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Preclinical Efficacy and Safety Comparison of CD3 Bispecific and ADC Modalities Targeting BCMA for the Treatment of Multiple Myeloma

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Abstract

The restricted expression pattern of B-cell maturation antigen (BCMA) makes it an ideal tumor-associated antigen (TAA) for the treatment of myeloma. BCMA has been targeted by both CD3 bispecific antibody and antibody–drug conjugate (ADC) modalities, but a true comparison of modalities has yet to be performed. Here we utilized a single BCMA antibody to develop and characterize both a CD3 bispecific and 2 ADC formats (cleavable and noncleavable) and compared activity both *in vitro* and *in vivo* with the aim of generating an optimal therapeutic. Antibody affinity, but not epitope was influential in drug activity and hence a high-affinity BCMA antibody was selected. Both the bispecific and ADCs were potent *in vitro* and *in vivo*, causing dose-dependent cell killing of myeloma cell lines and tumor regression in orthotopic myeloma xenograft models. Primary patient cells were effectively lysed by both CD3 bispecific and ADCs, with the bispecific demonstrating improved potency, maximal cell killing, and consistency across patients. Safety was evaluated in cynomolgus monkey toxicity studies and both modalities were active based on on-target elimination of B lineage cells. Distinct nonclinical toxicity profiles were seen for the bispecific and ADC modalities. When taken together, results from this comparison of BCMA CD3 bispecific and ADC modalities suggest better efficacy and an improved toxicity profile might be achieved with the bispecific modality. This led to the advancement of a bispecific candidate into phase I clinical trials.

Introduction

Multiple myeloma is a disease of malignant plasma cells and despite recent advances in treatment options, relapse is inevitable and patients become refractory to treatment (1–4), highlighting the clear need for additional treatment approaches. Two exciting approaches of current focus that may benefit myeloma patients are CD3 targeting bispecific antibodies and antibody–drug conjugates (ADC; refs. 5–12).

CD3 bispecific antibodies function by redirection of T lymphocytes, highly potent immune cells capable of killing cancer cells and virally infected cells through perforin/granzyme release (13–15). In general, antigen-induced cytotoxic T-cell immunity is dependent on target cell antigen presentation and exact recognition of the presented peptide/MHC by the T-cell receptor (TCR). One way to circumvent the need for this precise interaction is by T-cell redirection through the use of bispecific molecules that bridge TAAs and CD3 (9, 11). The FDA approval of blinatumomab (16) highlights the role of bispecifics as potentially transformative medicines. ADCs combine the specificity of monoclonal antibodies with the potency of cytotoxic payloads.

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Payloads and linkers such as DNA damaging agents, microtubule inhibitors, and cleavable or noncleavable linkers can be varied to afford ADCs' different characteristics (7, 17). FDA approval of brentuximab vendotin and inotuzumab ozogamicin validated this modality in the treatment of hematologic malignancies. Despite approvals, ADCs have limitations and off-target payload-related adverse events, such as lymphopenia/thrombocytopenia, limit maximum-tolerated dose (18). In addition, ADCs require a relatively high number of cell-surface TAAs per cell to achieve maximum efficacy (19, 20).

Finding a truly specific tumor associate antigen in a given indication is critical and in the case of myeloma, B-cell maturation antigen (BCMA also referred to as TNFRSF17 or CD269) is highly attractive. BCMA is a member of the tumor necrosis factor receptor (TNFR) superfamily and is selectively expressed in the B-cell lineage. *BCMA* RNA and protein expression is absent in nonhematopoietic tissue and naïve B cells but is upregulated upon B-cell differentiation into plasmablasts and in normal and malignant plasma cells (12, 21, 22). BCMA binds 2 ligands, B-cell activation factor (BAFF) and a proliferation inducing ligand (APRIL), which in turn promotes a signaling cascade that ultimately results in B-cell activation, maturation into plasma cells, and long-term plasma cell survival (23, 24). BCMA is the focus of much therapeutic interest and groups have reported BCMA-targeted therapies including CD3 bispecifics, ADCs, and chimeric antigen receptor (CAR) T cells (12, 25, 26). All modalities appear promising, but comparison is difficult when various antibodies with disparate affinities are tested in different models and systems. In this study, we developed a single BCMA antibody to characterize both CD3 bispecific and ADC activity in both *in vitro* and *in vivo* models and evaluated safety of both modalities in nonhuman primate studies. A BCMA CAR T utilizing a similar antibody was also evaluated and displayed robust activity, which is discussed in a separate manuscript (27). The efficacy and safety data supported development of both molecules, but the improvement in both potency and safety profile with the CD3 bispecific prompted advancement of this modality into the clinic (28).

Materials and Methods

Cell culture

Myeloma cell lines (MM.1S, MOLP8, JJN3, L363, KMS12BM, and OPM2) were obtained from ATCC or DSMZ. The time between thawing and use of cell lines did not exceed 1 month. Mycoplasma testing was performed upon receipt of cells and prior to each animal study. Cell line authentication was not conducted. All cells were cultured according to the supplier's recommendations (unless noted) and were maintained in a humidified chamber at 37°C, 5% CO_2. Cell lines were engineered to express luciferase and GFP using Luc2AGFP lentivirus (AMSbio). Peripheral blood mononuclear cells (PBMC) were sourced from Stanford Blood Center, Palo Alto, CA, and T cells were isolated using human Pan T Isolation Kit (Miltenyi).

In vitro cytotoxicity assays

Bispecific cytotoxicity assays were performed by mixing purified human $CD3^+$ T cells and luciferase-labeled myeloma cell lines, E:T of 5:1, and serial dilutions of bispecific antibody. After 2 days of incubation, viability of cells was assessed by One-Glo luciferase reagent (Promega).

ADC assays were performed by plating myeloma cell lines and adding serial dilutions of ADCs. After 5 days of incubation, viability of myeloma cells was assessed by using CellTiter-Glo reagent (Promega).

For cytotoxicity assays using patient T cells and myeloma cell lines, fresh bone marrow aspirates were acquired through UCSF (Grand MMTI). Myeloma and T-cell numbers were determined by flow cytometry and serial dilutions of bispecific or ADC were added. After 5 to 7 days of incubation, viability of cells was assessed by flow cytometry (Supplementary Materials and Methods).

T-cell activation and cytokine release

Assays were set up as above for cytotoxicity. Media was removed for cytokine analysis by MSD and T cells were removed and analyzed for CD25 and CD69 expression by flow cytometry (Supplementary Materials and Methods).

Orthotopic myeloma xenograft tumor models

Animal studies were carried out under protocols approved by the Pfizer Animal Care and Use Committee and performed in an AAALAC accredited facility. Either 5×10^6 MM.1S, 5×10^6 OPM2, 2×10^6 MOLP8, or 10×10^6 KMS12BM cells were intravenously injected into NSG mice (Jackson). After tumor establishment (Day 14 MM1.S, Day 6 or 8 MOLP8, and Day 15 OPM2) animals were randomized based on luminescent imaging and administered 2×10^7 expanded human T cells by intraperitoneal injection. Two days later, animals were administered a single dose of bispecific or nontargeting control antibody (CD3 variable and constant region combined with a truncated antibody consisting of only the hinge/constant region, thus a single arm with no antigen targeting) intravenously. Tumor growth was monitored via imaging measurements twice weekly. Imaging captured tumor burden in the whole animal and encompasses both bone marrow and extramedullary disease. ADC studies were performed similarly, without exogenous T cells. ADCs were injected on Day 22 or 23. NTC for ADCs was anti-BHV (Supplementary Materials and Methods).

Cynomolgus monkey studies

Protocols for the cynomolgus monkey toxicology studies were approved by the Animal Care and Use Committee of the AAALAC accredited institution. Female monkeys ($n = 2$ per group) were used in all studies. Animals were administered vehicle, 0.3 mg/kg NTC, or BCMA bispecific (0.1 or 0.3 mg/kg) intravenously on Days 1 and 8. Alternately, animals were administered vehicle, 6 or 9 mg/kg of cleavable BCMA ADC, or 6 or 12 mg/kg of noncleavable ADC by intravenously bolus on Days 1, 21, and 43.

Hematology samples were collected at baseline, Days 2, 4, 8, and 10 for the bispecific; baseline, Days 8, 15, 29, and 46 for ADCs. Cytokine samples were collected predose and 6 hours postdose. Immunophenotyping samples were taken on similar days (not Day 8 for bispecific). Coagulation and clinical chemistry samples were collected and assessed at baseline and necropsy (Day 10 for bispecific, Day 46 for ADCs). Pharmacokinetic analyses were performed for the bispecific or ADCs by either MSD or the Gyrolab platform.

PBMCs were isolated from blood samples at baseline, Day 4, and Day 10 for the BCMA bispecific study, or at baseline and on Days 15, 29, and 46 for the BCMA ADC study. Bone marrow samples were collected at baseline and at necropsy (Day 10 for the bispecific and Day 46 for the ADCs) and single cell suspensions were prepared. Plasma cells in PBMC and bone marrow

samples were enumerated using an IgG-specific ELISPOT assay (ELISpot^{BASIC}, Mabtech) according to the manufacturer's instructions (see Supplementary Materials and Methods, for details).

Animals were euthanized on Day 10 for the bispecific and Day 46 for ADCs. During necropsy, tissues were harvested, preserved in 10% neutral buffered formalin (except for eye in 3% glutaraldehyde), sectioned, processed to slides, and stained with hematoxylin and eosin for microscopic analysis. All slides were assessed by a board certified veterinary pathologist (Supplementary Materials and Methods).

Statistical analysis

Statistical analysis was performed using the Prism software package (GraphPad) and statistical tests used are indicated in the figure legends.

Results

Generation and characterization of anti-BCMA CD3 bispecifics and ADCs

Antibodies cross-reactive to human and cynomolgus (cyno) BCMA were generated by panning a synthetic human antibody phage display library (Supplementary Materials and Methods; ref. 29). The affinity was determined by surface plasmon resonance (SPR) and the target binding determined by flow cytometry against BCMA positive and negative cells lines (Fig. 1A). Highly specific, species cross-reactive antibodies with nanomolar affinities were considered for further evaluation. A highly specific CD3 antibody was generated in mice and humanized, resulting in an antibody with a binding affinity of 17 and 14 nmol/L toward human and cyno CD3ε, respectively. BCMA antibodies were formatted into CD3 bispecifics using an IgG2 Fc containing mutations, EEE and RRRR, that aid in heavy chain heterodimer formation (30) and G2ΔA/D265A Fc mutations that reduce Fcγ receptor binding (31, 32) to prevent potential antibody clustering on immune cells and nonspecific T-cell activation (W.C. and J.CR., manuscript in preparation; Supplementary Materials and Methods). BCMA antibodies were also formatted into ADCs with auristatin payloads and valine-citrulline proteolytically cleavable linkers, using site-specific transglutaminase conjugation (Supplementary Materials and Methods; ref. 33). Schematics of both molecule classes are shown (Fig. 1B). Antibodies formatted into CD3 bispecifics demonstrated effective binding to myeloma cell lines and human T cells, indicating that the bispecific format did not affect recognition of either epitope (Fig. 1C). Antibodies formatted as ADCs also retained binding to cells (Fig. 1D). To evaluate the effect of BCMA antibody epitope on potency, 3 BCMA antibodies with similar affinities, but unique epitopes, were tested for cytotoxic activity using the CD3 bispecific format (Fig. 1E). All antibodies displayed similar potency, demonstrating that BCMA epitope is not an important driver of activity. To independently evaluate the role of antibody affinity toward BCMA, the affinity of one BCMA antibody was modulated through mutagenesis, resulting in antibodies with low, medium, and high affinity (5.99, 0.54, 0.04 nmol/L, respectively). A comparison of their cytotoxic activity in the CD3 bispecific format indicated that their potency correlated with affinity toward BCMA (Fig. 1F). Based on these results, a high-affinity BCMA clone was selected for further studies as both BCMA CD3 bispecific and BCMA ADCs.

The BCMA CD3 bispecific and ADCs are both effective mediators of tumor cell lysis *in vitro*

A panel of multiple myeloma cell lines with 2,000 to 16,000 BCMA receptors/cells were selected for determining the relationship between receptor expression and efficient killing (Fig. 2A). Bispecific treatment led to T-cell activation (Fig. 2B) and cytokine release (Fig. 2C). In cytotoxicity assays, the bispecific was highly potent against BCMA-positive target cells with at least 5,500 receptors/cell, demonstrated by the ability to induce target lysis at extremely low concentrations (EC_{50} 3–244 pmol/L; Fig. 2D). A significant negative correlation was observed between BCMA cell-surface expression levels and EC_{50} ($r = -0.91$, $P = 0.011$; Fig. 2E). The BCMA ADC with a cleavable linker and auristatin payload with a drug to antibody ratio of 4 (DAR4) also demonstrated robust activity against myeloma cell lines, but with decreased potency compared with the bispecific (3.5, 5.2, and 40.4-fold less potent in the 3 cell lines tested; Fig. 2D and F). A significant negative correlation was observed between BCMA cell-surface expression levels and EC_{50} ($r = -0.99$, $P = 0.015$; Fig. 2G). A lower limit of BCMA expression required for ADC activity was not determined. Additional ADC formats were also examined but were at best equivalent or in some cases less potent (Supplementary Fig. S1A and S1B).

BCMA expressed on plasma cells and myeloma tumor can be shed, resulting in soluble BCMA in the serum of myeloma patients (median = 4.9 nmol/L, range = 1.6 to >24 nmol/L; Supplementary Fig. S2A). The BCMA ligand APRIL is also shed and can be found in myeloma patient serum (median = 0.05 nmol/L, range = 0.001 to >2 nmol/L; Supplementary Fig. S2B). *In vitro* cytotoxicity assays were performed with the bispecific and cleavable ADC in the presence and absence of soluble BCMA. At the median patient concentration of 5 nmol/L soluble BCMA, an approximately 20-fold ($EC_{50} = 0.132$ nmol/L vs. 2.6 nmol/L) decrease in potency was observed for the bispecific and an approximate 14-fold (EC_{50} = 0.518 nmol/L vs. 7.345 nmol/L) decrease was observed for the ADC (Supplementary Fig. S2C–S2D). Soluble APRIL decreased the potency of the bispecific minimally even at the ∼median patient serum concentration of 0.08 nmol/L (EC_{50} = 0.132 nmol/L vs. 0.153 nmol/L; Supplementary Fig. S2E).

The BCMA CD3 bispecific and ADCs were efficacious against established orthotopic myeloma tumors

An orthotopic myeloma model using luciferase-labeled MM.1S cells injected intravenously was chosen for *in vivo* studies (Supplementary Materials and Methods). In this model, cells home to the bone marrow as seen by luminescent imaging and establish tumors in a manner similar to the human disease. Imagining captures tumor burden in both the bone marrow and extramedullary disease. A single dose of BCMA CD3 bispecific resulted in initial activity at all doses tested (Fig. 3A). Tumor outgrowth was seen in a dose-dependent manner. A single injection of the BCMA ADC with a cleavable linker resulted in tumor regression at all doses and tumor outgrowth at the lowest dose (Fig. 3B). The BCMA ADC was also effective in the noncleavable format and displayed dose-dependence (Fig. 3C). The noncleavable ADC appears to be more potent than the cleavable ADC and did not have tumor outgrowth at the 0.75 mg/kg dose. Doses required for activity were approximately 10- to 20-fold greater for ADCs compared with the CD3 bispecific. Strong *in vivo* activity for both modalities was observed against additional orthotopic tumor models (Supplementary Fig. S3 and S4).

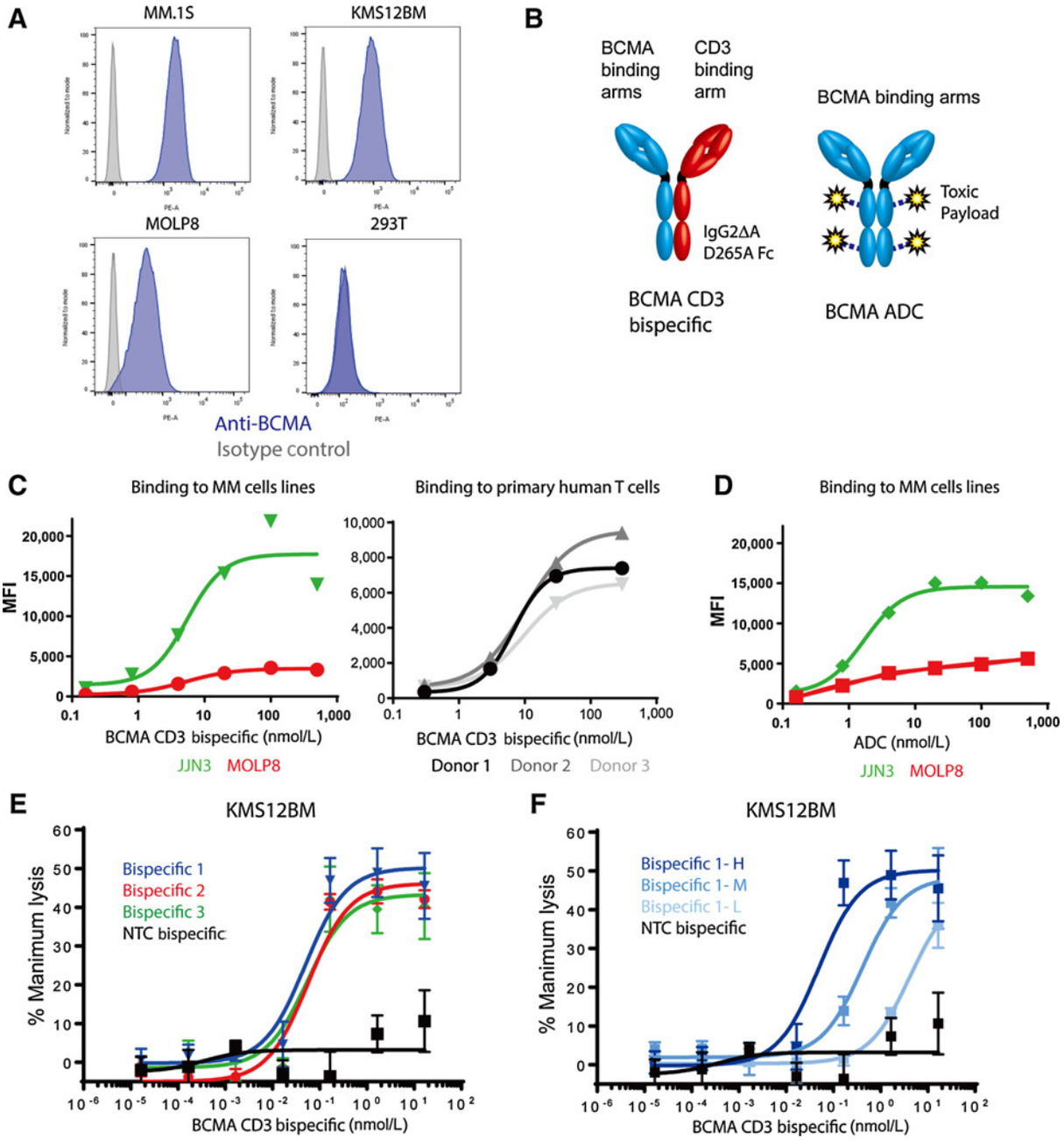


Figure 1.

Generation of anti-BCMA therapeutic molecules and characterization of cell binding and cytotoxicity. **A,** Antibodies with specific binding to BCMA, as seen in the representative plot, were selected for additional characterization. Antibodies were screened by flow cytometry against endogenously expressing myeloma cell lines MM.1S, KMS12BM, and MOLP8, and the BCMA-negative cell line, HEK293T. Blue and gray peaks represent staining with anti-BCMA and isotype control antibody, respectively. **B,** Schematic of the 2 anti-BCMA therapeutic molecule formats: a full-length fully human IgG2ΔA D265A bispecific generated by combining BCMA and CD3 targeting arms though hinge mutations and a BCMA ADC generated through site-specific conjugation of cleavable linkers and auristatin drug payloads with a drug-to-antibody ratio (DAR) of 4. **C,** The BCMA CD3 bispecific selected for additional analysis binds in a dose-dependent manner to 2 myeloma cell lines and to T cells from 3 donors, single points. **D,** The BCMA antibody selected for further BCMA ADC studies successfully binds to 2 BCMA-expressing myeloma cell lines when formatted as an ADC, single points. **E,** BCMA CD3 bispecific antibodies with different BCMA epitopes but comparable binding affinities showed similar cytotoxicity against KMS12BM. A NTC consisting of only the CD3 arm combined with a truncated Fc was used as a negative control. $n = 3$ technical replicates, results shown as mean ± SD. **F,** Higher affinity BCMA bispecifics against the same epitope are more potent in cellular toxicity assays. Three affinity variants with high (H), medium (M), and low (L) affinities were generated by affinity maturation of a parental antibody and formatted into CD3 bispecifics to evaluate effect of BCMA binding affinity on cytolytic activity. $n = 3$ technical replicates, results shown as mean ± SD.

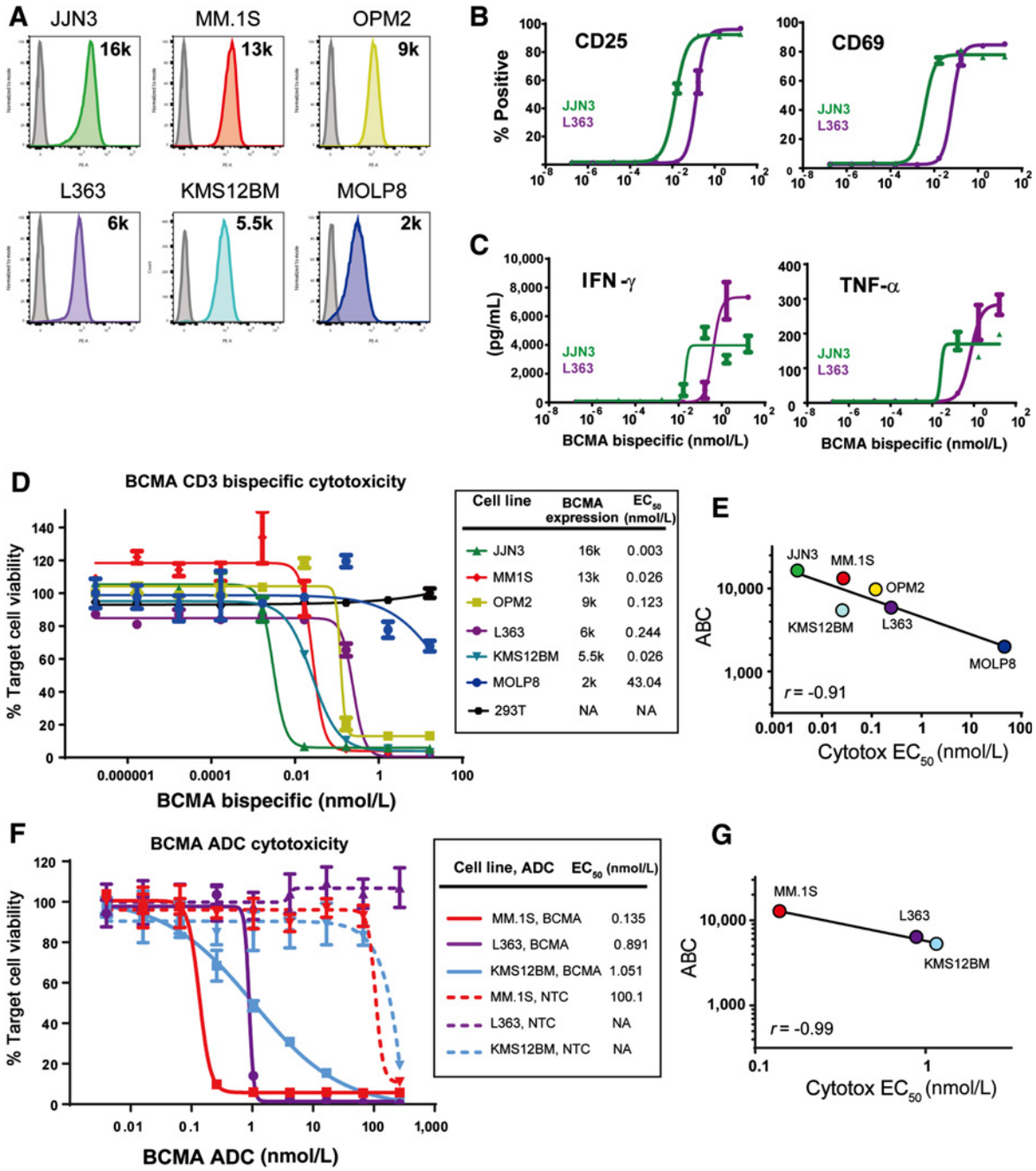


Figure 2.

BCMA CD3 bispecific induced cytokine release and was more potent *in vitro* than ADCs. **A,** Determination of BCMA cell-surface expression on a range of cell lines used for cytotoxicity assays. Receptor number indicated in thousands (k). **B,** BCMA CD3 bispecific activated T cells in the presence of BCMA target. Pan T cells were incubated with high (JJN3) or moderate (L363) BCMA expressing cell lines in the presence of BCMA CD3 bispecific at varying concentrations. Cells were analyzed for activation markers CD25 and CD69 by flow cytometry. **C,** BCMA CD3 bispecific mediated cytokine (IFNγ and TNFα) release in a dose-dependent manner, as seen by MSD analysis of co-culture supernatant. **D,** T cells potently lyse target cells in the presence of BCMA CD3 bispecifics. T cells and luciferase-labeled myeloma target cell lines were incubated at various concentrations of BCMA/CD3 bispecific for 48 hours, followed by One-glo luminescent measurement to assess target cell viability, $n = 3$. **E,** Potency of BCMA CD3 bispecific cytotoxicity negatively correlated with target cell BCMA cell-surface expression (ABC = antibody binding capacity; $r = -0.91$; $P = 0.011$ Pearson 2-tailed test). **F,** BCMA ADC with cleavable linker and auristatin toxic payload at DAR4 mediated myeloma target cell killing relative to NTC ADC (anti-BHV ADC). Three myeloma cell lines were incubated at varying concentrations of either BCMA ADC (solid lines) or NTC ADC (dashed lines) for 7 days. **G,** Correlation analysis of potency of BCMA ADC cytotoxicity and target cell BCMA cell surface expression ($r = -0.99$; $P = 0.015$, Pearson 2-tailed test). For activation, cytokine, and cytotoxicity assays, 3 biological replicates with 3 technical replicates each were performed. Data are shown for 1 representative biological replicate. Results shown as mean ± SEM.

The BCMA CD3 bispecific is more effective at mediating complete lysis of patient with primary multiple myeloma samples than BCMA ADCs

In vitro cytotoxicity assays utilizing myeloma primary patient bone marrow aspirates were developed (Supplementary Materials and Methods). Aspirates contain myeloma tumor cells and various other cell types, including T cells. The ratio of effector T cells to target myeloma tumor cells ranged from 3:1 to 1:2. In all 4 patient samples evaluated, the bispecific was able to effectively redirect patient T cells and drive near complete lysis of myeloma tumor cells (Fig. 4A). Potency was comparable to that seen with myeloma cell lines and healthy donor T cells [0.021–0.276 nmol/L EC_{50} in primary samples (Table 1) compared with 0.002–1.510 nmol/L EC_{50} in cell lines (Fig. 2D)]. The bispecific was able to eliminate greater than 95% of tumor cells in all patient samples, and >99% in 3 of the 4 patient samples.

BCMA ADCs with cleavable and noncleavable linkers were tested against 3 of the 4 primary patient samples. Both ADCs killed the majority of primary tumor cells from the first 2 patients, with EC_{50} in responsive patients ranging from 0.47 to 2.04 nmol/L (Fig. 4B and C). Unlike the bispecific, ADCs were only able to eliminate 84%, 74%, and 0% of myeloma cells in the 3 patient samples. BCMA expression on the cell surface of myeloma cells from all 4 patients was confirmed by flow cytometry, demonstrating that differences in activity were not because of the absence/presence of the target (Fig. 4D).

BCMA CD3 bispecific and ADCs are efficacious with an acceptable toxicology profile in cynomolgus monkeys

Cynomolgus monkey studies were performed to evaluate both activity and toxicity of the bispecific and ADC modalities (Supplementary Materials and Methods). PK parameters were assessed, and all molecules had the extended half-life expected of antibody therapies (Supplementary Fig. S5A–S5D; Supplementary Table S1). Both ADCs had stable conjugation of linker-drug to antibody as seen by comparison of total antibody and ADC (Supplementary Fig. S5C and S5D).

In the bispecific study, ELISPOT analysis of peripheral blood showed clear and rapid depletion of the majority of peripheral plasma cells in all bispecific-treated animals by Day 4 and total depletion by Day 10 (Fig. 5A; Supplementary Table S2). In the ADC studies, near complete elimination of all plasma cells in peripheral blood was seen on days 15, 29, and 46 for the cleavable ADC at all doses (Fig. 5B). The noncleavable ADC depleted greater than 90% of peripheral plasma cells by day 15, but was not capable of complete elimination, as was seen for the bispecific or cleavable ADC (Fig. 5C).

Unlike in humans, BCMA in cynomolgus monkeys is highly expressed on peripheral CD20-positive B cells (12). An approximate 10- to 12-fold depletion of blood B cells was observed by flow cytometry in bispecific-treated animals in both treatment groups at Day 4 and near complete depletion was seen at Day 10. No loss of B cells was observed in NTC-treated animals (Fig. 5D). BCMA ADCs were also able to eliminate peripheral B cells, but to a lesser extent (Fig. 5E and F). Peripheral T-cell dynamics were evaluated. No significant declines in T cells were seen at the end of studies with the bispecific or noncleavable ADC and a modest decrease was seen with the cleavable ADC (Supplementary Fig. S6A–S6F).

Plasma cell numbers in bone marrow were enumerated by ELISPOT analysis. All bispecific treated animals exhibited robust depletion of plasma cells on Day 10 (~200-fold decrease; Fig. 5G). However, in the bone marrow of ADC treated animals, no statistically significant depletion of plasma cells was seen with the cleavable ADC and modest/marked depletion of plasma cells was observed with the noncleavable ADC on day 46 (19-fold decrease at 12 mg/kg; Fig. 5H and I).

In the bispecific study, IFNγ, IL6, IL10, and IL2 concentration were measured prior to dosing and 6 hours postdose on Day 1 and Day 8 (Fig. 6A–D). Cytokines spiked in the bispecific-treated groups at 6 hours post the first dose. Cytokine concentrations returned to baseline prior to the second dose and no cytokine release was observed following the second dose in any of the groups, similar to published observations (34). Cytokines were not increased after administration of NTC antibody and cytokine analysis was not performed in the ADC studies.

The bispecific and ADCs were generally well tolerated with respect to clinical findings. Key bispecific findings included minimal mononuclear cell infiltrates in the liver and decreased lymphocytes in spleen, lymph node, and tonsil germinal centers, and decreased bone marrow cellularity (Fig. 6E). There were no findings for animals treated with NTC. Key toxicities for the cleavable ADC were generally more severe at the higher dose. Findings such as decreased cellularity in the bone marrow and decreased lymphocytes in the spleen and tonsils overlapped with those seen for the bispecific and were likely related to the killing of BCMA-expressing cells. Nonoverlapping findings were edema, inflammation, and interstitial fibrosis of the lung and single cell necrosis and corneal atrophy in the eye. These events were likely findings related to ADC payload toxicity because there is no known expression of BCMA in these tissues (18). For animals treated with the noncleavable ADC, on-target findings such as increased mitosis/single cell necrosis in the spleen and tonsils were observed. Notable additional findings in these animals were degeneration/single cell necrosis and glomerularnephropathy in the kidney and single cell necrosis of crypts and or glands of the stomach, duodenum and ileum (Fig. 6E). Findings in the stomach were accompanied by ulcers. These events were all seen at the higher dose of 12 mg/kg and were believed to be payload related. Animals treated with either the bispecific or the ADCs had minimal to mild injection site findings.

Discussion

This study is the first to perform a head to head comparison of CD3 bispecifics and ADCs. Because epitope and affinity can play a large role in activity of a CD3 bispecific (35–37), both were evaluated. Three antibodies with similar affinity, but varying epitope had similar cytolytic activity, suggesting that antibody epitope is less important in the case of BCMA. This may be a result of the extracellular domain comprising a mere 54 amino acids, meaning all epitopes are spatially close to the cell membrane, an important factor for CD3 bispecifics (35). When a single BCMA-specific antibody was affinity-tuned through mutagenesis, the high-affinity CD3 bispecifics and ADCs were more potent on BCMA-low cell lines. BCMA expression on patient tumors is reported to be low, with a median of ~1,500 (12), thus a high affinity antibody was selected for further comparison between the CD3 bispecific and ADCs (Supplementary Fig. S7).

The bispecific and ADCs successfully lysed myeloma cells with varying levels of BCMA cell-surface expression, with lower EC_{50} being observed against cell lines with higher cell-surface

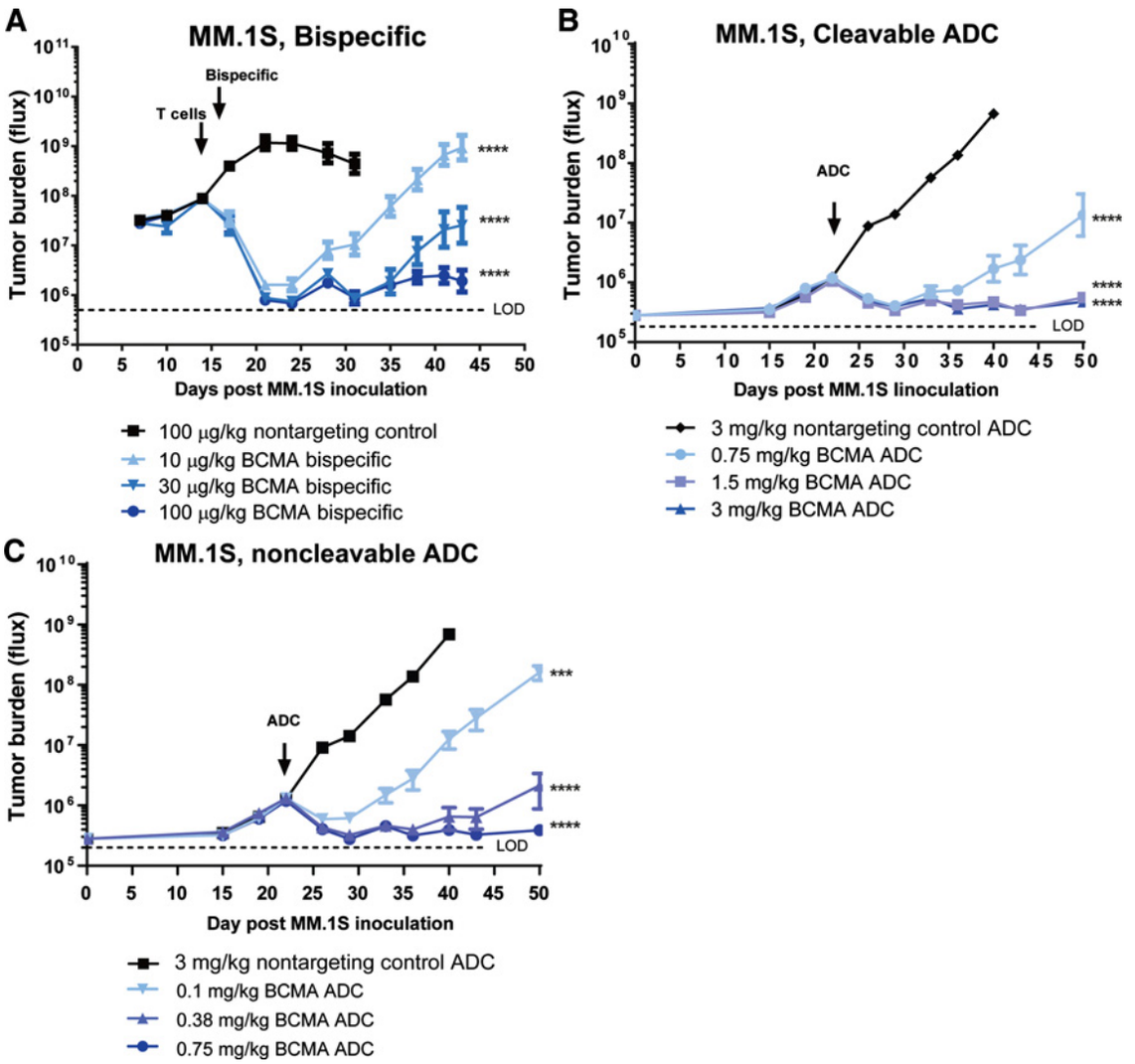


Figure 3.

BCMA CD3 bispecific and BCMA ADCs are highly active against established orthotopic xenograft tumors. **A,** BCMA CD3 bispecific was efficacious at all doses tested against an orthotopic MM.1S myeloma model expressing high levels of cell-surface BCMA. Luciferase-labeled MM.1S myeloma cells were inoculated intravenously. Fourteen days later, activated and expanded human T cells were injected intraperitoneally, followed 2 days later by a single intravenous dose of BCMA CD3 bispecific or NTC. **B,** BCMA cleavable ADC demonstrated dose-dependent efficacy against an established orthotopic MM.1S tumor cell model. Luciferase-labeled MM.1S myeloma cells were inoculated intravenously. Animals were administered a single intravenous dose of BCMA ADC or NTC ADC 22 days after tumor inoculation. ADCs utilized a cleavable linker and were DAR4. **C,** BCMA noncleavable ADC demonstrated dose-dependent efficacy against an established orthotopic MM.1S tumor cell model. Luciferase-labeled MM.1S myeloma cells were inoculated intravenously. Animals were administered a single intravenous dose of BCMA ADC or NTC ADC 22 days after tumor inoculation. ADCs utilized a noncleavable linker and were DAR4. All studies were performed in immune compromised NSG mice with $n = 7$–10 mice per group. *Y*-axis represents whole body tumor burden. All data shown as mean ± SEM. Tumor growth was monitored by luminescent imaging twice weekly on an IVIS imager. Statistics represent RMANOVA with Dunnett *post-hoc* test, all groups compared to NTC (****, $P < 0.0001$; **, $P < 0.01$), and Wilcoxon signed-rank test, **C,** single dose compared with double dose (*, $P = 0.012$).

expression levels of BCMA, consistent with BCMA expression being a driver of activity in both modalities. Kinetics of lysis varies greatly between CD3 bispecifics and ADCs because of a difference in mechanism of action. Optimal assay timing was determined and used for each modality (2 days with bispecific and 7 days with ADCs) so as to show optimal performance of each molecule and not bias toward one modality or the other. Against 3 cells lines, the bispecific was markedly more potent than the same antibody formulated as ADCs. The bispecific was further evaluated against additional cell lines once it was selected for development.

Both BCMA and its ligand, APRIL, are shed in patients with myeloma and can interfere with BCMA-based therapeutics through direct competition/blocking as reported by others (9). We found that soluble BCMA at median patient levels, but not soluble APRIL, decreased bispecific potency approximately 20-fold. Despite this shift in potency, the bispecific was still able to completely eliminate target cells. Soluble BCMA decreased

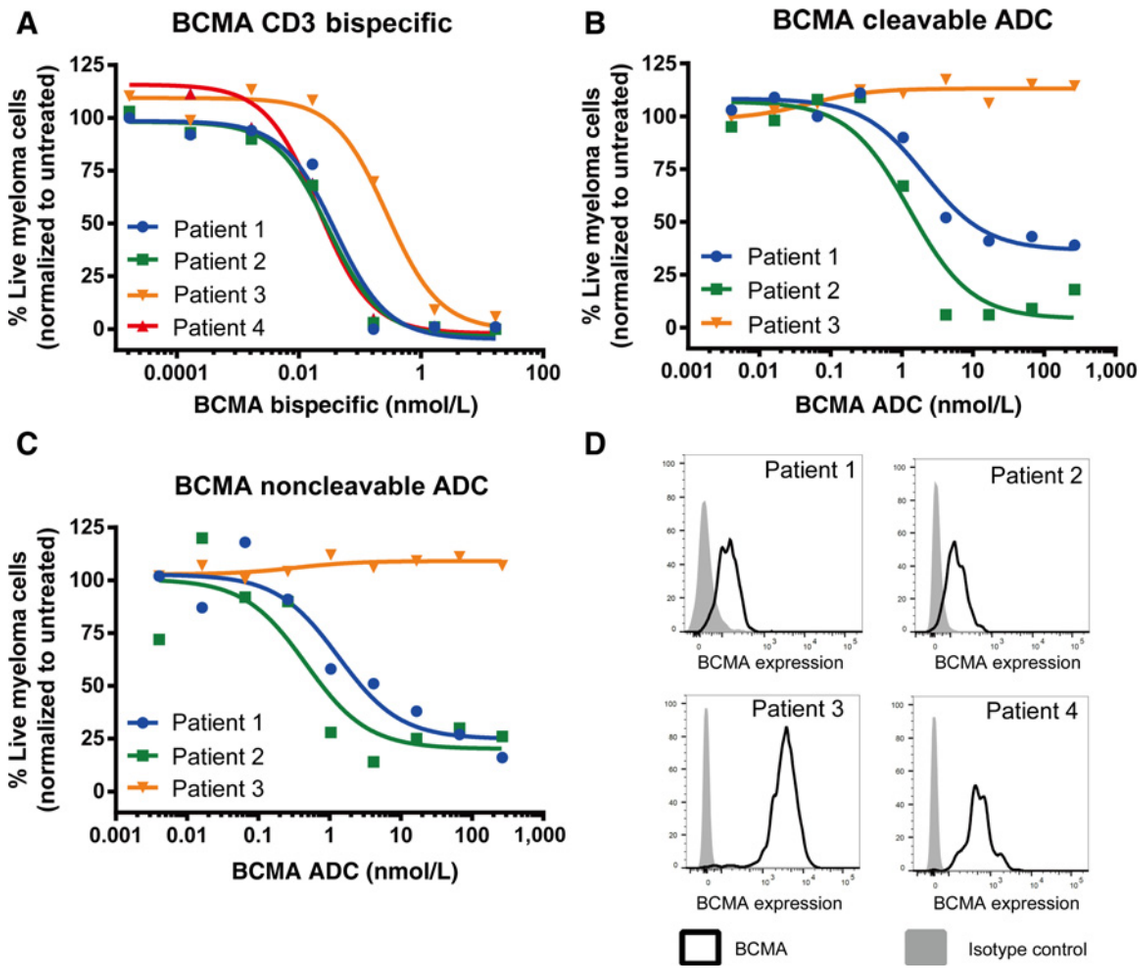


Figure 4.

BCMA CD3 bispecific effectively targeted primary tumor cells from myeloma patients and was more potent than BCMA ADCs. **A,** BCMA CD3 bispecific effectively lysed primary patient myeloma tumors cells from all patients tested. Fresh total bone marrow mononuclear cells were acquired from 4 myeloma patients and incubated with serial dilutions of BCMA CD3 bispecific. Target cell viability was determined by flow cytometry ($CD138^+$ staining). **B,** BCMA cleavable DAR4 ADC was active against 2 of 3 primary patient tumor samples. Fresh total bone marrow mononuclear cells were tested from 3 of the 4 patients with myeloma tested in **A** and incubated with serial dilutions of BCMA ADC. Target cell viability determined as in **A**. **C,** BCMA noncleavable DAR4 ADC was active against 2 of 3 primary patient tumor samples. Fresh total bone marrow mononuclear cells from the 3 patients tested in **B** were incubated with serial dilutions of BCMA ADC. Target cell viability determined as in **A**. **D,** Resistance of patient 3 tumor cells to either BCMA CD3 bispecific or BCMA ADCs is not accounted for by low BCMA expression. Flow cytometry analysis of BCMA cell surface expression in $CD38^+$ $CD138^+$ patient cells.

potency of the BCMA ADC to a similar extent as that seen with the bispecific. BCMA is cleaved/shed by γ-secretase so inhibition of this enzyme may be a way to mitigate the effects of soluble BCMA in patients (38). Soluble APRIL had a minimal effect on bispecific potency, likely because of its 800-fold lower affinity to BCMA relative to the bispecific (16 nmol/L vs. 0.02 nmol/L; ref. 39). Effects of the soluble BCMA ligand, BAFF, were not evaluated, but are predicted to be minimal because of its low affinity for BCMA (1,500 nmol/L; ref. 39).

Table 1. *In vitro* E:T ratio, potency, and maximal killing with patient samples

			EC_{50} (maximal killing)	
Patient	E:T ratio	Bispecific	Cleavable ADC	Noncleavable ADC
Patient 1	1:1	0.039 nmol/L (99%)	2.038 nmol/L (61%)	1.33 nmol/L (84%)
Patient 2	2:1	0.031 nmol/L (100%)	1.289 nmol/L (82%)	0.47 nmol/L (74%)
Patient 3	1:2	0.276 nmol/L (94%)	NA (0%)	NA (0%)
Patient 4	3:1	0.021 nmol/L (100%)	Not tested	Not tested

To evaluate bispecific and ADC efficacy *in vivo*, we developed orthotopic xenograft models utilizing human myeloma cell lines and human T cells in immune compromised mice. During bispecific model development, varying amounts of human T cells were injected and the tumor cell to T-cell ratio in the bone marrow was evaluated. Injection of 2×10^7 T cells per mouse resulted in a relevant ratio of approximately 1:1. This ratio is similar to that found in primary patient samples and thus all bispecific studies were run with injection of 2×10^7 T cells per mouse. Both bispecific and ADC modalities had significant efficacy, but the bispecific was more potent compared to the ADCs and showed

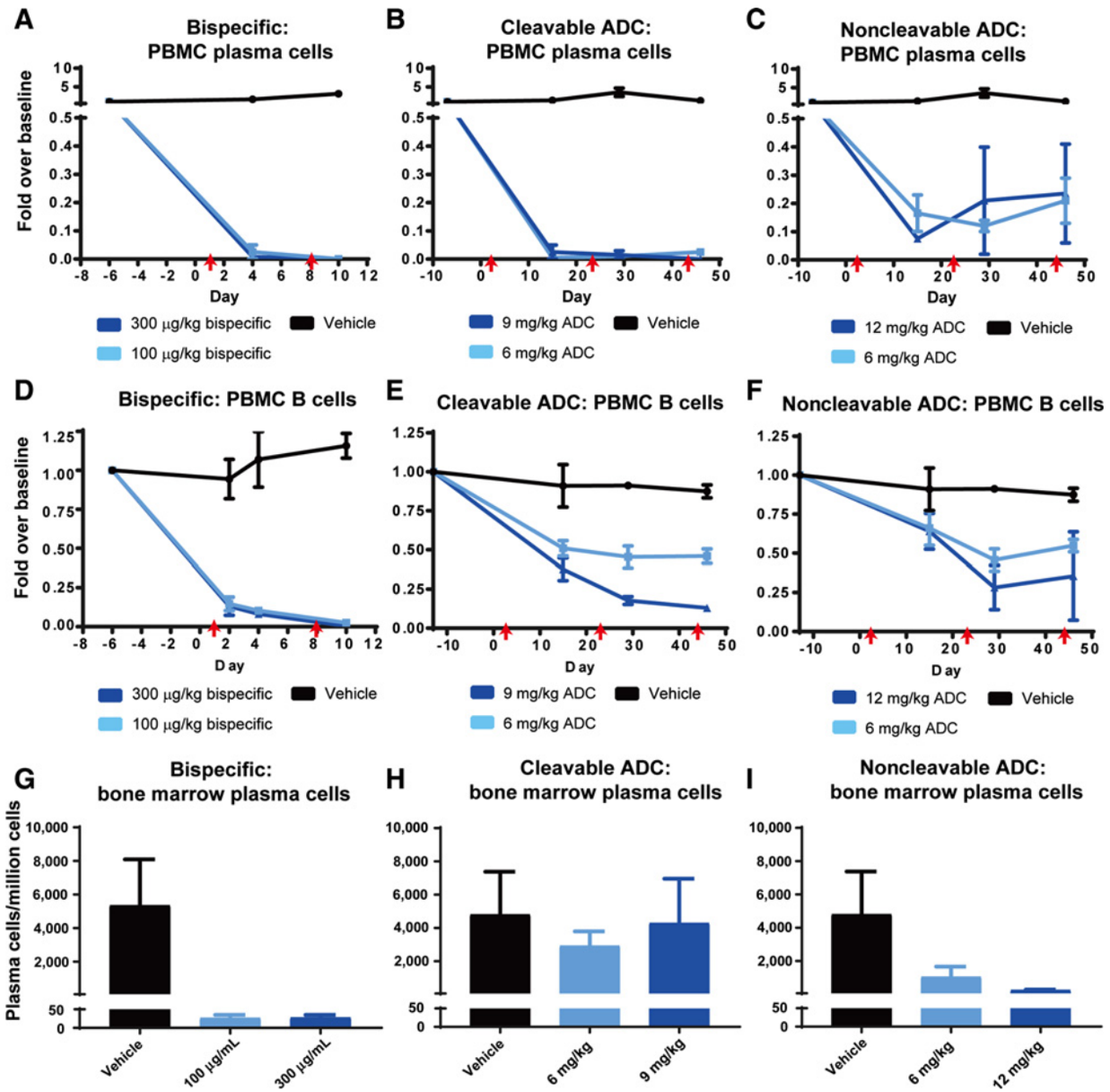
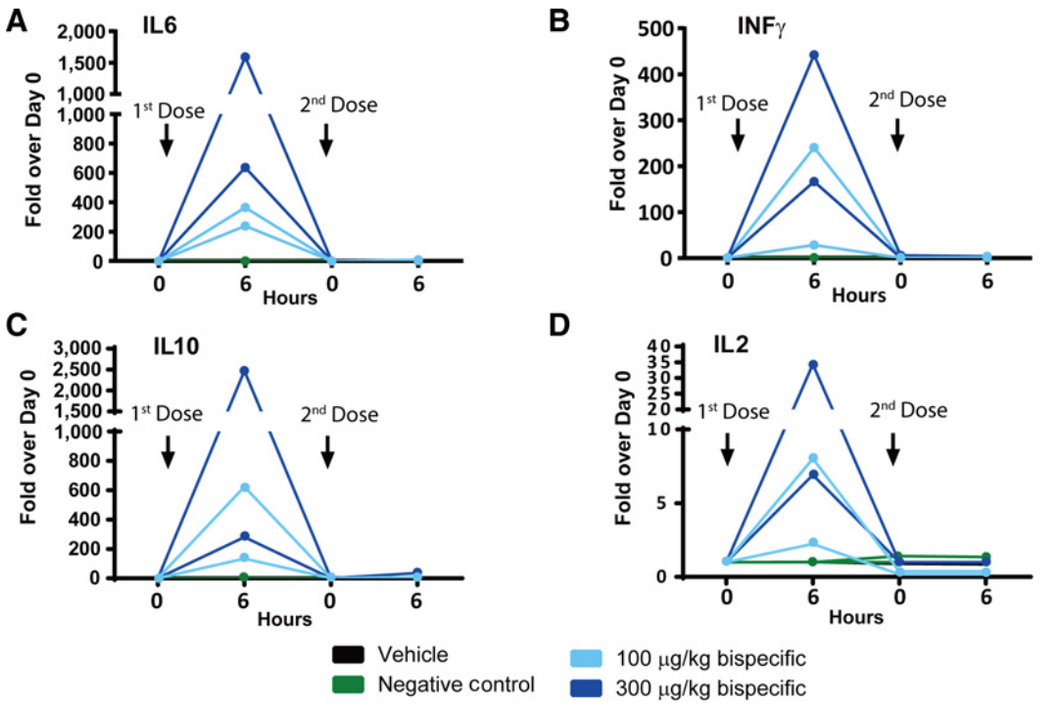


Figure 5.

BCMA CD3 bispecific and BCMA ADCs mediate depletion of plasma cells and B cells in the bone marrow and peripheral blood of cynomolgus monkeys. **A–C,** Peripheral plasma cell counts were decreased by BCMA CD3 bispecific, cleavable ADC, or noncleavable ADC treatment. Monkeys were dosed intravenously with test articles on either days 1 and 8 (bispecific) or days 1, 22, 43 (ADCs; red arrows). Plasma cells in blood samples were enumerated by ELISPOT on either days 4 and 10 (bispecific) or days 15, 29, and 46 (ADCs). **D–F,** BCMA CD3 bispecific, cleavable ADC, or noncleavable ADC treatment depleted peripheral B cells. B cells ($CD3^-$, $CD20^+$) in blood samples from monkeys were measured by flow cytometry prior to dosing and on days 2, 4, and 10 (bispecific) or days 15, 29, and 46 (ADCs). **G–I,** BCMA CD3 bispecific, cleavable ADC, or noncleavable ADC treatment depleted plasma cells in the bone marrow. Plasma cells were enumerated in bone marrow of monkeys by ELISPOT at necropsy on day 10 (bispecific) or day 46 (ADCs) All results shown as mean ± SEM, $n = 2$ animals per group for all studies.

tumor lysis even at a dose of 10 μg/kg, with full tumor control at 100 μg/mL. Tumor control was not seen with the same dose of 100 μg/kg noncleavable ADC or 750 μg/kg cleavable ADC. The noncleavable ADC appears to be more potent than the cleavable format and showed no tumor outgrowth at the 750 μg/kg dose, potentially because of increased stability of the molecule in the mice and greater drug exposure at the tumor site. However, studies were not run at the same time and exact comparisons of potency cannot be made. Myeloma is generally considered an indolent disease with a low proliferation index (40–42), thus use of antimitotic agents such as auristatins used with ADCs may not be the optimal mechanism of action to treat this cancer type. In



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Key Target Toxicities	BCMA CD3 Bispecific		BCMA ADC (Cleavable)		BCMA ADC (Noncleavable)	
	0.1 mpk	0.3 mpk	6 mpk	9 mpk	6 mpk	12 mpk
Bone Marrow: Decreased cellularity and/or increased megakaryocyte	1	2	1-2	1-2		
Lymphoid Tissue (spleen, lymph nodes and tonsil): Decreased Lymphocytes at germinal center and/or increased mitoses/single cell necrosis	1-2	1-3	1-2	2-3	1-2	1-3
Liver: increased cellularity sinusoids, increased mitosis/single cell necrosis of sinusoids, infiltrating mononuclear cells	1	1				1
Lung: Edema/Inflammation, interstitial fibrosis			1-2	1-3		
Eye: increase mitosis/single cell necrosis, corneal atrophy, deposition pigment in cornea			1-2	2-3		
Kidney: degeneration/regeneration tubular epithelium, glomerularnephropathy, increased mitosis/single cell necrosis tubular epithelium						1-2
GI Tract (stomach, duodenum and ileum): increased mitosis/single cell necrosis glands and/or crypts					1	1-3
Injection Site inflammation/edema, perivascular thrombosis, increased mitoses and single cell necrosis, hyperplasia in epidermis			1	1-3	1-2	1-2

No findings in: heart, colon, jejunum, optic nerve, peripheral nerve, lacrimal gland gall bladder or skin.

Figure 6.

Comparison of toxicologic findings between different BCMA targeting modalities. **A–D,** BCMA CD3 bispecific treatment induced cytokine release. Cynomolgus monkeys were dosed intravenously with either BCMA CD3 bispecific, NTC, or vehicle on day 1 and day 8, and the serum concentrations of IL6 (**A**), IFNγ (**B**), IL10 (**C**), or IL2 (**D**) were measured by electrogenerated chemiluminescence (ECL) 6 hours post-dosing. Each animal is plotted individually and colored according to dose ($n = 2$ animals per group). The vehicle line (black) is directly behind the NTC line (green) and not visible. **E,** Pathology assessment of cynomolgus monkeys treated with BCMA CD3 bispecific and ADCs revealed decreased lymphocytes or increased single cell necrosis/mitosis in spleen, tonsil, or lymph nodes across all treatment groups, lung and eye toxicity in cleavable ADC treated animals, and kidney and stomach findings in noncleavable ADC treated animals. Color coding represents severity of toxicity as assessed by a board-certified veterinary pathologist on a grading scale of 1 to 5 representing minimal (1), mild (2), moderate (3), marked (4), and severe (5) with the colors representing the highest severity score in the tissue: light yellow = minimal, yellow = mild, orange = moderate and green = no findings relative to control tissues.

contrast to primary myeloma cells, the cell lines used in the in vivo models are highly proliferative and susceptible to microtubule inhibitors, thus the antitumor activity observed with the ADCs may be overestimated.

The bispecific and ADC molecules were tested against primary myeloma patient bone marrow aspirates to model response in the clinic. Such aspirates can be regarded as a complex representation of a tumor microenvironment containing both effector T cells and other enhancing or suppressive cells found in the bone marrow. Most samples were from patients who received multiple prior lines of therapy and were relapse/refractory, thus patients with high unmet need (Supplemental Table S3). To be clinically relevant, only patient T cells present in the aspirates were used as the effector cells in the assays. The bispecific effectively lysed greater than 95% of tumor cells in all patient samples tested, demonstrating activity against patient myeloma cells and patient T-cell functionality. The ADCs killed myeloma cells from 2 patients and had decreased maximal killing and potency relative to the bispecific. Tumor cells from the ADC-resistant patient expressed cell-surface BCMA, but were resistant to additional toxic payloads, suggesting a broad resistance to the ADC modality. The limited number of primary samples tested does not allow conclusive declaration of a superior modality, but a clear trend is seen toward greater potency and maximal killing with the BCMA bispecific. Inability of the bispecific molecule to eliminate MOLP8 cells (BCMA expression of ~2,000/cell), suggests the bispecific may be inefficient against primary myeloma patient samples (median expression of ~1,500/cell). However, the primary cell killing data and similar results with BCMA bispecific molecules observed previously by other groups demonstrate strong activity of this modality against primary samples (12). Inability to kill MOLP8 cells may be because of additional resistance mechanisms beyond low expression of BCMA. Correlation of clinical outcome and BCMA expression data will help determine if a diagnostic BCMA expression test will aid in patient selection.

Cynomolgus monkey studies were performed to evaluate toxicity of both modalities. Acceptable PK parameters to support therapeutic development were observed for all molecules. Clear elimination of plasma cells from peripheral blood and bone marrow of bispecific and ADC-treated animals was seen, with near complete elimination in both compartments for the bispecific. The cleavable ADC was best able to clear plasma cells from the periphery, whereas the noncleavable ADC displayed better activity in the bone marrow. One hypothesis is that while the stability of both ADCs is high in the periphery, the noncleavable ADC is more stable in the bone marrow and this stability results in greater active drug in this compartment. Cynomolgus monkey B cells also express BCMA, and a robust depletion of these cells was observed (12). Depletion of plasma cells illustrated that the bispecific molecule can eliminate very rare cell populations (0.049% in blood), whereas prevalent peripheral B-cell elimination demonstrated the ability to clear large cell populations similar to what might be seen with a high tumor burden.

Administration of the bispecific was generally well tolerated in cynomolgus monkeys and lack of unanticipated findings supported the absence of BCMA expression in normal tissues outside the B-cell lineage. Of note, cytokine release was only observed after the initial dose of the bispecific. This has been described previously in primate models (34) and in the clinic (43) and suggests an initial low dose followed by higher doses may decrease the risk of cytokine release and toxicity in the clinic. Toxicity consistent with BCMA expression was also seen with the ADC molecules and included deceased cellularity/lymphocytes in the bone marrow and spleen. However, additional findings were observed and considered to be related to the toxic payload. Key payload-related findings were lung inflammation and necrosis/atrophy in the eye for the cleavable ADC and kidney degeneration and stomach inflammation/erosion for the noncleavable ADC. Different toxicity profiles between ADCs suggest linker/payload stability also plays an important role in safety.

Of the ADCs tested, the noncleavable ADC showed improved efficacy and was better tolerated *in vivo*. It should be noted that alternate ADC formats exist with additional payloads/linkers and these may show improved activity; however, improved potency is generally accompanied by increased toxicity (18). Evaluation of the bispecific and ADC modalities for pharmacodynamic activity and toxicity in the cynomolgus monkey demonstrate that the bispecific modality has the potential to be more efficacious based on ability to reduce plasma cells in peripheral blood and bone marrow and has a better toxicity profile in which findings are generally limited to BCMA expression.

In summary, both modalities appear promising. A BCMA ADC has indeed already proven clinically successful with the robust data and FDA fast-track designation of GSK2857916 (44). A wave of BCMA CD3 bispecifics are now entering the clinic and their success is currently unknown, but the nonclinical efficacy and safety data presented in this manuscript help tie the exciting ADC clinical data to what may be expected with the bispecific modality and suggest bispecifics should perform equally well or better than the ADC, particularly in patients with decreased expression of BCMA target. Given all of the above, the bispecific candidate described here has been advanced into phase I clinical trials (28).

Disclosure of Potential Conflicts of Interest

T.C. Kuo is a Director of Biology at ALX Oncology. Y. Zhang is a principal scientist at Allogene Therapeutics; and has ownership interest (including patents) in Allogene Therapeutics. L. Aschenbrenner is a Drug Development Leader at Covance Inc. C. Kamperschroer is an Associate Fellow at Pfizer, Inc.; and has ownership interest (including patents) in Pfizer, Inc. Russell G Dushin has ownership interest (including patents) in Pfizer Stock. T.J. Van Blarcom is a Head of Protein Engineering at Allogene Therapeutics; and has ownership interest (including patents) in Patent. M. Mirsky is an Associate Research Fellow at Pfizer. B. Buetow has ownership interest (including patents) in Pfizer. Thomas G. Martin reports of receiving commercial research grant from Sanofi; and is a consultant/advisory board relationship member of Roche. D. Shelton has ownership interest (including patents) in Pfizer; and is a consultant/advisory board relationship member of Pfizer. A. Rajpal has ownership interest (including patents) in Pfizer and BMS. P. Strop has ownership interest (including patents) in Pfizer. B.J. Sasu is a consultant at Allogene Therapeutics; and has ownership interest (including patents) in Allogene Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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