM tumour mass is indirect; and, thus, response to

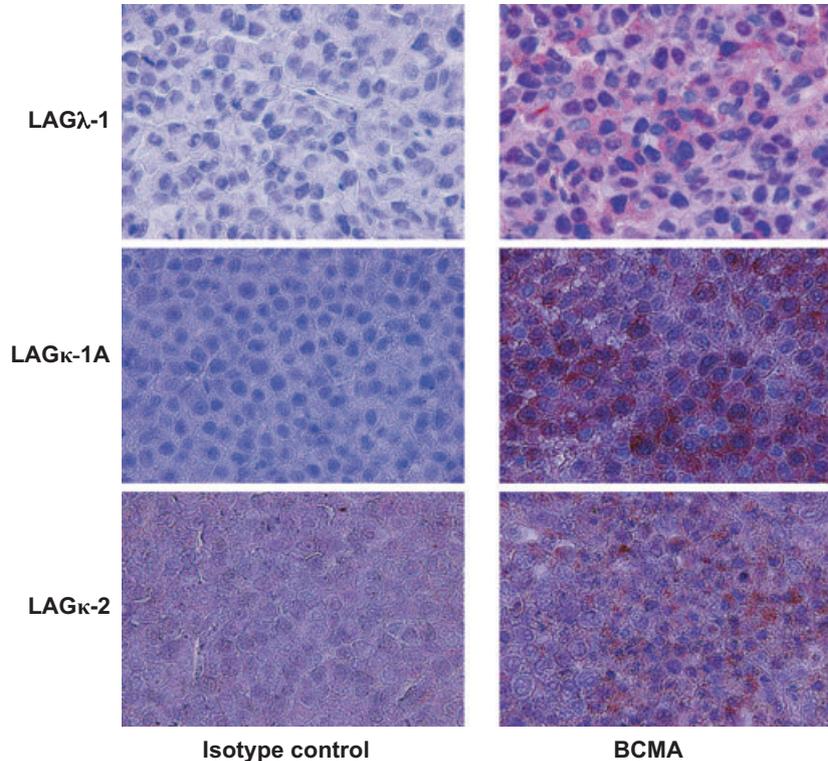


Fig 4. Positive immunohistochemical staining with anti-BCMA antibodies in human MM xenografts removed from CB17 SCID mice. Slides (original magnification  $\times 100$ ) were analysed using the Mircrosuite Biological Suite Program (Olympus BX51).

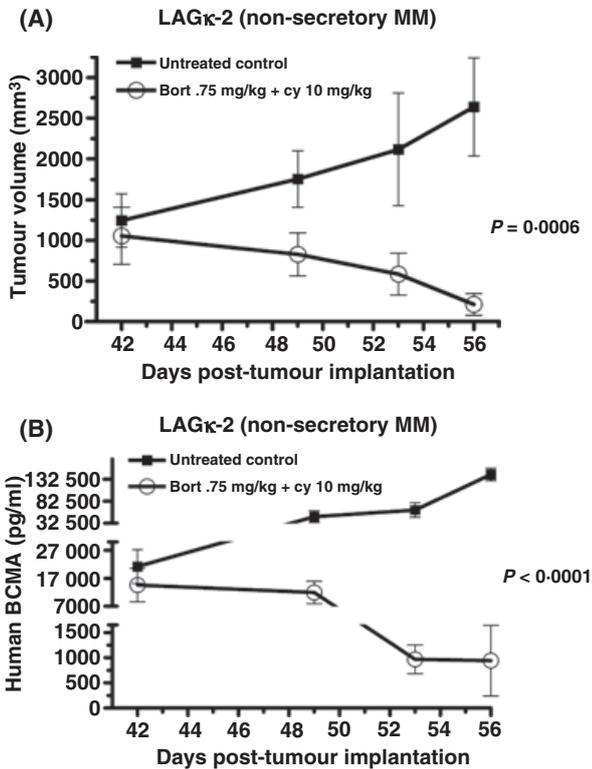


Fig 5. BCMA is produced by live MM cells in tumour-bearing mice. To determine if the observed serum BCMA was the result of membrane-bound BCMA from dead MM cells, mice bearing 1000 mm<sup>3</sup> LAGκ-2 tumours were dosed with bortezomib (bort) at 0.75 mg/kg plus cyclophosphamide (cy) at 10 mg/kg. Twelve hours following treatment, human BCMA levels were determined. (A) Tumour volumes of LAGκ-2-bearing mice were markedly smaller (*P* = 0.0006) when compared to untreated mice. (B) Serum BCMA levels in LAGκ-2-bearing mice were also markedly lower (*P* < 0.0001) when compared to untreated mice.

therapy is often difficult to determine. Besides blood and urine monoclonal Ig levels, other blood markers have included: haemoglobin, urea nitrogen, calcium, albumin, creatinine, monoclonal protein, β2M, IL-6, C-reactive protein, soluble IL-6 receptor, lactate dehydrogenase, thymidine kinase, and α<sub>1</sub>-antitrypsin (Kyle, 1994). However, these markers are not produced directly by MM cells. IL-6 and its soluble receptor have been used to follow human MM growth in mice (Tassone *et al*, 2005). However, assessment of these proteins to follow MM patients has proven less useful, probably due to their widespread presence in many other cell types (Jones *et al*, 2001). Thus, they have not proven to be reliable predictors of response (Kyle, 1994).

Classically, the amounts of monoclonal Ig have been used to assess disease status and response to treatment of individual MM patients. The homogenous synthesis of plasma globulin (or Ig) by malignant plasma cells but not normal host tissues was first demonstrated with mouse plasma cell tumours (Nathans *et al*, 1958). An excellent correlation was observed between total MM cell number and Ig synthesis

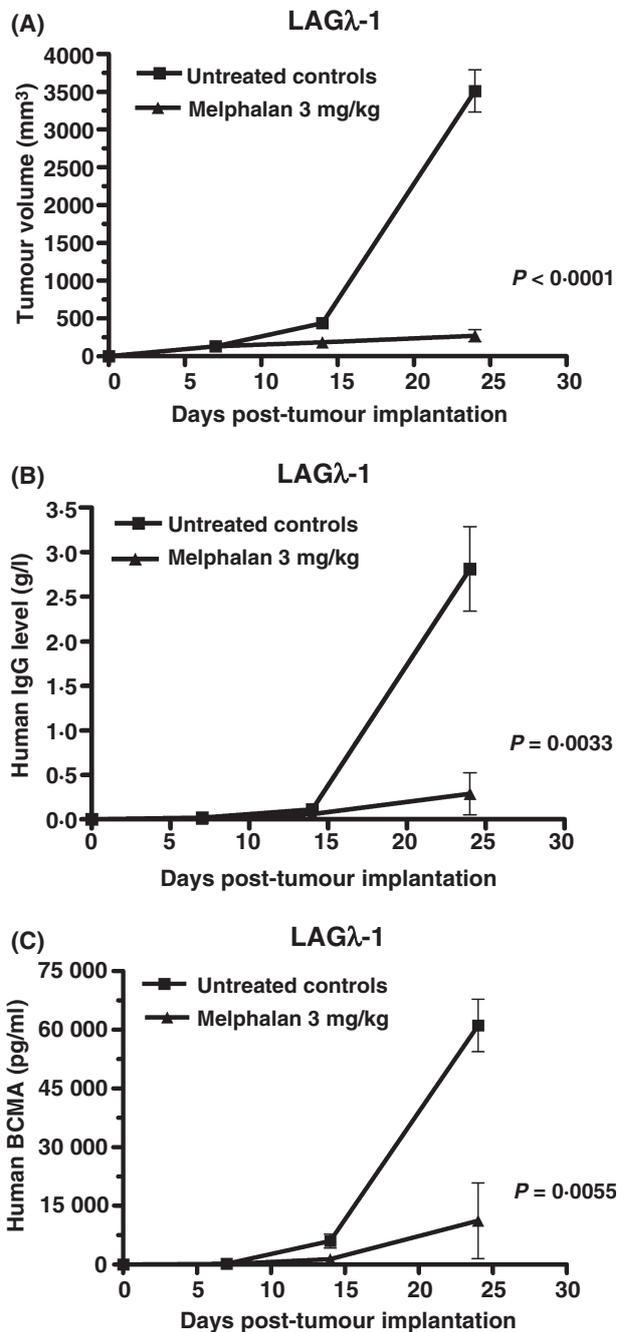


Fig 6. SCID mice bearing LAGλ-1 treated with melphalan show a significant reduction in tumour size and IgG and serum human BCMA levels. Mice bearing this human MM xenograft model, that shows secretory disease of IgGλ type, were dosed with melphalan (3 mg/kg) twice weekly via i.p. injection. (A) Untreated mice (*n* = 4) had large tumour volumes whereas melphalan-treated mice (*n* = 7) had small tumour volumes (*P* < 0.0001). (B) Levels of serum IgG were significantly reduced among drug-treated mice when compared to controls (*P* = 0.0033). (C) Levels of serum BCMA were significantly reduced among drug-treated mice when compared to controls (*P* = 0.0055). Results presented are group means ± standard error of the mean.

rates in both mouse MM models and MM patients (Salmon & Smith, 1970; Sullivan & Salmon, 1972; Ghanta & Hiramoto, 1974). Monoclonal Ig levels are used as a prognostic factor as part of the Durie-Salmon staging system (Durie & Salmon, 1975), which was widely used until recently (Larson *et al*, 1997). Moreover, changes in these protein levels are used to follow the disease course and response to treatment in MM patients. However, assessment of Ig does not always accurately reflect changes in MM tumour growth (Sullivan & Salmon, 1972; Kawano *et al*, 1993; Zaanen *et al*, 1998). Changes in Ig metabolism may lead to a significant underestimation of the body's tumour burden (Sullivan & Salmon, 1972). In our own studies, mice containing human MM xenografts treated with PIs often show marked reductions in serum human paraprotein levels despite the presence of large, growing tumours (Sanchez *et al*, 2010). PIs are more active against MM tumour cells that contain higher Ig concentrations (Meister *et al*, 2007) and so the reduction in Ig levels may overestimate the decrease in tumour burden. Interferon- $\alpha$  has been shown to have inhibitory effects on Ig secretion from plasma cells, so that reductions in monoclonal Ig levels may overestimate the actual reduction in MM tumour burden (Tanaka *et al*, 1989).

We have grown human MM tumours in SCID mice from patients who show serum monoclonal Ig; however, mice containing certain MM tumours eventually do not show circulating human Ig (Shaw, 2006). The immature, proliferative cell population that is responsible for maintaining the MM clone does not appear to express Ig whereas the mature myeloma cells display a low proliferative activity with secretion of large amounts of Ig (Kawano *et al*, 1993). Thus, measurement of changes in Ig levels may fail to assess important parts of the MM clone. Some MM patients do not produce monoclonal Ig; and, thus, following these patients' response to therapy is particularly difficult. Although our study suggests that serum BCMA levels may be a useful way to follow the response of MM patients to therapy and predict overall survival, much larger studies will need to be performed to confirm these initial observations and determine whether this level can assess changes in clinical status and outcome more accurately than assessment of monoclonal Ig. Whether this TNFR family member represents a new prognostic factor for MM and MGUS will need further evaluation with studies involving larger numbers of patients from samples that have been previously stored or prospectively evaluated.

The clinical importance of BCMA in MM has recently been documented (Bellucci *et al*, 2005). Patients who relapsed after allogeneic haematopoietic stem-cell transplantation who received donor lymphocyte infusions and achieved CR developed serum anti-BCMA antibodies (Bellucci *et al*, 2005), which reacted with the membrane-bound BCMA and induced complement-mediated lysis and antibody-dependent cellular cytotoxicity (ADCC) of transfected cells and primary MM cells (Bellucci *et al*, 2005). Another group confirmed the anti-MM effects of anti-BCMA mAbs, which

were shown to have ADCC against BCMA-expressing MM lines (Ryan *et al*, 2007).

Interestingly, the addition of soluble BCMA-Fc, which binds APRIL and BAFF both as free ligands and when membrane-bound, to nude mice bearing human colon or lung carcinoma cell lines, resulted in inhibition of tumour growth (Rennert *et al*, 2000). Normal mice exposed to soluble BCMA-Ig showed a dramatic reduction in B-cell numbers in the blood and peripheral lymphoid organs (Thompson *et al*, 2000). Similarly, splenic B cell reductions occurred in an *in vitro* mouse splenocyte proliferation assay following *in vivo* administration of BCMA-Fc to normal mice (Pelletier *et al*, 2003). Anti-BCMA antibodies are in clinical development (Ryan *et al*, 2007). Whether our new observation, that serum BCMA is found in the blood of MM patients and at higher levels among those with progressive disease, means that these antibodies may be bound by this circulating BCMA and thus limit their anti-MM effects, remains to be determined. This circulating receptor may bind its respective ligands, BAFF and APRIL, and stimulate tumour growth as reported with other circulating ligand-receptor complexes (Jones *et al*, 2001). We are currently evaluating whether this protein is cleaved off the cell surface in its complete or a truncated version, or actively secreted from the cell.

Notably, BCMA has also been shown to be present on the cell surface of the malignant cells from other types of B-cell malignancies, but serum levels have not been reported in these patients (Elsawa *et al*, 2006; Chiu *et al*, 2007; Maia *et al*, 2011). It will also be interesting to determine whether serum BCMA levels are also elevated in auto-immune disorders characterised by mature B-cell dysfunction, such as SLE and rheumatoid arthritis and, if so, whether these levels correlate with disease activity.

Given that BCMA binds BAFF, APRIL and anti-BCMA antibodies, it is possible that serum BCMA bound to these proteins may not be detected with this ELISA and thus, lead to a lower measured level of serum BCMA than is actually present. Further studies will reveal the structure of this circulating form of BCMA and whether this protein will become an important prognostic factor and additional marker with which to follow patients with MM and other B-cell disorders.

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## Conflict of interest

The authors have no competing interests.

## Author contributions

ES wrote the manuscript, designed and oversaw execution of the *in vivo* experiments; ML performed serum BCMA ELI-

SAs; AK collected the clinical data from patients charts and graphed the serum data, survival data and the lack of correlation between treatment regimens and serum BCMA levels; JL stained BMMC from MM patients, performed the percent plasma cells *versus* supernatant BCMA levels; CSW performed the bone marrow cultures and collected the supernatants for the BCMA ELISA; DTK collected the clinical data from patients charts; OY reviewed clinical data; CMN dosed and bled mice, ran and graphed the *in vivo* BCMA ELISAs; MPD performed serum BCMA ELISAs; CPA performed serum BCMA ELISAs; AR, EM, GNW and RAS reviewed clinical charts; BB and RAV reviewed the manuscript; RB collected and provided bone marrow samples and reviewed the manuscript; JC reviewed statistics; HC assisted in writing the manuscript, designed experiments, analysed data; JRB provided PB and BM samples, designed research experiments, analysed data, interpreted data, and assisted in writing the manuscript.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig S1.** Membrane bound BCMA expression on primary MM cells.

**Fig S2.** Serum and supernatant BCMA level correlation.

**Fig S3.** Changes in serum BCMA levels were found to correlate with changes in individual patient's clinical status in response to anti-MM treatment.

**Fig S4.** Kaplan–Meier survival of MM patients with serum BCMA levels in the highest quartile (25%).

**Table SI.** Serum BCMA levels do not correlate with the use of specific anti-MM agents.

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