Cancer Cell

Target Expression, Generation, Preclinical Activity, and Pharmacokinetics of the BCMA-T Cell Bispecific Antibody EM801 for Multiple Myeloma Treatment

Graphical Abstract

Highlights
- BCMA is an excellent target in myeloma, including high-risk/refractory patients
- Our IgG-based BCMA-TCB EM801 shows high single-agent activity
- EM801 shows no significant toxicity and can be administered weekly i.v. or s.c.

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In Brief
Seckinger et al. show that B cell maturation antigen (BCMA) is universally and specifically expressed in normal and malignant plasma cells. They construct a BCMA-CD3 bispecific antibody that efficiently induces myeloma cell death by autologous T cells and depletes BCMA+ cells in cynomolgus monkeys.

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Target Expression, Generation, Preclinical Activity, and Pharmacokinetics of the BCMA-T Cell Bispecific Antibody EM801 for Multiple Myeloma Treatment

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SUMMARY

We identified B cell maturation antigen (BCMA) as a potential therapeutic target in 778 newly diagnosed and relapsed myeloma patients. We constructed an IgG-based BCMA-T cell bispecific antibody (EM801) and showed that it increased CD3⁺ T cell/myeloma cell crosslinking, followed by CD4⁺/CD8⁺ T cell activation, and secretion of interferon-γ, granzyme B, and perforin. This effect is CD4 and CD8 T cell mediated. EM801 induced, at nanomolar concentrations, myeloma cell death by autologous T cells in 34 of 43 bone marrow aspirates, including those from high-risk patients and patients after multiple lines of treatment, tumor regression in six of nine mice in a myeloma xenograft model, and depletion of BCMA⁺ cells in cynomolgus monkeys. Pharmacokinetics and pharmacodynamics indicate weekly intravenous/subcutaneous administration.

INTRODUCTION

Multiple myeloma is a malignant hematological disease characterized by the accumulation of clonal plasma cells in the bone marrow and associated clinical signs and symptoms, especially those related to displacement of normal hematopoiesis and generation of osteolytic bone disease (Kyle and Rajkumar, 2004). With more than 80,000 new diagnoses worldwide per year, myeloma is the second most common hematological malignancy (Becker, 2011). Despite recent approval of multiple agents, e.g., daratumumab, elotuzumab, carfilzomib, and pomalidomide (Lokhorst et al., 2015; Lonial et al., 2015; San Miguel et al., 2013; Stewart et al., 2015), it is rare to eradicate all myeloma cells and the vast majority of patients ultimately become either refractory to all available compounds or can no longer be treated effectively due to the toxicity of previous treatments, and thus succumb to the disease. For this reason, alternative immunotherapeutic options represent an attractive approach.

Significance

Despite recent approval of multiple agents, the majority of myeloma patients ultimately become refractory or can no longer be treated effectively due to the toxicity of previous therapies. Our study raises hope of overcoming this, at least in part, by our immunotherapeutic BCMA-T cell bispecific antibody approach: EM801 shows promising single-agent activity, being active also in high-risk patients and non-cross-resistant with previous lines of treatment. Together with the spectrum of side effects validating in vitro and in vivo target specificity this offers the opportunity for use in combination regimen and, together with a weekly administration schedule, for prolonged treatment. EM801 emerges therefore as attractive compound for multiple myeloma treatment, and our study gives the rationale for clinical testing.
strategy to cover this unmet medical need. One of the most advanced options is the recruitment of innate immunity to kill myeloma cells by immunoglobulin G (IgG) monoclonal antibodies targeting antigens expressed on normal as well as malignant plasma cells (e.g., anti-CS1 or -CD38) (Deckert et al., 2014; Lokhorst et al., 2015; Lonial et al., 2019). Another approach is to (re)direct T cells toward myeloma cell killing. This can be achieved via chimeric antigen receptor (CAR)- or T cell receptor (TCR)-modified T cells. Both have shown preclinical or early clinical activity, but require ex vivo engineering and expansion of patient-specific T cells (Carpenter et al., 2013; Garfall et al., 2015; Klebanoff et al., 2016; Rapoport et al., 2015; San Miguel et al., 2015). Alternatively, T cell bispecific antibodies (TCBs) that simultaneously bind a surface target on tumor cells and an associated TCR chain (CD3 epsilon chain [CD3ε]) present on all T cells have shown the capacity to induce a potent T cell-mediated killing of tumor cells carrying the target (Batievi et al., 2016; Lameris et al., 2014; Ramadoss et al., 2015; Staerz et al., 1985), and are in principle applicable to all patients.

For our TCB approach, we selected as target the TNF receptor superfamily member B cell maturation antigen (BCMA), which is the receptor for the myeloma cell survival factors B cell activating factor (BAFF) and a proliferation-inducing ligand. BCMA was selected because it is essential for the survival of long-lived bone marrow plasma cells (Moreaux et al., 2004; O’Connor et al., 2004) and that its expression had previously been found on primary myeloma cells in small cohorts of newly diagnosed myeloma patients (Bellucci et al., 2005; Carpenter et al., 2013; Claudio et al., 2002; Moreaux et al., 2004, 2009; Ng et al., 2004; Novak et al., 2004; Tai et al., 2006, 2014).

The aims of our study were to assess BCMA as target for T cell immunotherapy for myeloma and to generate and evaluate the IgG1-based BCMA-TCB EM801 for the treatment of multiple myeloma.

RESULTS

Expression of BCMA
BCMA is expressed in malignant plasma cells from all samples of previously untreated or relapsed myeloma patients as assessed by gene expression profiling (n = 712) and RNA sequencing (n = 263) (Figure 1A), including patients with highly proliferative disease or high-risk features according to chromosomal aberrations or gene expression-based risk scores (Figure S1). Surface BCMA antigen expression is found using multidimensional flow cytometry on malignant plasma cells of all previously untreated (n = 31) or relapsed myeloma patients (n = 12) (Figure 1B). The BCMA antigen density on malignant (median of 1,479 specific antibody-binding capacity [SABC] units; range, 42–14,055) and normal (median of 673 SABC units; range, 189–1,713) (Figures 1B and 1C) plasma cells was significantly (p < 0.001) higher than on all other normal bone marrow cell subsets (median of ≤65 SABC units; range, 0–213). In the latter subsets, BCMA expression was absent (Figure 1A). Flow cytometry confirmed the restricted expression of BCMA on both normal and malignant plasma cells (Figures 1B and 1C).

During B cell development, BCMA is expressed in normal plasma cells and their highly proliferative precursors, polyclonal plasmablasts, but absent in earlier stages such as pro- and pre-B cells, naïve and memory B cells, as well as resting and activated B cells (Figures 1A–1C).

BCMA is thus an attractive TCB target in both previously untreated and relapsed myeloma patients.

Constructing the BCMA-tcb EM801
EM801 is constructed as an asymmetric two-arm IgG1-based human antibody based on a previously developed TCB platform (Bacac et al., 2016), it binds bivalently to BCMA and monovalently to CD3ε (Figures 2A and S2A). To minimize unspecific T cell activation in the absence of concomitant binding to BCMA, binding to CD3ε on T cells is monovalent with a low affinity of KD = 70–100 nM (surface plasmon resonance [SPR] measurement), while binding to BCMA expressed on plasma cells is of higher affinity (KD = 10 nM by SPR) and is bivalent for an avidity effect to facilitate preferential binding to BCMA+ myeloma cells and in vivo tumor targeting (Figure S2B). Molecule asymmetry is generated via a heterodimeric Fc region. Correct association of the light chains of the antibody during production is facilitated by introduction of a VH-VL crossover into the internal CD3ε binding Fab (Schaefer et al., 2011), whereas correct heavy chain association is facilitated via the knob-into-hole technology (Ridgway et al., 1996). For extended elimination half-life, EM801 carries a heterodimeric Fc region with intact FcRn binding to enable at least once a week intravenous (i.v.) or subcutaneous (s.c.) administration. To minimize the risk of infusion reactions, binding to Fc gamma receptors expressed on innate immune cells and to complement component (C1q), is abolished by engineering of the heterodimeric Fc with P329G, L234A, and L235A mutations (Schlothauer et al., 2016). EM801 does not bind to TACI or BAFF-R, two receptors with high homology to BCMA (Mackay and Ambrose, 2003) (data not shown). The intended mechanism of action of BCMA-TCB is shown in Figure 2B. EM801 BCMA-TCB can be easily produced with high purity and was biophysically and biochemically stable with no tendency for aggregation.

Mechanism of Action
Simultaneous binding of EM801 to CD3ε on T cells and BCMA on myeloma cells resulted in a strong CD3+ T cell–tumor cell interaction as visualized using immunofluorescence and electron scanning microscopy (Figure 3A). In a concentration-dependent manner, EM801 couples T cells and myeloma cells as measured by atomic force microscopy with a median binding strength of 538 ± 210 pN (1.5- to 6-fold of control) for myeloma cell lines (p < 0.001; n = 10) and 549 ± 149 pN (2- to 7-fold of control) for primary myeloma cells (p < 0.001; n = 10) (Figure 3B). The binding interaction between T cells and myeloma cells led to TCR/CD3 crosslinking and activation of the CD3 downstream signaling pathway (Figure 3C). T cell activation and increased T cell function was observed by upregulation of CD69 and CD25 expression, as well as release of granzyme B and proinflammatory cytokines, e.g., interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-2 (IL-2) (Figures 3D and 3E). At 120 hr incubation, BCMA-TCB induced concentration-dependent CD4+ and CD8+ T cell proliferation as measured by carboxy-fluorescein succinimidyl ester (CFSE) dilution (Figure 3F). Lack of need for TCR:major histocompatibility complex (MHC) recognition through activation of the CD3
downstream signaling pathway induced by EM801 allowed us to measure T cell-induced killing of myeloma cell lines in a 24 hr co-culture assay with human peripheral blood mononuclear cells (PBMCs) and myeloma cells at an effector:target (E:T) ratio of 10:1. Using EM801 as single agent, killing of NCI-H929, L363, and RPMI 8226 myeloma cells was observed with median effective concentration (EC50) ranging from 7 to 35 pM as measured by lactate dehydrogenase (LDH) release (Figure 3G). To prove that the observed killing is dependent on CD3+ T cells and furthermore can, in agreement with direct CD3 activation, be performed by both CD4+ and CD8+ T cells, we exposed myeloma cells plus EM801 with total, CD3-, CD4-, or CD8-depleted peripheral blood leukocytes. In each experiment, equal numbers of leukocytes were used. Whereas minimal activity was seen in CD3-depleted samples, both CD8+ and CD4+ T cells remaining in the leukocyte sample alone (i.e., in the CD4- and CD8-depleted samples, respectively) were able to achieve similar levels of activity during 48 hr exposure as the undepleted sample (Figures 3H and S3).

**Activity of EM801**

In bone marrow aspirates (n = 19), increasing concentrations of EM801 lead to higher T cell activation, with significantly increased expression levels of CD69 (p < 0.001), CD25 (p < 0.001), and HLA-DR (p = 0.001) in both CD4+ and CD8+ T cells compared with control TCB (binding to CD3ε, but not to

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**Figure 1. Expression of B Cell Maturation Antigen in Normal and Malignant Plasma Cells as well as Other Cells of the Bone Marrow Microenvironment**

(A) The left panel shows gene expression profiling using DNA microarrays (206641.at) of monocytes (CD14, n = 5), T cells (CD3, n = 5), hematopoietic stem cells (CD34, n = 5), mesenchymal stromal cells (MSC, n = 8), as well as in vitro differentiated osteoblasts (OB, n = 8) and osteoclasts (OC, n = 7), followed by normal plasma cell precursors, i.e., memory B cells (MBC, n = 5) and in vitro generated polyclonal plasmablastic cells (PPC, n = 5), as well as normal bone marrow plasma cells (BMPC, n = 10), malignant plasma cells from patients with newly diagnosed multiple myeloma (MM, n = 630) and relapsed/refractory myeloma MM (MMR, n = 82), and human myeloma cell lines (HMCL, n = 54). The dashed red line depicts the threshold for being predicted as expressed (above) or not expressed (below) according to the PANP algorithm. The right panel shows confirmation using RNA sequencing of PPC (n = 4), MBC (n = 4), normal (n = 10) and malignant plasma cells (n = 263), and cell lines (n = 19), BM, bone marrow.

(B) Illustrative results of flow cytometric detection of BCMA expression on normal and malignant plasma cells from a myeloma patient. APC, allophycocyanin.

(C) Specific antigen-binding capacity (SABC) ± SD per cell as determined by flow cytometry in normal (n = 25) and malignant (n = 35) plasma cells, as well as HMCL (n = 7; left panel) compared with other bone marrow subsets. See also Figure S1.
BCMA) (Figures 4A, S4A, and S4B). EM801 significantly and dose dependently induced secretion of cytokines and cytolytic proteins, i.e., IFN-γ from median 4 ± 194 pg/mL in the medium control to 733 ± 1,069 pg/mL at 10 nM EM801 (p < 0.001; n = 24), granzyme B 42 ± 408 versus 644 ± 801 pg/mL (p < 0.001), and perforin 225 ± 2,007 versus 672 ± 1,295 pg/mL (p < 0.01) as measured by ELISA (Figure 4B). EM801 induced significant primary myeloma cell death by autologous T cells in bone marrow aspirates in 24/31 (77%) previously untreated and 10/12 (83%) relapsed/refractory myeloma patients at concentrations ranging from 10 pM to 30 nM (Figure 4C; Table 1). Activity was also seen in samples from heavily pretreated patients as shown for patient no. 21 (Figure 4D). The E:T ratio mirrored the measured ratios as present in the bone marrow aspirates with a median of 2.9 (range, 0.2–21) versus 1.0 (range, 0.03–4.3) in responding versus non-responding patients (p = 0.18), and median SABC units were 1,267 (range, 42–6,400) versus 1,522 (range, 487–14,055) in responding versus non-responding patients (p = 0.87) (Table 1). No toxicity was detected in cells of the bone marrow microenvironment (Figures 4E and 4S4C).

In vivo antitumor activity was first studied in NCI-H929 s.c. xenograft in human PBMC-transferred NOG mice. Treatment was initiated when median tumor volume reached 287 ± 135 mm³ (similar for all groups; Table S1). At a dose of 2.6 nM/kg (0.5 mg/kg) of EM801, given once a week for 3 weeks, tumor growth inhibition was already observed after the second i.v. injection in six of nine animals, and the tumors regressed to 16 ± 3 mm³ on day 47 (Figure 5). Non-responding mice had significantly higher tumor volumes (p = 0.048) above 375 mm³ at start of treatment (Table S1). An equimolar dose of control TCB showed no effect (Figure 5).

To further investigate in vivo activity of our TCB in an autologous setting, we choose a cynomolgus monkey model. As in humans, cynomolgus plasma cells express surface BCMA, but unlike humans, the majority of cynomolgus peripheral blood B cells also express BCMA (Figure S5A). The model thus allows serial assessment of BCMA⁺ B cell depletion in peripheral blood and BCMA⁺ plasma cell infiltration in the bone marrow, as well as the impact on IgG and IgM levels. The higher number of BCMA⁺ cells also mirrors more closely the situation in myeloma patients regarding their typical tumor burden.

As the binding affinity of EM801 to cynomolgus BCMA is approximately ten times lower than to human BCMA, and to mirror as closely as possible the human situation, we constructed a surrogate BCMA-TCB2 with the same structure, the same affinity to human and cynomolgus CD3, but comparably high affinity with cynomolgus BCMA. We first confirmed cynomolgus BCMA⁺ cell lysis by BCMA-TCB2 in vitro (Figure S5B). Next, a single i.v. injection of BCMA-TCB2 in the dose range from 0.01 to 1 mg/kg already caused a decrease in BCMA⁺ cells in blood 24 hr after injection and this was sustainable for up to 168 hr (Figure 6A). Blood B cell rebound associated with
B cell-depleting agents was observed 3 weeks post injection (data not shown). In the bone marrow, a decrease in total B cells and BCMA+ bone marrow cells was observed in all animals 96 hr after injection of BCMA-TCB2 (Figures 6B and 6C). A dose-dependent decrease in serum IgG levels up to 56.1% compared with baseline was observed 168 hr post injection (Figure 6D). A similar response was observed with serum IgM, indicating that plasma cells are depleted by BCMA-TCB2. A transient increase in activated CD8+ T cell absolute cell count was also detected in the bone marrow of all animals 96 hr after injection; in agreement with the observed induction of CD8 proliferation, CD25+CD8+ absolute T cell count returned to baseline level 3 weeks post injection (Figure 6E). Similar results were observed for activated CD25+CD4+ helper T cells in three of six animals, especially when treated with the highest doses (Figure 6E). The male animal treated with 0.1 mg/kg already had a high number of CD25+CD4+ cells pre-dose. A modest and transient increase in cytokine release was measured 30 min after injection of BCMA-TCB2; cytokine levels in plasma returned to baseline 8–24 hr after injection (Figure 6F).

Pharmacokinetic Studies in Monkeys and Mice

Figures 7A and 7B show the concentrations measured in serum and bone marrow aspirates after 0.01, 0.1, and 1 mg/kg i.v. and 0.01 and 0.1 mg/kg s.c., respectively, in cynomolgus monkeys. Pharmacokinetic (PK) parameters were determined by non-compartmental analysis (Tables S2 and S3). PK is dose linear, elimination half-life is approximately 4 days and bioavailability after s.c. dosing is above 90% (Table S3). BCMA-TCB concentrations measured in bone marrow aspirates were similar to those measured at the same time points in serum. These PK data suggest the possibility of convenient i.v. or s.c. dosing once a week or once every second week. PK results in immunodeficient mice also suggest dose linearity and a long elimination half-life (Figure 7C).

Observed Side Effects and Safety Aspects

In bone marrow aspirates from myeloma patients, no toxicity was detected in cells of the microenvironment including B cell subpopulations, T lymphocytes, or natural killer (NK) cells (Figures 4E and S4C). In cynomolgus monkeys, a single i.v. or s.c. injection of BCMA-TCB2 up to 1 mg/kg was well tolerated in all animals. A modest and transient increase in cytokine release was observed, with plasma concentrations returning to baseline levels within 24 hr (Figure S5C). A 4 week toxicity trial in cynomolgus monkeys including, among other groups, a 1 mg/kg/week group and a 2 mg/kg/week group (three females and three males in each group) at an average serum drug concentration, over the 4 weeks at 2 mg/kg/week, close to 100 nM, i.e., ten times higher than therapeutic exposure, showed no pathological findings on macroscopic and histopathological examination of all organs (data not shown).

DISCUSSION

BCMA is an excellent immunotherapeutic target for myeloma for several reasons. First, BCMA is expressed in all investigated malignant plasma cell samples in our large cohort of previously untreated or relapsed/refractory myeloma patients, in agreement with earlier data obtained from myeloma cell lines and small patient cohorts (Bellucci et al., 2005; Carpenter et al., 2013; Claudio et al., 2002; Moreaux et al., 2004, 2009; Ng et al., 2004; Novak et al., 2004; Tai et al., 2006, 2014). BCMA is likewise expressed in patients with adverse prognosis. Second, BCMA is essential for the survival of normal long-lived bone marrow plasma cells (O’Connor et al., 2004), making resistance by downregulation during treatment unlikely, in contrast to other antigens such as CD38 (Nijhof et al., 2015). Despite malignant plasma cells showing a homogeneous pattern of positive BCMA expression by flow cytometry, we cannot completely exclude the possibility of a minor subclone expressing it at levels below those needed for it to be a viable target for EM801, thus potentially giving rise to disease progression. We could, however, show EM801 activity against cells expressing the lowest measured number of cell surface BCMA molecules, 42, i.e., 35-fold lower than the median of 1,479. This implies that our TCB is potentially able to overcome low BCMA expression. Data with anti-BCMA CAR-T cells also showed progression most frequently related to the lack of persistence of BCMA+ CAR-T cells, not the emergence of BCMA- tumor cells (Ali et al., 2016). Taken together, this makes a clonal escape scenario for BCMA-targeted treatment unlikely. Third, in agreement with a previous report (Novak et al., 2004), BCMA expression being absent in early B cell development should allow for a rapid re-emergence of B cell immunity.
after cessation of anti-BCMA treatment. Fourth, the absence of BCMA expression in other populations in the bone marrow indicates a favorable toxicity profile.

As intended, EM801 effectively couples T cells and myeloma cells leading into a 3–4× increased binding, followed by T cell activation, proliferation, and myeloma cell killing. This implies that crosslinking alone can induce myeloma cell killing, independently of, or even overcoming, inhibitory mechanisms such as loss of T cell repertoire or increased presence of accessory cells, inhibiting immune response (Rabinovich et al., 2007) in terms of primary myeloma cells, as well as of self-tolerance mechanisms in place in cynomolgus monkeys. EM801 exerts its effect, as TCB antibodies do in general, by redirecting and engaging T cells via CD3, the common component of the TCR complex in both CD4+ and CD8+ T cells, overcoming the need for antigen recognition in MHC class II and I molecules from immune or tumor cells, respectively. This explains why both CD4+ and CD8+ T cell rapidly become active and proliferate, and why each subset alone is able to eliminate myeloma cells. The contribution of both CD4+ and CD8+ T cell subsets to redirected lysis of tumor cells has been reported, with a description of the mechanism of action of, e.g., CD19xCD3 or PSCAxCD3 TCBs (Brischwein et al., 2006; Feldmann et al., 2012; Haas et al., 2009). These studies have also shown that, similarly to our observations with EM801, there is equal T cell lysis with only CD4+ or CD8+ T cells (Brischwein et al., 2006; Feldmann et al., 2012; Haas et al., 2009). Whereas similar levels of T cell-induced killing in CD4+- or CD8+-depleted samples compared with the full T cell content may seem surprising, as the activity of the depleted T cell fraction should be lacking, this should only be expected in an experimental setting with an excess of target cells; with the number of eliminated myeloma cells primarily being dependent on the number of effector cells. The opposite is true for our experiments with the number of effector cells exceeding the number of myeloma cells (E:T ratios > 1:1, including the CD4+- and CD8+-depleted samples). Under comparable conditions, Feldmann et al. (2012) described that even a high increase in T cells implies only limited effect on tumor cell lysis, i.e., 60%, 70%, or 80% average lysis at E:T ratios of 2:1, 5:1, and 10:1. In other words, in the E:T range used in our experiments, and with both CD4+ and CD8+ T cells contributing to a similar extent, although with different kinetics to tumor cell lysis, a depletion of either population does not change the E:T ratio enough to bear high impact on the number of eliminated myeloma cells.

In terms of activity, we first showed the ability of EM801 as a single agent at low nanomolar concentrations to significantly induce killing of primary myeloma cells by autologous T cells, reaching 90% reduction of myeloma cells after 48 hr even in aspirates with a very low E:T ratio. We deliberately did not manipulate E:T ratios in order to reproduce the high heterogeneity in T cell numbers found in the bone marrow of myeloma patients, and the demonstration of efficacy among patients with very low E:T ratios warrants investigating if EM801 is clinically meaningful in such patients. This is in agreement with previous observations using CAR-T cells (Carpenito et al., 2009; Kalos et al., 2011). In the sense of a “serial killing,” each infused CAR-T cell was assumed to have eradicated more than 1,000 leukemic cells based on calculations using the estimated initial tumor burden and the number of infused engineered T cells (Kalos et al., 2011). To this end, the potential of each T cell to kill more than one myeloma cell was validated by time-lapse microscopy. Given that non-responders also showed T cell activation on the background of limited time of exposure of primary myeloma cells, we hypothesize that longer incubation might have turned non-responders into responders. Based on our ex vivo data, EM801 may thus overcome inhibitory immune mechanisms such as T cell anergy.

In vivo activity of EM801 was demonstrated in two animal models, i.e., the NOD mouse model with human myeloma xenograft (allogeneic) and a cynomolgus monkey model. In the former, given the high proliferation rate of the myeloma cell line used being much higher than those observed in the vast majority of myeloma patients (Hose et al., 2011), EM801 is thus able to eradicate even highly proliferating myeloma cells. Mice not responding had a significantly higher initial tumor mass. As the amount of myeloma cells which can be killed by CD3+ T cells per time is limited (see above), and fast growth would especially impact at higher initial numbers of myeloma cells, we see the likely explanation that, in these cases, CD3+ T cells were not able to cope with the number of myeloma cells. With the cynomolgus monkey model, we could show activity in an autologous setting in vivo, i.e., reduction of the number of plasma cells in the bone marrow as well as BCMA+ B cells in bone marrow and peripheral blood, including a decrease of serum IgG and IgM levels.

**Figure 4. T Cell Activation and Myeloma Cell Killing in Bone Marrow Aspirates from Myeloma Patients Induced by EM801**

(A) T cell activation as measured by flow cytometry for the surface markers CD69, CD25, and HLA-DR in both CD4+ and CD8+ T cells (n = 19 bone marrow samples).

(B) Secretion of IFN-γ, granzyme B, and perforin by T cells using ELISA in bone marrow samples (n = 24) upon treatment with the indicated concentrations of EM801. Boxplots summarizing the data are shown; see below for illustrative cases.

(C) Killing of primary myeloma cells in bone marrow aspirates ex vivo induced by EM801 in the presence of autologous T cells, and corresponding T cell activation regarding secretion of IFN-γ, granzyme B, and perforin. Exemplary plots are shown for a newly diagnosed (no. 16, left) and a relapsed patient (no. 17, right).

(D) Myeloma cell killing with corresponding T cell activation regarding secretion of IFN-γ, granzyme B, and perforin (top four graphs) and clinical course (bottom) of a heavily treated patient (no. 21). Red arrows highlight the time point at which ex vivo activity of EM801 in a bone marrow sample of the patient was analyzed. The blue line indicates the M-protein; units are shown on the left y axis (g/L). The orange line indicates the value of the involved light chain (FLC) with units shown on the right y axis (mg/L). Color bars indicate type and duration of different treatment regimens. VCD, bortezomib (Velcade), cyclophosphamide, and dexamethasone; CAD, cyclophosphamide, adriamycin, and dexamethasone; HD7, high-dose chemotherapy; Len, lenalidomide consolidation; VD, bortezomib and dexamethasone; Pom/dex, pomalidomide and dexamethasone; pan-PIM inh., pan-PIM kinase inhibitor; dex, dexamethasone; FVD, panobinostat, bortezomib, and dexamethasone.

(E) Toxicity on cells of the bone marrow microenvironment is shown for the same patients as in (C); measurements referred to medium control. BMME, bone marrow microenvironment. Error bars in each case represent the SD.

*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.
Table 1. Activity of EM801 and Characteristics of Patients and Samples Included in the EM801 Ex Vivo Study

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<th>Patient Sample No.</th>
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<th>E:T Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Myeloma Cell Lysis at 10 nM at 48 hr (If Not Stated Otherwise)&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>641</td>
<td>3.92</td>
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<sup>a</sup>SABC, specific antigen-binding capacity.
<sup>b</sup>E:T ratio, effector to target ratio.
<sup>c</sup>Values depict responding samples (i.e., statistically significant cell death); underlined values depict non-responding samples. A minus sign depicts an increase in cell number compared with the medium control. NA, not applicable.
<sup>d</sup>Killing at 3 nM.
<sup>e</sup>Twenty-four hour exposure.
<sup>f</sup>Killing at 30 nM.
Taken together, our data give convincing evidence for the activity of our TCB, thus warranting clinical testing. Regarding the PKs, maintaining the Fc region leads to extended elimination half-life and convenient weekly i.v. or s.c. dosing intervals. This is, for example, in contrast to BCMA-BiTE which requires continuous infusion via a pump that patients have to carry for weeks of treatment (Hipp et al., 2015). As an antibody, EM801 has several advantages over CAR-T cell or TCR approaches. First, it is completely eliminated from circulation within 1–2 months after cessation of treatment, in contrast to the previously described long-term persistence of engineered T cells (Kalos et al., 2011; Rapoport et al., 2015). Second, the TCB approach and the molecular format of EM801 is such that, in contrast, e.g., to CAR-T cells, no patient-specific generation ex vivo is necessary (Ali et al., 2016; Carpenter et al., 2013), allowing for its potential use in almost all patients. In terms of side effects and safety, histopathological assessment in cynomolgus monkeys after repeated dosing did not show any pathological findings, and observed cytokine release in these animals was modest and transient.

In conclusion, EM801 emerges as an attractive non-cross-resistant compound with high potential for use as a single agent, in combination, or in long-term maintenance treatment in multiple myeloma.

EXPERIMENTAL PROCEDURES

Patients, Healthy Donors, and Samples

Patients with previously untreated, therapy-requiring (n = 684) and relapsed (n = 94) myeloma as well as healthy donors (n = 20) were included in the study, which was approved by the ethics committees of the University of Heidelberg (no. 229/2003 and no. S-152/2010), as well as the University of Navarra (no. 115/2014) after written informed consent.

Normal bone marrow plasma cells and myeloma cells were purified using anti-CD138 microbeads (Miltenyi Biotec), and aliquots of CD138 + malignant plasma cells were subjected to in situ fluorescence hybridization (Hose et al., 2009; Neben et al., 2013; Seckinger et al., 2009). T cells for microscopic analyses were purified using autoMACS Pro (Miltenyi Biotec). For further cell populations and myeloma cell lines used, see the Supplemental Information.

BCMA Expression

Gene Expression Profiling

Gene expression profiling was performed using U133 Plus 2.0 Arrays (Affymetrix) as published (Hose et al., 2011; Seckinger et al., 2009).

RNA Sequencing

Full-length, double-stranded cDNA was generated from 5 ng of total RNA and amplified using the SMARTer Ultra Low RNA Kit (Illumina). Library preparation was performed from 10 ng of fragmented cDNA using the NEBNext ChIP-Seq Library Prep protocol (New England Biolabs). Libraries were sequenced on an Illumina HiSeq 2000 with 2 × 50-bp paired-end reads.

Flow Cytometry

Cells were stained with anti-CD138-PE (Miltenyi Biotec), anti-CD38-FITC (Becton Dickinson), and anti-BCMA-APC (provided by EngMab) for analysis of the myeloma cell compartment. T cell analysis was performed using CD4-FITC (Beckman Coulter), anti-CD45RA-APC, anti-CD45RO-PE (both from SouthernBiotech), anti-CD28-PE, anti-CD25-PE, anti-CD95-APC, anti-CD3-PerCP, and anti-CD8-APC-Cy7 (all from Becton Dickinson). Cells stained with corresponding isotype antibodies were used as control. Analysis was performed on a FACSARia or FACSComp using FACSDiva software (Becton Dickinson) and FlowJo version x.0.7 (FlowJo, LLC).

Multidimensional flow cytometry immunophenotyping of bone marrow aspirates was performed using an eight-color antibody combination and a direct immunofluorescence stain-and-then-lyse technique: anti-CD45-V450 (Becton Dickinson), anti-CD27-BV510 (BioLegend), anti-CD34-PerCP-Cy5.5 (Becton
Figure 6. In Vivo Activity in Cynomolgus Monkeys
(A and B) Total B cell numbers (BCMA+CD45+CD20+CD16−) from blood (A) and bone marrow (B) after single i.v. injection of 0.01, 0.1, and 1.0 mg/kg of BCMA-TCB2. M, male; F, female, with three animals in each group.
(C) Numbers of bone marrow plasma cells (BCMA+CD20−CD38hiCD16−). Green bars, females; blue bars, males.
(D) Serum IgG (left) and IgM levels (right) relative to baseline (%) in cynomolgus monkeys post single i.v. injection of 0.01, 0.1, or 1.0 mg/kg of BCMA-TCB2.
(E) Absolute cell counts of CD4+CD25− (left) and CD8+CD25−-activated T cells (right) in bone marrow post treatment with 0.01, 0.1, or 1.0 mg/kg of BCMA-TCB2. Green bars, females; blue bars, males. See also Figure S5.
### Figure 7. Pharmacokinetics in Cynomolgus Monkeys and Mice

(A) PK in cynomolgus monkeys upon i.v. administration of BCMA-TCB2 at a single dose of 0.01, 0.1, or 1.0 mg/kg. Concentrations measured in serum (full lines) and bone marrow aspirates (dotted lines). Each vertical dotted line represents 1 week, the green area shows the effective concentration range of BCMA-TCB2 in bone marrow aspirates from myeloma patients. F, female; M, male with three animals in each group. PK parameters were obtained by non-compartmental analysis.

(B) Concentrations measured in serum and bone marrow aspirates after 0.01 and 0.1 mg/kg s.c.

(C) PK in mice after single i.v. dose with the indicated concentrations of EM801. The horizontal dotted line indicates the limit of quantification levels. See also Tables S2 and S3.

### Binding Capacity

BCMA density was assessed using QIFIKIT (Dako) as per the manufacturer’s instructions. The SABC was calculated as the number of BCMA antibodies per cell after correction for background fluorescence. Data analysis was performed using FACSDiva software.

### EM801 Design, Production, and Purification

**Generation of Fc-Containing 2+1 Asymmetric BCMA-TCB EM801**

EM801 vectors were generated first. The IgG1-derived bispecific molecules consisted of two antigen-binding moieties capable of binding specifically to two distinct antigenic determinants, CD3ε and BCMA. The antigen-binding moieties were Fab fragments composed of a heavy and light chains, each comprising a variable and a constant region. The Fab fragment binding to CD3ε was a CrossFab fragment, wherein the variable domains of the Fab heavy and light chains were exchanged. The bispecific molecule design was monovalent for CD3ε and bivalent for BCMA, in which one Fab fragment is fused to the N terminus of the inner CrossFab (2+1) specific to CD3ε. The bispecific molecule contained an engineered silent Fc region.

**Production**

Molecules were produced by co-transfecting HEK293-expressing Epstein-Barr virus nuclear antigen (EBNA; American Type Culture Collection, CRL-10852) growing in suspension with the Preparation of a 2+1 CrossFab-IgG constructs, cells were transfected with four corresponding expression vectors: (1) “vector Fc(knob),” (2) “vector light chain,” (3) “vector light chain CrossFab,” and (4) “vector heavy chain CrossFab,” using an optimal expression vector ratio.

**Surface Plasmon Resonance**

Affinity to human BCMA was measured using SPR (Supplemental Information).

**Microscopy**

The binding forces between myeloma cells and T cells were assessed by atomic force microscopy; visualization of T cell-myeloma cell interaction was

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Dickinson, anti-CD19PE-Cy7 (Beckman Coulter), anti-BCMA-APC (provided by EngMab), anti-CD38-FITC, anti-CD56-PE, and anti-CD138-APC.C750 (all from Cytognos). This combination allowed to evaluate surface expression of BCMA on malignant and normal plasma cells discriminated through antigen underexpression (CD19, CD27, CD38, or CD45) or antigen overexpression (CD56). B cell precursors (CD19⁺CD34⁺CD19⁺CD38⁺⁺), mature B cells (CD19⁺CD34⁺CD38⁺⁺⁺), T cells (CD19⁺CD56⁺FSC⁺SSC⁺⁺), NK cells (CD19⁺CD56⁻FSC⁺SSC⁺⁺), eosinophils (identified by FSC/SSC and autofluorescence pattern), neutrophils (CD45⁺SSC⁺⁺), monocytes (CD45⁺SSC⁺⁺CD38⁺⁺⁺), and erythroblasts (CD45 SSC⁺⁺⁺). Cell acquisition was performed on a FACSCanto II using FACSDiva software, and files were analyzed using Infinicyt (Cytognos).
performed using fluorescence and scanning electron microscopy (see the Supplemental Information).

**T Cell Activation**

**Jurkat NFAT Reporter Cell Assay**

Analysis of the CD3 downstream signaling pathway was measured after 16 hr of co-culture of GloResponse Jurkat NFAT-RE-luc2P (Promega) and NCI-H929 myeloma cells at an E:T ratio of 2.5:1 and increasing concentrations of EM801, according to the manufacturer’s instructions. Relative luminescence units, corresponding to the intensity of luciferase expression downstream of CD3, was measured using a plate reader with glow-type luminescence reading capabilities (Promega). Concentration-response curves and the EC50 values of antibody response were fitted and calculated using Prism software (GraphPad).

**Flow Cytometry**

T cell activation was measured after 24–48 hr of co-culture of myeloma cell lines with human PBMCs (E:T ratio = 10:1) or patient bone marrow aspirates containing autologous T cells and myeloma cells with increasing concentrations of EM801. Activation markers CD69, CD25, and HLA-DR (BD Biosciences) were measured on CD4+ and CD8+ T cells.

T cell proliferation was measured using CFSE (Life Technologies) staining (100 μM) and CFSE dilution analysis, after 5 days of co-culture of PBMCs with RPMI 8226 myeloma target cells or MKN45 control cells (E:T ratio = 10:1).

**ELISA**

The amount of the secreted IFN-γ (BD OptEIA ELISA sets, Becton Dickinson) granzyme B, and perforin (both from Mabtech) in supernatants from bone marrow cultures was quantified using ELISA and analyzed with an automated plate reader (Tecan) using Magellan V2.22 software. Experiments were performed in triplicates.

**Cytometric Bead Array**

Cytokines/cytolytic enzymes, i.e., granzyme B, IFN-γ, TNF-α, and IL-2 were measured using a CBA Human Soluble Protein Flex Set (BD Biosciences), according to the manufacturer’s instructions. Supernatants were collected from 48 hr co-cultures of PBMCs with myeloma cell lines as described below. Data were acquired using FACSCanto II and EC50 values calculated using Prism software (GraphPad).

**EM801 Activity**

**Killing of Human Myeloma Cell Lines**

Killing of human myeloma cell lines NCI-H929, L363, and RPMI 8226, expressing high, medium, and low levels of surface BCMA, respectively, was measured by quantification of LDH release by dead cells into supernatant (LDH Detection Kit, Roche Applied Science). In brief, unstimulated human PBMCs were co-cultured with myeloma cell lines at an E:T ratio of 10:1 for 24–48 hr (37°C, 5% CO2) together with graded concentrations of EM801 or control TCB, i.e., 0.000128, 0.00064, 0.0032, 0.016, 0.08, 0.4, 2, and 10 nM in triplicates. Maximal lysis of the target cells (80%) was achieved by incubation of target cells with 1% Triton X-100. Minimal lysis (0%) refers to target cells incubated with effector cells without EM801. The percentage of specific cell lysis was calculated as [sample release – spontaneous release] / [maximum release – spontaneous release] × 100.

**T Lymphocyte Depletion**

Unstimulated peripheral blood leukocytes from two healthy donors were co-cultured with NCI-H929 cells (1:5 ratio) for 24 hr under different conditions: (1) total leukocytes; (2) leukocytes depleted of total CD3+ T lymphocytes plus EM801 (50 pM); (3) leukocytes depleted of CD4+ T lymphocytes plus EM801 (50 pM); and (4) leukocytes depleted of CD8+ T lymphocytes plus EM801 (50 pM). For conditions 2, 3, and 4, we depleted total or CD4 and CD8 T lymphocytes using a FACsAria IIb (BD Biosciences). In each experiment, equal numbers of leukocytes were used. The percentage of tumor cell lysis was measured by [absolute number of annexin V-negative myeloma cells/μL in condition 1 – absolute counts annexin V-negative myeloma cells/μL in conditions 2, 3, or 4] / absolute counts annexin V-negative myeloma cells/μL in condition 1) × 100. The absolute number of myeloma cells was determined using Perfect-Count Microspheres (Cytognos) following the manufacturer instructions.

**Primary Myeloma Cells Cultured together with Their Bone Marrow Microenvironment**

Primary myeloma cells cultured together with their bone marrow microenvironment (n = 24) were exposed to EM801 at graded concentrations as published previously (Seckinger et al., 2009). Compound exposure was performed once on day 0 and cells were cultured for 48 hr. Each dose point was done in triplicate. Data analysis was performed using FACSDiva software. Cell culture supernatants were harvested for subsequent ELISA measurements.

**Whole Bone Marrow**

Whole bone marrow samples (n = 19) were cultured in RPMI 1640 medium with 10% fetal bovine serum, 0.5% gentamicin, and 1% L-glutamine for 24 hr and/or 48 hr with increasing concentrations of EM801 (0.0003, 0.0003, 0.003, 0.03, 0.1, 0.3, 1, 3, 10, and 30 nM), control TCB (10, 30 nM), or without any drug (control). After incubation, cells were lysed and stained with antibodies against CD45, CD5, CD38, CD56, annexin V, CD19, BCMA, and CD138 to measure cytotoxicity in myeloma cells and other bone marrow subsets.

**Xenograft Model**

Animal housing and experimental procedures were realized according to the French and European Regulations and the NRC Guide for the Care and Use of Laboratory Animals. The animal facility is authorized by the French authorities (agreement no. B 21 231 011 EA). All procedures using animals were submitted to the Animal Care and Use Committee Oncomet, agreed by French authorities (CNREEA agreement no. 91). In vivo efficacy was studied in an s.c. NCI-H929 xenograft model in human PBMC-transferred NOG (NOD.Cg-PkdcscidIl2rgtm1Sug/J-Tac, Taconic) mice. NOG mice aged 6–8 weeks were injected s.c. with NCI-H929 cells (5 × 106 cells) and tumor volume measured under blind conditions twice/week by caliper. Mice were injected intraperitoneally with human PBMC (20 × 106 cells) 15 days after tumor cell injection, then randomized into treatment and control groups using Vivo Manager software (Biosystems). Antibody treatment started 4 days after PBMC transfer (i.e., 19 days after tumor cell injection), followed by i.v. injection of EM801 (2.6 nM/kg), control TCB, or vehicle once a week for 3 weeks (n = 9 mice/group).

**Cynomolgus Monkeys: Macaca fascicularis**

The study protocol for the cynomolgus monkeys was approved by the local authorities after review by the Animal Care and Use Committee of the AAALAC-certified institution. All the animal procedures and ethical revision were performed according to the current Italian legislation (Legislative Decree March 4th, 2014, no. 26) enforcing the 2010/63/UE Directive on the protection of animals used for biomedical research and agreed by the Italian authorities. Due to the low affinity to cynomolgus BCMA, EM801 is not an ideal molecule for studies in cynomolgus monkeys. Therefore, we used surrogate BCMA-TCB2, with high affinity to cynomolgus BCMA. Effects were measured after i.v. injection of 0.01, 0.1, and 1.0 mg/kg of BCMA-TCB2. Absolute cell counts for total B cells (CD45+CD20+CD21+16), plasma cells (CD20+CD38+CD138+CD21+low/med, as well as activated helper (CD25+CD45+CD21+low/med) and cytotoxic T cells (CD25+CD56+CD28+CD21+low/med) were calculated using flow cytometry (FACSAnalyt C) combined with a hematology analyzer (ADIVA 120, Bayer). Serum IgG levels were measured by ELISA (Life Diagnostics). Cytokine release was measured by multiplexed bead-based immunoassay (Luminex).

**PKs**

**Mouse**

The study protocol was approved by the local authorities after review by the Animal Care and Use Committee of the AAALAC-certified institution. The procedures and facilities comply with the requirements of Directive 2010/63/EU from September 22, 2010, and subsequent amendments, as well as the national legislation defined in the German animal protection law “TierSchG (first version July 24, 1972, actual version May 18, 2006 [BGB1. I S. 1206, 1313]” in its current amended version. The experimental design was reviewed and approved by the German authorities; project authorization registration no. AZ 55.2-1-54-2532.2-7-13.

For details on PK studies in mice and monkeys, see the Supplemental Information.

**Statistical Analysis**

See the Supplemental Information for details. For boxplots, the box expands from the first/upper quartile (Q1), over the median to the third/upper quartile (Q3). The inter-quartile range (IQR) is defined as IQR = Q1–Q3. The lower whisker starts at Q1 – 1.5 × IQR, if this is above the lowest data point,
otherwise it starts at the lowest data point; the upper whisker ends at Q3 + 1.5 × IQR.

ACCESSION NUMBERS

The accession numbers for the expression data reported in this paper are ArrayExpress: E-MTAB-4715, E-MTAB-4717, E-MTAB-5212, E-TABM-937, E-TABM-1088.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2017.02.002.

AUTHOR CONTRIBUTIONS


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