The Monoclonal Antibody nBT062 Conjugated to Cytotoxic Maytansinoids Has Selective Cytotoxicity Against CD138-Positive Multiple Myeloma Cells *In vitro* and *In vivo*

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Abstract

Purpose: We investigated the antitumor effect of murine/human chimeric CD138-specific monoclonal antibody nBT062 conjugated with highly cytotoxic maytansinoid derivatives against multiple myeloma (MM) cells *in vitro* and *in vivo*.

Experimental Design: We examined the growth inhibitory effect of BT062-SPDB-DM4, BT062-SMCC-DM1, and BT062-SPP-DM1 against MM cell lines and primary tumor cells from MM patients. We also examined *in vivo* activity of these agents in murine MM cell xenograft model of human and severe combined immunodeficient (SCID) mice bearing implant bone chips injected with human MM cells (SCID-hu model).

Results: Anti-CD138 immunoconjugates significantly inhibited growth of MM cell lines and primary tumor cells from MM patients without cytotoxicity against peripheral blood mononuclear cells from healthy volunteers. In MM cells, they induced G_2-M cell cycle arrest, followed by apoptosis associated with cleavage of caspase-3, caspase-8, caspase-9, and poly(ADP-ribose) polymerase. Nonconjugated nBT062 completely blocked cytotoxicity induced by nBT062-maytansinoid conjugate, confirming that specific binding is required for inducing cytotoxicity. Moreover, nBT062-maytansinoid conjugates blocked adhesion of MM cells to bone marrow stromal cells. The coculture of MM cells with bone marrow stromal cells protects against dexamethasone-induced death but had no effect on the cytotoxicity of immunoconjugates. Importantly, nBT062-SPDB-DM4 and nBT062-SPP-DM1 significantly inhibited MM tumor growth *in vivo* and prolonged host survival in both the xenograft mouse models of human MM and SCID-hu mouse model.

Conclusion: These results provide the preclinical framework supporting evaluation of nBT062-maytansinoid derivatives in clinical trials to improve patient outcome in MM.

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The cell surface proteoglycan CD138 (syndecan-1) is an integral membrane protein acting as a receptor for the extracellular matrix. Within the normal human hematopoetic compartment, CD138 is expressed on differentiated plasma cells and is a primary diagnostic marker of multiple myeloma (MM; ref. 1). The large extracellular domain of CD138 binds via its heparin sulfate chains to soluble extracellular molecules, including the growth factors epidermal growth factor, fibroblast growth factor, and hepatocyte growth factor, and to insoluble extracellular molecules, such as collagen and fibronectin (2, 3). CD138 also mediates cell-cell adhesion through interactions with heparin-binding molecules. Studies of plasma cell differentiation show that CD138 is a differentiation antigen (4) and a coreceptor for MM growth factors (5).

Several monoclonal antibodies (mAb; i.e., B-B4, BC/B-B4, B-B2, DL-101, 1 D4, MI15, 1.BB.210, 2Q1484, 5F7, 104-9, 281-2) specific for CD138 have been reported. B-B4, 1D4, and MI15 antibodies, which bind to similar or closely related epitopes,

Translational Relevance

CD138 is expressed on differentiated plasma cells and is a primary diagnostic marker of multiple myeloma (MM). In this study, we investigated the antitumor effect of murine/human chimeric CD138-specific monoclonal antibody nBT062 conjugated with highly cytotoxic maytansinoid derivatives against MM cells *in vitro* and *in vivo*. We first examined the growth inhibitory effect of BT062-SPDB-DM4, BT062-SMCC-DM1, and BT062-SPP-DM1 against MM cell lines and primary tumor cells from MM patients. We then examined *in vivo* activity of these agents in murine of human MM xenograft model and SCID-hu mice model, in which human MM cells injected into fetal bone chips implanted s.c. in severe combined immunodeficient mice. Importantly, nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 all have antitumor activity against MM cells and can overcome the protective effects of cytokines and bone marrow stromal cells. Our results, therefore, provide the preclinical framework for clinical trials of these agents to improve patient outcome in MM.

recognize both the intact CD138 molecule and the core protein (with the heparin sulfate chains removed) and target the same or closely related epitopes. B-B4 preferentially binds to membrane-bound versus soluble CD138 (6). It is a murine IgG1 mAb that binds to a linear epitope between residues 90 to 95 of the core protein on human syndecan-1. Consistent with the expression pattern of CD138, B-B4 strongly reacts with the MM cell line RPMI8226, but not with endothelial cells. Moreover, B-B4-saporin immunotoxin is highly cytotoxic to RPMI8226 cells (7).

mAbs are emerging as an important cancer therapy (8), particularly for the treatment of hematologic malignancies. For example, anti-CD20 mAb rituximab, which induces lysis and apoptosis of normal and malignant human CD20-positive B cells by complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, is now broadly used to treat CD20-positive cancers. Additional mAb-based therapies have been developed for treatment of non–Hodgkin lymphomas, including the radioimmunotherapeutics 90Y-ibritumomab tiuxetan and ^{131}I-tositumomab (9). These treatment options have distinctive mechanisms of action, with rituximab mediating complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity whereas radioimmunoconjugates represent a targeted systemic radiation approach.

The recent progress in therapeutic mAbs has both established these agents as a cornerstone of targeted therapies for cancer and renewed interest in the development of mAbs conjugated to potent cytotoxic agents. The first of these new targeted agents to be approved by the U.S. Food and Drug Administration was gemtuzumab ozogamicin (10), an immunoconjugate of an anti-CD33 mAb chemically linked to the potent cytotoxic calicheamicin, which can achieve complete remission in ~15% of patients with acute myelogenous leukemia in first relapse (11, 12). More recent efforts are directed at improving selectivity and potency of the immunoconjugates. One such advance is the development of the antibody-maytansinoid conjugates, which are structurally composed of a mAb that binds to a target antigen and a small maytansinoid cytotoxic agent stably linked to the antibody. Such conjugates are much more selective in their cytotoxicity than the parent cytotoxic maytansine. Here, we show the antitumor efficacy of three novel anti-CD138 antibody-maytansinoid conjugates, nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1, which vary in the linkage between the maytansinoid moiety and mAb. The nBT062-SMCC-DM1 linkage contains a thioether bond, which is not cleavable by disulfide exchange, whereas the nBT062-SPDB-DM4 and nBT062-SPP-DM1 conjugates contain disulfide linkages, which can be cleaved by disulfide exchange, resulting in liberation of active maytansinoid agent. The anti-CD138 antibody nBT062 is a murine/human chimeric form of B-B4, with identical specificity for CD138 as the parent murine antibody. The observed preclinical antitumor activity of the nBT062-maytansinoid conjugates provides the framework for clinical development of these agents to improve patient outcome in MM.

Materials and Methods

Cell culture. Dexamethasone-sensitive (MM.1S) and resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University). RPMI8226, MOLP-8, and U266 human MM cell lines were obtained from American Type Culture Collection. Doxorubicin-resistant (RPMI-DOX40) and Melphalan-resistant (LR5) cells were kindly provided by Dr. William Dalton (Lee Moffitt Cancer Center). OPM1, INA-6, and OPM2 plasma cell leukemia cell lines were kindly provided by Dr. Edward Thompson (University of Texas Medical Branch). All MM cell lines were cultured in RPMI 1640 (Sigma), and bone marrow (BM) stromal cells were cultured in DMEM (Sigma) containing 10% fetal bovine serum, 2 mmol/L L-glutamine (Life Technologies), 100 units/mL penicillin, and 100 mg/mL streptomycin (Life Technologies). The interleukin-6 (IL-6)–dependent INA-6 cell line was cultured in the presence of 1.0 ng/mL of human recombinant IL-6 (R&D Systems). Blood samples collected from healthy volunteers were processed by Ficoll Paque centrifugation to obtain peripheral blood mononuclear cells (PBMC). Patient MM and normal donor plasma cells were obtained from BM samples after informed consent was obtained per the Declaration of Helsinki and approval by the Institutional Review Board of the Dana-Farber Cancer Institute. Normal donor BM mononuclear cells were separated by Ficoll Paque density sedimentation, and plasma cells were purified (>95% CD138+) by positive selection with anti-CD138 magnetic activated cell separation micro beads (Miltenyi Biotec). Tumor cells were purified from the BM of patients with MM using the RosetteSep negative selection system (StemCell Technologies). RosetteSep antibody cocktail is added to BM samples, and CD138-negative cells are crosslinked to RBC (rosetted) with RosetteSep reagents, followed by incubation for 20 min at room temperature and separation by Ficoll density centrifugation, as described previously (13).

Immunofluorescence. Cells grown on glass coverslips were fixed in cold absolute acetone for 10 min. After fixation, cells were washed in PBS and then blocked for 60 min with 5% fetal bovine serum in PBS. Slides were then incubated with anti-CD138 antibody (Santa Cruz Biotechnology) for 24 h at 4°C, washed in PBS, and incubated with FITC-conjugated goat anti-mouse IgG for 1 h at 4°C. Slides were analyzed using Nikon E800 fluorescence microscopy, as previously described (14, 15).

Growth inhibition assay and proliferation assay. The growth inhibitory effect of nBT062-SMCC-DM1, nBT062-SPDB-DM4, nBT062-SPP-DM1, and dexamethasone on growth of MM cell lines, PBMCs, and BM stromal cells (BMSC) was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Chemicon International) dye absorbance,

as previously described (16). One antibody molecule has attached to it ~3.5 molecules DM4. The molecular weight of the antibody is not significantly increased by the attachment of the DM4 molecules.

The ability for nBT062-SPDB-DM4 to mediate antigen-dependent bystander killing of proximal CD138-negative cells was evaluated. CD138-positive MM OPM2 cells (1×10^{-4} per well) and CD138-negative Namalwa cells (3×10^{-3} per well) were plated separately or mixed in 96-well round-bottomed plates and exposed to nBT062-SPDB-DM4 for 120 h. Cell viability was then assessed using WST-8 reagent. To evaluate growth inhibitory effects of immunoconjugates against MM cells in the BM milieu, MM cells (2×10^4 per well) were cultured for 48 h in BMSC (1×10^4 per well) coated 96-well plates (Costar) in the presence or absence of the drugs. DNA synthesis was measured by [^{3}H]thymidine (Perkin- Elmer) uptake, with [^{3}H]thymidine (0.5 μCi/well) added during the last 8 h of 48-h cultures. All experiments were done in quadruplicate.

Cell cycle analysis. MM cells (1×10^6) were incubated with or without agents, washed with PBS, permeabilized by a 30-min exposure to 70% ethanol at −20°C, incubated with propidium iodide (50 μg/mL) in 0.5 mL PBS containing 20 units/mL RNase A (Roche Diagnostics) for 30 min at room temperature, and analyzed for DNA content by using flow cytometry.

Detection of apoptotic cells and caspase inhibitor. MM cells (1×10^6) were incubated with or without agents, washed with PBS, stained with PE-conjugated Apo 2.7 antibody (7A6, Beckman Coulter, Inc.), and analyzed on RXP Cytomics software on an Epics flow cytometer (Beckman Coulter, Inc.). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk; Bachem Bioscience, Inc.), a pan-caspase inhibitor was added in culture medium to reach a final concentration of 50 μmol/L. OPM1 cells were treated with or without *z*-VAD-fmk for 1 h before drug treatment.

In vivo *human MM MOLP-8 xenograft model.* Severe combined immunodeficient (SCID) mice were inoculated s.c. with MOLP-8 cells (1.5×10^7/mouse) in a mixture of serum-free media and Matrigel. Mice were randomized when tumors reached ~100 mm^3 and treated by bolus i.v. injection, as indicated. Mouse body weight was monitored as a sign of toxicity, and tumors were measured twice weekly in three dimensions using a caliper. The tumor volume was expressed in mm^3 using the formula V = length × width × height × 1/2. Mice were sacrificed when the tumors reached ~2,000 mm^3 or if the tumor became necrotic.

Green fluorescent protein–positive human MM xenograft mouse model and SCID-hu mouse model. OPM1 cells were transfected with green fluorescent protein (OPM1^{GFP+}) using a lentiviral vector, as previously described (17). CB17 SCID mice (48-54 days old) were purchased from Charles River Laboratories. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber Cancer Institute. Mice were inoculated s.c. with 5×10^6 OPM1^{GFP+} MM cells in 100 μL RPMI 1640. When tumors became palpable, mice were assigned into the treatment group receiving 200 μg conjugate per mouse via tail vein injection weekly or the control group receiving vehicle alone. Caliper measurements of the longest perpendicular tumor diameters were done every alternate day to estimate the tumor volume using the following formula representing the three-dimensional volume of an ellipse: 4 / 3 × (width / 2)^2 × (length / 2). Animals were sacrificed when tumors reached 2 cm or when moribund. Survival was evaluated from the first day of treatment until death. Tumor growth was evaluated using caliper measurements from the first day of treatment until day of sacrifice, day 10 for control, and day 21 for the nBT062-SPDB-DM4 treatment group. Mice were monitored by whole-body fluorescence imaging using Illumatool Bright Light System LT-9900 (Lightools Research) after shaving the tumor area. The images were captured with a Canon IXY digital 700 camera. *Ex vivo* analysis of tumor image was captured with a LEICA DM IL microscope connected to the LEICA DFC300 FX camera at 40 units/0.60 (Leica).

Human fetal long bones were implanted into CB17 SCID mice (SCID-hu), as previously described (18). Briefly, 4 wk after bone implantation, 2.5×10^6 INA-6 cells in a final volume of 100 μL of RPMI 1640 were injected directly into the human BM cavity in the SCID-hu mice. An increase in the levels of soluble human IL-6 receptor (shuIL-6R), which is released by INA-6 cells, was used as a parameter of MM cell growth and burden of disease in SCID-hu mice. Mice developed measurable serum shuIL-6R ~4 wk after INA-6 cell injection and then received 0.176 mg conjugate or vehicle control via tail vein injection weekly for 7 wk. After treatments, blood samples were collected and assayed for shuIL-6R levels by an ELISA (R&D Systems).

Flow cytometry. For CD138 staining, tumor cells were harvested, stained with anti-CD138-PE (Sigma Chemical), and analyzed using an Epics flow cytometer (Coulter Immunology).

Western blotting. MM cells were cultured with or without nBT062-SMCC-DM1, nBT062-SPDB-DM1, or nBT062-SPP-DM1, harvested, washed, and lysed using radioimmunoprecipitation assay buffer containing 2 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and 5 mg/mL complete protease inhibitor, as described previously (19, 20). Whole-cell lysates (20-40 μg/lane) were subjected to SDS-PAGE, transferred to pure nitrocellulose membranes (Bio-Rad Laboratories), and immunoblotted with antibodies against poly(ADP-ribose) polymerase (PARP), caspase-8, caspase-3, and caspase-9 (Cell Signaling Technology), as well as α-tubulin and CD138 (Santa Cruz Biotechnology).

Soluble CD138 ELISA. Supernatants from the cell cultures and BM plasma from MM patients was assayed for soluble (s)-CD138 using a solid phase sandwich ELISA kit (Cell Science) according to manufacturer's instructions. Samples were measured in triplicate, and assay range is 8 to 256 ng/mL.

Cell adhesion assay. BMSCs (1×10^4/well) were seeded in 96-well plates and cultured overnight at 37°C. On the following day, MM cells were washed thrice with PBS and resuspended in serum-free RPMI. Cells (2×10^5) in 100 μL media, with or without immunoconjugate, were added to each well. Each sample was run in triplicate. After 2 h of culture at 37°C, floating cells were removed by manual pipetting. The remaining adherent cells were cultured with 10% fetal bovine serum RPMI and pulsed with [^{3}H]thymidine added (0.5 μCi/well) for the last 8 h to measure DNA synthesis.

Statistical analysis. The statistical significance of differences observed in drug-treated versus control cultures was determined using

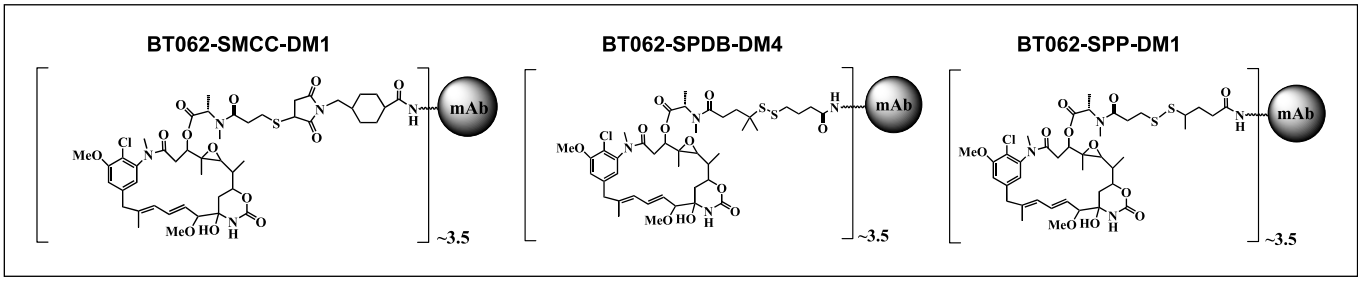

Fig. 1. Structure of nBT062-maytansinoid conjugates. nBT062-SMCC-DM1 contains a thioether-linkage that is not a substrate for disulfide exchange reactions. nBT062-SPDB-DM4 and nBT062-SPP-DM1 contain hindered disulfide linkages. Expression of CD138 in MM cell lines. CD138 expression in MM cell lines was determined by flow cytometry using anti-CD138–specific antibodies.

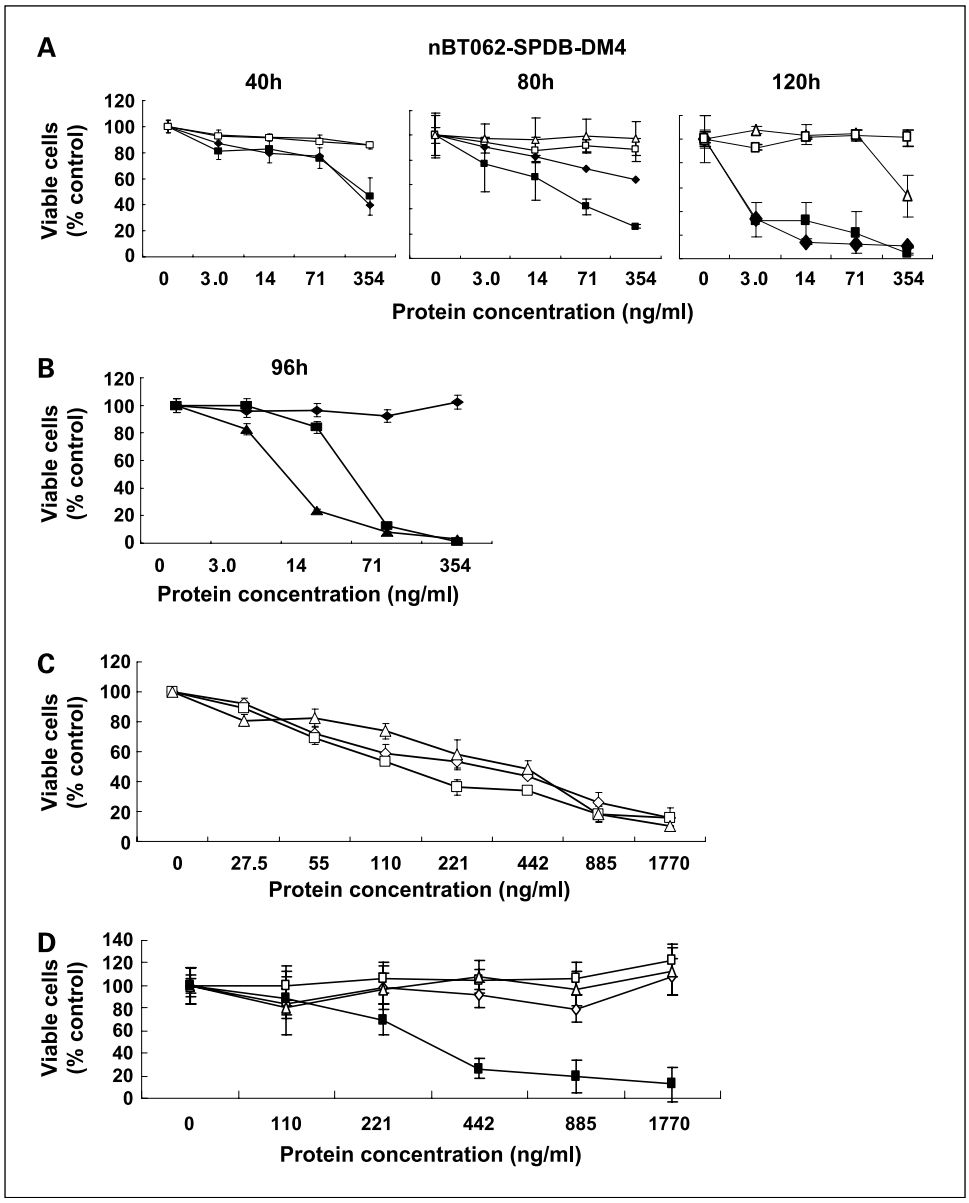

Fig. 2. nBT062-maytansinoid conjugates have selective cytotoxicity toward CD138-positive cell lines. *A,* CD138-positive OPM1 (■) and RPMI8226 (♦), and CD138-low expression MM cell lines DOX40 (□) and MM1S (△) were treated with nBT062-SPDB-DM4 for the indicated time periods. *B,* OPM1 cells were cultured with DM4 (■), nBT062 (♦), and BT062-SPDB-DM4 (▲) for the indicated time periods. *C,* primary tumor cells from MM patients were isolated by negative selection and cultured with nBT062-SMCC-DM1 (◊), nBT062-SPDB-DM4 (□), and nBT062-SPP-DM1 (△) for 48 h. *D,* PBMCs isolated from normal donors were cultured with nBT062-SMCC-DM1 (◊), nBT062-SPDB-DM4 (□), nBT062-SPP-DM1 (△) for 72 h, with OPM1 cells (■) serving as a positive control. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Points, mean of triplicate cultures, expressed as percentage of untreated controls; bars, SD.

Dunn's multiple comparison tests. The minimal level of significance was $P < 0.05$. For *in vivo* experiments, tumor volumes were compared using one-way analysis of Dunn's multiple comparison tests. Survival was assessed using Kaplan-Meier curves and log-rank analysis.

Results

Expression of CD138 in MM cell lines. We first evaluated the expression of CD138 in MM1S, OPM1, OPM2, RPMI8226, DOX40, MM1R, LR5, U266, MOLP-8, and INA-6 MM cell lines. Western blot analysis (Supplementary Fig. S1A) and flow cytometric analysis (Supplementary Fig. S1B) showed that CD138 is expressed on all MM cell lines tested except LR5 and DOX40. Immunofluorescence analysis (Supplementary Fig. S2) showed weak expression of CD138 on MM.1S, DOX40, and LR5 cells. All other cell lines showed high CD138 expression.

***Selective cytotoxicity of nBT062-maytansinoid conjugate against CD138-positive MM cell lines* in vitro.** The *in vitro* cytotoxicity of the anti-CD138 antibody nBT062 conjugated with maytansinoids DM1 and DM4 was next evaluated. The nBT062 conjugates tested vary in the chemical linker used to attach the maytansinoid molecule to the antibody. The chemical structures of nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 are shown in Fig. 1. nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 (3-354 ng/mL) showed cytotoxicity against OPM1 and RPMI8226 cells (CD138 positive) in a dose-dependent fashion. In contrast, minimal cytotoxicity was noted in low CD138 expression cell lines (Fig. 2A; Supplementary Fig. S3A and B). We also examined the cytotoxicity of free toxin DM4 and naked BT062 antibody compared with nBT062-SPDB-DM4. nBT062-SPDB-DM4 induced significantly greater cytotoxicity against OPM1 cells than free DM4 ($P < 0.05$) Moreover, naked BT062 antibody is not cytotoxic against OPM1 cells (Fig. 2B).

Importantly, these agents were also cytotoxic against primary tumor cells from MM patient isolated by negative selection,

with IC_{50} values of ~1 nmol/L (111-442 ng/mL) at 48 h (Fig. 2C). However, no cytotoxicity was observed against primary tumor cells from MM patients isolated by CD138-positive selection (Supplementary Fig. 4A) suggesting that CD138-binding is important for immunoconjugate-mediated cytotoxicity and that binding is blocked by the anti-CD138 antibody used in the positive selection procedure. Indeed, nonconjugated nBT062 completely abrogated cytotoxicity induced by nBT062-SPDB-DM4 in OPM2 cells (Supplementary Fig. S4B). Importantly, the maytansinoid conjugates did not induce cytotoxicity in PBMCs from healthy volunteers at concentrations as high as 12 nmol/L (1770 ng/mL), further showing the specificity of these agents for CD138-positive cells (Fig. 2D).

CD138-specific immunoconjugates induce cell cycle arrest, followed by caspase and PARP cleavage in OPM1 cells. Maytansinoids are antimitotic agents that inhibit tubulin polymerization and microtubule assembly, and the maytansinoids DM1 and DM4 induce growth arrest in tumor cells in the G_2-M phase of the cell cycle (21). We, therefore, next examined the cell cycle profile of OPM1 cells after nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 treatment. As shown in Fig. 3A and Supplementary Fig. S5, treatment of OPM1 cells with 1,000 ng/mL of these three agents for 24 hours induced a time-dependent increase in G_2-M phase cells. nBT062-SPDB-DM4 had the most potent effect. To determine whether the cytotoxicity induced by these agents is via an apoptotic mechanism, we carried out Apo 2.7 staining and assessed cleavage of caspases and PARP. Treatment of OPM1 cells with the maytansinoids significantly increased Apo 2.7-positive cells in a time-dependent fashion (Fig. 3B), associated with induction of cleavage of caspase-8, caspase-9, caspase-3, and PARP (Fig. 3C). Conversely, the pan-caspase inhibitor z-VAD-fmk (50 μmol/L) blocked nBT062-SPDB-DM4–induced caspase and PARP cleavage in OPM1 cells (Fig. 3D). These results indicate that cytotoxicity triggered by these maytansinoid conjugates is mediated via caspase-dependent (both intrinsic and extrinsic) apoptotic pathways.

The immunoconjugates overcome the protective effects of growth factors and BMSCs. Because IL-6 and insulin-like growth factor-I (IGF-I) promote MM cell survival by inhibiting apoptosis, we next examined whether these immunoconjugates can overcome this protective effect. Neither IL-6 nor IGF-I was able to block the cytotoxicity induced by nBT062-SPDB-DM4 (Fig. 4A and B). We also examined the cytotoxicity triggered by the conjugates in the context of BMSCs. Although BMSCs significantly inhibited dexamethasone-induced growth inhibition, they were not able to protect against immunoconjugate-induced cytotoxicity in OPM1 cells (Fig. 4C).

nBT062-SPDB-DM4 and nBT062-SPP-DM1 inhibit adhesion of MM1S cells to BMSCs. Many studies have revealed that

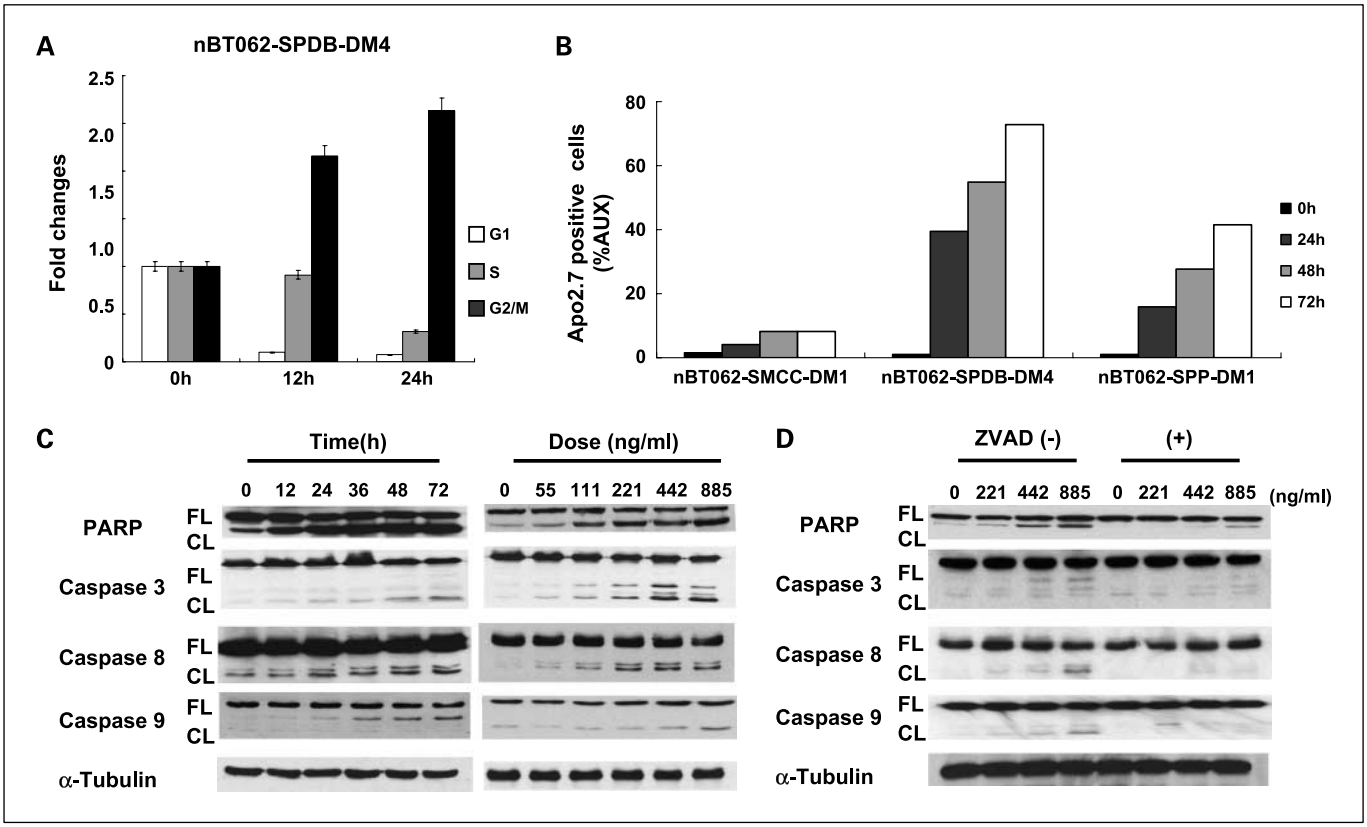

Fig. 3. nBT062-maytansinoid conjugates induce G_2-M growth arrest, followed by caspase-dependent apoptosis. *A,* cell cycle analysis; OPM1 cells were treated with nBT062-SPDB-DM4 for 0, 12, and 24 h and then subjected to propidium iodide staining. *B,* OPM1 cells were cultured with nBT062-maytansinoid (885 ng/mL) for 0 h (■), 24 h (▩), 48 h (▨), and 72 h (□). Apoptotic cells were assessed by Apo 2.7 staining using flow cytometric analysis. *C,* OPM1 cells were cultured with nBT062-SPDB-DM4 (885 ng/mL) for the indicated time periods. Cells were treated with increasing concentrations of nBT062-SPDB-DM4 (0-885 ng/mL) for 48 h. *D,* OPM1 cells were preincubated with Z-VAD-fmk (50 μmol/L) for 60 min before treatment with 24 h nBT062-SPDB-DM4. Total cell lysates were subjected to immunoblotting using anti–caspase-3, caspase-8, caspase-9, PARP, and α-tubulin antibodies. FL, full-length protein; CL, cleaved protein.

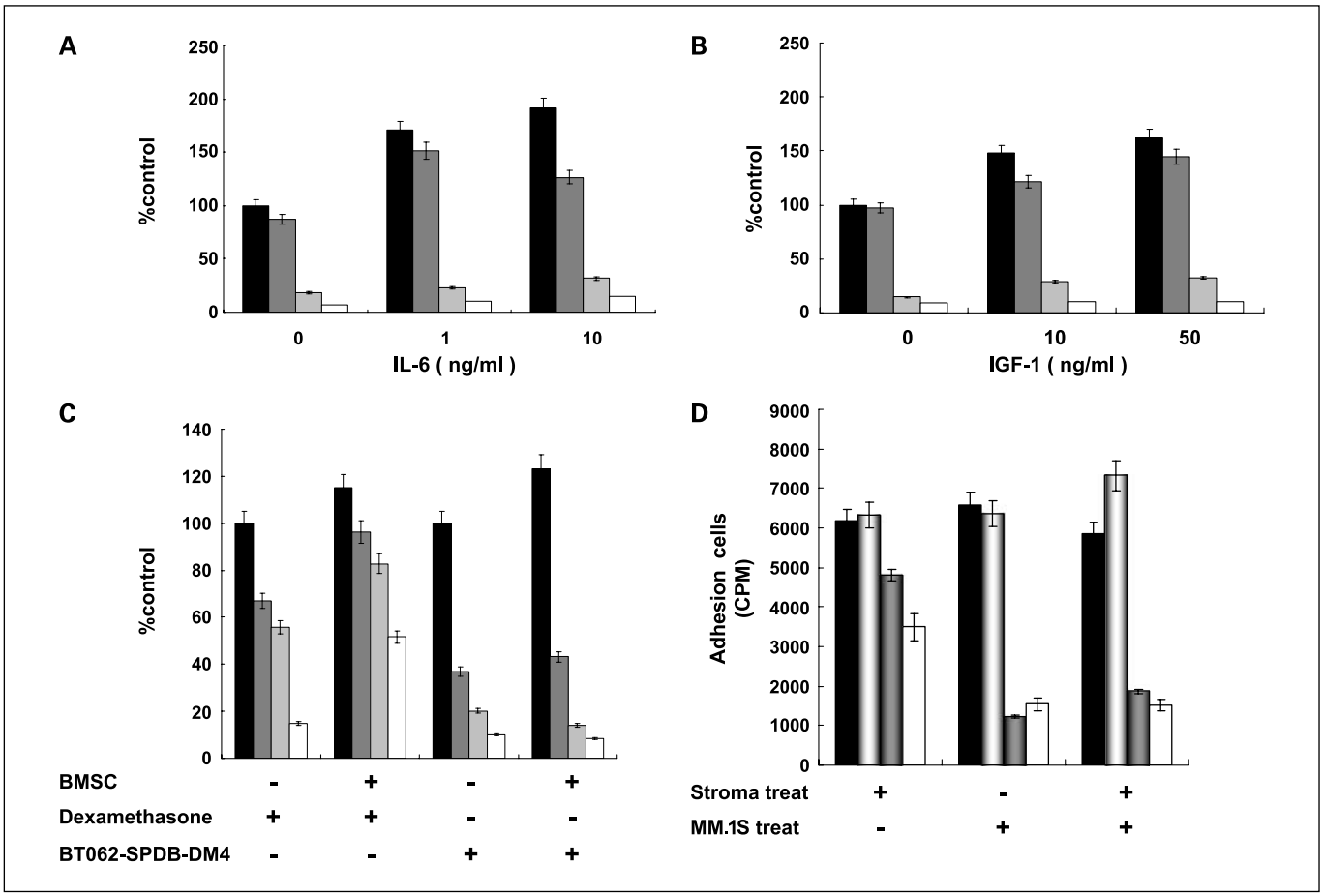

Fig. 4. Effect of growth factors and BMSCs on the sensitivity of MM cells to nBT062-maytansinoid immunoconjugates. OPM1 cells were cultured for 48 h with control media (■) or with nBT062-SPDB-DM4 at 55 ng/mL (▩), 111 ng/mL (▨), 221 ng/mL (□), in the presence or absence of IL-6 (*A*; 1 and 10 ng/mL), IGF-I (*B*; 10 and 50 ng/mL), or BMSCs (*C*). In BMSC coculture, cells were incubated for 48 h with control media (■) and 250 nmol/L (▩), 500 nmol/L (▨), 1,000 nmol/L (□) dexamethasone, in the presence or absence of BMSCs for 48 h, as a positive control for drug resistance. DNA synthesis was determined by measuring [^{3}H]thymidine incorporation during the last 8 h of 72 h cultures. Columns, means of triplicate cultures; bars, SD. *D,* MM1S cells and/or BMSCs were incubated with control media (■), nBT062-SMCC-DM1 (▮), nBT062-SPDB-DM4 (▩), and nBT062-SPP-DM1 (□) at 885 ng/mL for 2 h before adhesion. Adherent cells were assessed by measuring [^{3}H]thymidine uptake. Values represent the mean [^{3}H]thymidine incorporation (cpm) of triplicate cultures.

syndecan-1 (CD138) mediates interactions between cells and extracellular matrix proteins to function as an adhesion molecule (3, 4, 22). We, therefore, next evaluated whether these conjugates could inhibit MM cell adhesion to BMSCs. Pretreatment of BMSCs with nBT062-SPDB-DM4 had only a modest inhibitory effect on MM1S and OPM1 cell adhesion to BMSCs; however, pretreatment of MM1S cells with nBT062-SPDB-DM4 almost completely blocked MM1S cell adhesion to BMSCs, suggesting that CD138 mediates MM cell adhesion, which can be blocked by immunoconjugates (Fig. 4D).

Soluble CD138 levels are greater in MM cell culture supernatants than in BM plasma of MM patients. sCD138 can be cleaved by the action of secretases (23) and released from the cell surface, which may inhibit binding of anti-CD138 immunoconjugates to the MM cell surface. We, therefore, next measured sCD138 levels in MM cell culture supernatants and BM plasma from MM patients. As shown in Fig. 5, soluble CD138 concentrations in BM plasma from MM patients were lower than levels in culture supernatants from RPMI8226 and OPM1 MM cells. Because the immunoconjugates are cytotoxic against both RPMI8226 and OPM1 MM cells, these results suggest that levels of circulating sCD138 in BM plasma of MM patients will not inhibit binding of anti-CD138 immunoconjugate to MM cells.

nBT062-maytansinoid conjugates inhibit tumor growth in a human MM xenograft model and SCID-hu model. The *in vivo* efficacy of nBT062-SPDB-DM4, nBT062-SMCC-DM1, and nBT062-SPP-DM1 was next evaluated in SCID mice bearing established CD138-positive MOLP-8 human MM cells. A single i.v. administration of the immunoconjugates caused significant dose-dependent tumor growth inhibition and tumor regression at concentrations that were well tolerated, evidenced by stable body weight. nBT062-SPDB-DM4 was the most active conjugate tested in this model (Fig. 6A). In addition, weekly dosing of the nBT062-SMCC-DM1 (six doses of 13.8 μg/kg) completely blocked tumor growth during the dosing period (Supplementary Fig. S6A).

In a second study, the importance of antigen-targeting for the antitumor activity of nBT062-SPDB-DM4 and nBT062-SPP-DM1 was evaluated by comparing the activity of unconjugated maytansinoid DM4, native unmodified nBT062 antibody, and a nontargeting (irrelevant) huIgG1-SPDB-DM4 conjugate. Treatment with a single bolus i.v. injection of

nBT062-SPDB-DM4 and nBT062-SPP-DM1 (at a dose of ~14 mg/kg) inhibited the growth of the MOLP-8 xenografts (Fig. 6B); nBT062-SPDB-DM4 was the most active conjugate. In contrast, minimal antitumor activity was observed with free DM4, nBT062 antibody, and the nonbinding DM4 conjugate, showing the importance of specific targeting by the nBT062-maytansinoid conjugates for their *in vivo* efficacy.

The efficacy of nBT062-SPDB-DM4 and nBT062-SPP-DM1 was also examined in mice bearing s.c. fluorescent OPM1 MM cells ($OPM1^{GFP+}$; Supplementary Fig. S7). Treatment of OPM1 MM tumor-bearing mice with nBT062-SPDB-DM4 (0.176 mg conjugate per mouse; ~6 mg/kg) significantly inhibited MM tumor growth compared with control animals treated with control vehicles (Dunn's multiple comparison test; control vehicle versus nBT062-SPDB-DM4 at 10 days after treatment, $P < 0.01$; Fig. 6C). Similar to the results observed in the MOLP-8 model, nBT062-SPP-DM1 was not as effective as nBT062-SPDB-DM4 (Dunn's multiple comparison test; nBT062-SPP-DM1 versus nBT062-SPDB-DM4 at 10 days after treatment, $P < 0.05$). nBT062-SPDB-DM4 inhibited tumor growth (Supplementary Fig. S8A). Moreover, Kaplan-Meier and log-rank analysis revealed a mean overall survival of 13.6 days in the control cohort (95% confidence interval, 10-19 days) versus 26 days (95% confidence interval, 23-42 days) in groups treated with nBT062-SPDB-DM4 (Supplementary Fig. S8B). *Ex vivo* analysis of tumors excised from mice showed significantly increased apoptosis in the mice treated with nBT062-SPDB-DM4 versus control cohorts (Supplementary Fig. S9) Importantly, treatment with these agents did not affect body weight (Supplementary Fig. S10)

To examine the activity of nBT062-SPDB-DM4 and nBT062-SPP-DM1 on MM cell growth in the context of the human BM microenvironment *in vivo*, we next used a SCID-hu model, in which IL-6–dependent INA-6 cells are directly injected into a human bone chip implanted in SCID-mice. These SCID-hu mice bearing human bones engrafted with INA-6 cells were treated via tail vein with nBT062-SPDB-DM4, nBT062-SPP-DM1, or vehicle alone weekly for 7 weeks. The serum shuIL-6R levels released by INA-6 cells reflects tumor burden in this model. As shown in Fig. 6D, nBT062-SPDB-DM4 and nBT062-SPP-DM1 treatment caused significant inhibition of tumor growth compared with vehicle control.

Bystander killing. Antibody-maytansinoid conjugates similar to nBT062-SPDB-DM4 have been shown to be able to kill antigen-negative cells proximally to antigen-positive tumor cells (bystander killing; ref. 24). To determine whether nBT062-SPDB-DM4 mediates bystander killing, CD138-positive OPM2 cells and CD138-negative Namalwa cells, either cultured separately or together, were treated with nBT062-SPDB-DM4 for 120 hours. Whereas nBT062-SPDB-DM4 was inactive against CD138-negative Namalwa cells cultured alone, significant killing of the CD138-negative cells by nBT062-SPDB-DM4 was observed when cultured with CD138-positive OPM2 cells (Supplementary Fig. S11).

Discussion

CD138 is highly expressed on MM cells and is involved in their development and/or proliferation (2, 25, 26), making CD138 an attractive therapeutic target. CD138 may be a suitable target for an antibody-directed immunoconjugate, although the use of a murine antibody in prior studies has precluded their clinical development (27). In the current study, we have evaluated the antitumor activity of a series of immunoconjugates composed of the murine/human chimeric anti-CD138 antibody nBT062 conjugated with potent cytotoxic maytansinoid moieties. The immunoconjugates tested, nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1, vary in the nature of the disulfide linkage that attaches the cytotoxic agent to the antibody.

A series of MM cell lines were tested for CD138 expression using flow cytometry and immunoblotting, and these cell lines were used for the evaluation of the activity of the nBT062 conjugates. The nBT062-maytansinoid conjugates were highly active

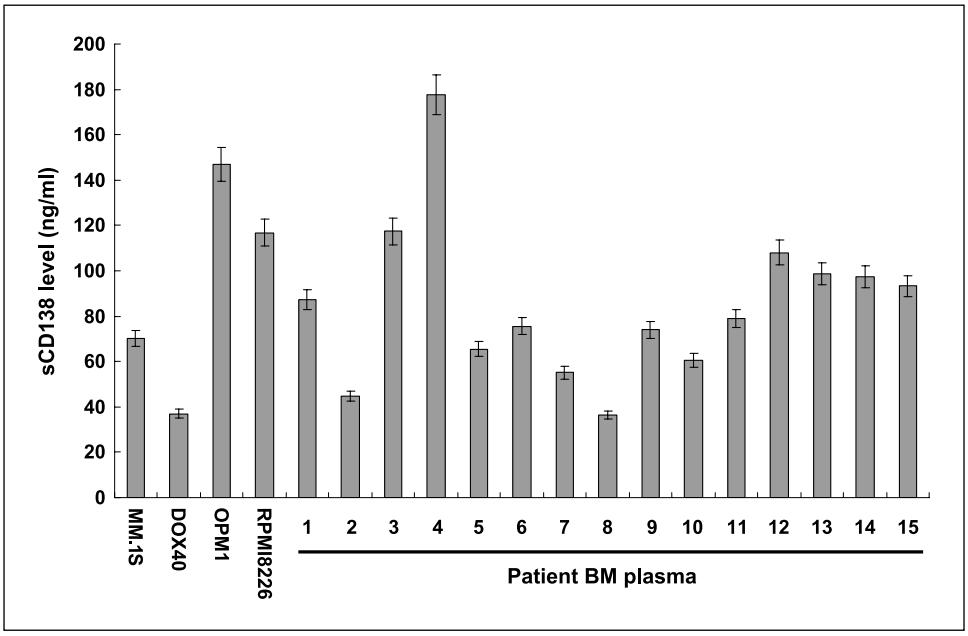

Fig. 5. Quantifications of sCD138 levels in cell culture supernatants from MM cell lines and BM plasma of MM patients, sCD138 levels in cell culture supernatants from MM.1S, RPMI8226, OPM1, and DOX40 MM cell lines, as well as from 15 BM plasma of MM patients, were measured by ELISA. Error bars, SD.

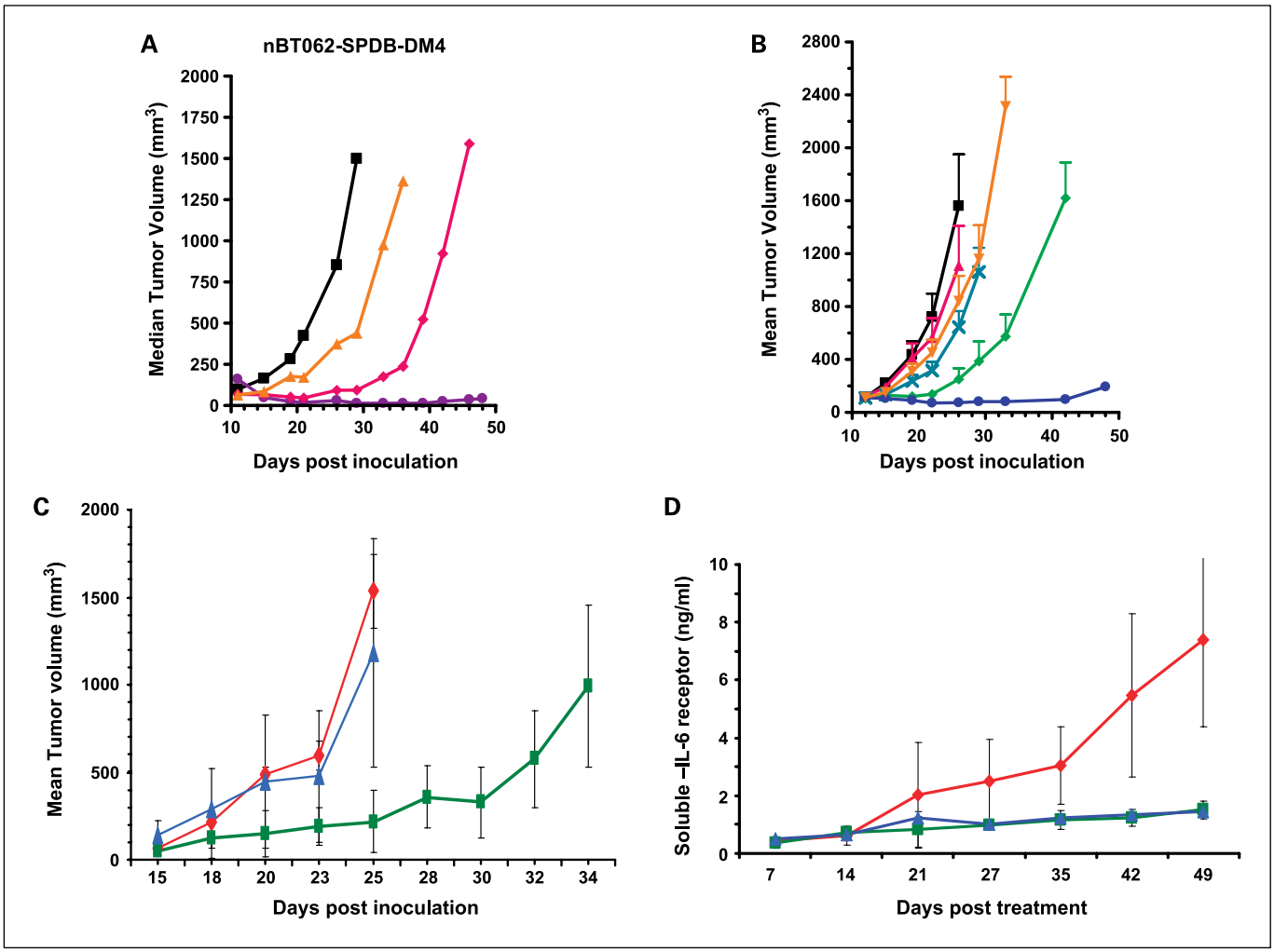

Fig. 6. *In vivo* efficacy of nBT062-SPDB-DM4 conjugates against human MM xenografts in SCID mice. *A,* mice bearing established (~100 mm^3) MOLP-8 tumor xenografts were treated with a single i.v. administration at day 11 after inoculation of PBS (■) of nBT062-SPDB-DM4 at doses of 100 μg/kg (▲), 250 μg/kg (◆), and 450 μg/kg (●), expressed as linked maytansinoid. *B,* mice bearing MOLP-8 xenografts were treated on day 12 with a single i.v. dose of control vehicle (■) or with 250 μg/kg (linked maytansinoid) of nBT062-SPP-DM1 (◆), nBT062-SPDB-DM4 (●), and huIgG-SPDB-DM4 (▼). Groups were also treated with a single injection of the free maytansinoid DM4 (×) at 250 μg/kg and unmodified nBT062 antibody (▲) at 13.8 mg/kg, an antibody dose equivalent to the amount of antibody in the conjugate treatment groups. *C,* mice injected with 5×10^6 $OPM1^{GFP+}$ cells were treated with control vehicle (◆), nBT062-SPDB-DM4 (■), and nBT062-SPP-DM1 (▲). Mean tumor volume was calculated as in Materials and Methods. Error bars, SD. *D,* SCID-hu mice engrafted with INA-6 cells in human bone chips were monitored for tumor growth by serial serum measurements of shuIL-6R. Mice were treated with nBT062-SPDB-DM4 (■), nBT062-SPP-DM1 (▲), or vehicle (♦), and the shuIL-6R levels were determined every week.

against MM tumor cell lines and patient MM cells that expressed CD138, with nBT062-SPDB-DM4 being the most potent of the three conjugates tested. Importantly, little or no cytotoxicity was observed upon treatment of CD138-negative cell lines and PBMCs from healthy volunteers, suggesting that the immunoconjugates are selective for CD138-expressing cells. *In vivo* studies with MM tumor xenografts in immunocompromised mice showed that nBT062-SPDB-DM4 is the most efficacious of the conjugates tested and that the antitumor activity in mice is dependent on specific targeting of the nBT062 conjugate.

In vitro mechanistic studies also showed that nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 inhibited the proliferation of MM cells by inducing G_2-M cell cycle arrest followed by apoptotic cell death, evidenced by dose-dependent cleavage of caspases and PARP, as well as increased APO2.7-positive cells. We and others have previously reported that IL-6 triggers proliferation of MM cells and protects against dexamethasone-induced apoptosis via activation of PI3K/Akt, MEK/ERK, and JAK2/STAT3 signaling cascades (28-30). IGF-I also promotes MM cell proliferation and survival; however, neither IL-6 nor IGF-I protect against nBT062-SPDB-DM4–induced cytotoxicity, suggesting that these immunoconjugates can overcome the protective effects of these cytokines in the BM milieu. We further evaluated the effect of the BM microenvironment on the antitumor activity of these immunoconjugates using MM cells cocultured with isolated BMSCs. Whereas coculture with BMSCs significantly inhibits the antiproliferative effects of dexamethasone, there was no effect on the cell killing activity of the nBT062-maytansinoid conjugates.

Previous studies have shown that IL-6 can bind to the soluble heparin sulfate side chain of proteoglycans, such as CD138 (syndecan-1). These heparin sulfate proteoglycans can function as coreceptors for the growth factors, thereby leading to increased cell growth, survival, and adhesion (31-33). Within

the BM milieu, induction of IL-6 secretion from BMSCs is triggered by direct MM cell–BMSC contact mediated by adhesion molecules, such as integrins and CD44 on the surface of MM cells (15, 34, 35). Interestingly, nBT062-SPDB-DM4 and nBT062-SPP-DM1 can block the adhesion of MM cells to BMSCs, suggesting that the immunoconjugates may also function to overcome cell adhesion–mediated drug resistance to conventional therapies.

Our experiments suggest that free nBT062 can block the cytotoxicity of nBT062-SPDB-DM4, confirming selectivity. However, antibody-maytansinoid conjugates similar to nBT062-SPDB-DM4 can have potent cell killing effects not only on antigen-positive cells but also on antigen-negative cells in close proximity to the tumor cells. Importantly, the presence of antigen-positive cells is required for this so-called bystander killing. A general mechanism of cytotoxicity for disulfide bond-linked antibody-maytansinoid conjugates includes binding of the conjugate to target cells, internalization into the target cell, cleavage of the conjugate disulfide bond, and release of the maytansinoid moiety, which is then capable of killing the target and nearby nontarget cells. We carried out studies that showed bystander killing of CD138-negative Namawla cells in the presence of CD138-positive OPM2 cells by nBT062-SPDB-DM4 (Supplementary Fig. S5). Bystander killing of nontarget cells in close proximity to MM cells would be expected to (*a*) provide an advantage for the eradication of tumor cells that heterogeneously express CD138, such as the putative CD138 negative myeloma stem cell (36); (*b*) kill tumor stroma cells, thereby destroying the tumor microenvironment; and/or (*c*) prevent selection of BT062-resistant tumor cells.

The enzyme responsible for the shedding of CD138 (syndecan-1) from the cell surface has not been identified. The proteolytically released extracellular domain (ectodomain) of syndecan-1 retains its biologically active heparin sulfate chains. Therefore, shed sCD138 in the BM plasma of MM patients could interfere with the function of an nBT062-maytansinoid by blocking access to the surface of MM tumor cells or by increasing the plasma clearance of the conjugate. Importantly, we showed that the level of sCD138 in BM plasma of MM patients is less than in MM cell line supernatants, wherein the immunoconjugates show potent cell killing activity. Therefore, sCD138 levels in BM plasma of MM patients should not interfere with binding of immunoconjugates.

In summary, nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 have *in vitro* and *in vivo* antitumor activity against CD138-positive MM cells and can overcome the protective effects of cytokines and BMSCs. Our results provide the preclinical framework for clinical trials of the most potent of the immunoconjugates tested, nBT062-SPDB-DM4 (so-called BT062), to improve patient outcome in MM.

Disclosure of Potential Conflicts of Interest

C. Uherek, B. Dälken, S. Aigner, and F. Osterroth, employment, Biotest. R.J. Lutz, employment, Immunogen. K.C. Anderson, consultant, Biotest, Immunogen. T. Hideshima, consultant, Immunogen.

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