Original Article
Maytansinoid immunoconjugate IMGN901 is cytotoxic in a three-dimensional culture model of multiple myeloma

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Abstract: Environmental-mediated drug-resistance (EM-DR) presents a major challenge for therapeutic development. Tissue microenvironment in the form of extracellular matrix, soluble factors, and stroma contribute to EM-DR. In multiple myeloma (MM), drug-resistance has hindered treatment success with 5-year survival rates remaining <50%. Here we evaluated IMGN901, a maytansinoid immunoconjugate, for its ability to overcome EM-DR alone or in combination with lenalidomide or dexamethasone. We show that while adhesion of MM cells to the extracellular matrix reduces potency of IMGN901, it remains cytotoxic with an average LC_{50}=43 nM. However, only a combination of IMGN901, lenalidomide, and dexamethasone was able to overcome drug-resistance arising from the direct contact between MM and stromal cells. We demonstrate that multi-drug resistance protein-1 (MDR-1) was upregulated in MM cells grown in contact with stroma, likely responsible for the observed resistance. This study emphasizes the importance of incorporating the elements of tumor microenvironment during preclinical testing of novel therapeutics.

Keywords: Multiple myeloma, environment-mediated drug resistance, tumor-stromal interactions, extracellular matrix, MDR-1

Introduction

While 5-year survival rates for patients with multiple myeloma (MM) have increased somewhat over the last decade, not much additional progress has been made in improving the overall survival of patients since agents such as bortezomib and lenalidomide have entered the market. Thus, MM remains incurable and is responsible for over 10,000 deaths annually [1]. The current standard of care for MM is chemotherapy, bone marrow (BM) transplant, and biologic agents, such as proteasome inhibitors and immunomodulatory compounds [2, 3]. MM arises from a clonal expansion of malignant plasma cells (PCs) in the BM, and is characterized by elevated levels of monoclonal immunoglobulin in the blood and urine, anemia, hypercalcemia, and lytic bone lesions [1]. The majority of MM cells are confined to the BM until the final, stage of the disease, PC leukemia, when clonal PCs enter systemic circulation. While in the bone marrow (BM), MM cells interact with the microenvironment composed of the extracellular matrix (ECM) proteins, soluble factors, and stromal cells. The cross-talk between malignant PCs and the BM stroma affects the behavior of the tumor cells and the non-malignant tissue leading to drug-resistance, immune evasion, and lytic bone lesions [4-6].

Environmental-mediated drug resistance (EM-DR), a term coined to describe resistance to therapeutic agents induced by the tissue microenvironment, has been a major hindrance toward development of robust new therapies for MM [7]. Four major types of interactions are responsible for inducing drug-resistance *in vivo*: 1) adhesion of tumor cells to the ECM of the tissue [8-12]; 2) signaling induced in the tumor cells by the soluble factors present in the microenvironment [6, 13, 14]; 3) cell-cell interactions between adjacent tumor cells [15, 16]; and 4) contacts between tumor cells and the surrounding stroma [6, 17-21].

The goal of the present study was to evaluate the maytansinoid immunoconjugate, IMGN901, as a potential anti-MM therapeutic that can overcome EM-DR. To target CD56-positive MM cells, lorvotuzumab (huN901), a humanized anti-CD56 antibody was conjugated to a cytotoxic derivative of maytansine (DM1), creating IMGN901 (huN901-SPP-DM1, lorvotuzumab mertansine) [22, 23]. CD56 is expressed on malignant plasma cells in approximately 70% of MM cases, and has been shown to be associated with poor prognosis [24]. Thus, an anti-body-drug conjugate directed against CD56 not only allows specific targeting of malignant PCs in the majority of MM patients; it also provides a potential therapeutic advantage against the most aggressive forms of MM. Here we show that the potency of IMGN901 was greatly reduced due to EM-DR arising from cell-ECM and cell-cell interactions. This resistance was overcome by treating MM cells with a triple combination of IMGN901, lenalidomide, and dexamethasone. Furthermore, we show that contact with stroma induced higher levels of multidrug resistance protein 1 (MDR-1) in MM cells, suggesting that the upregulation of the ABC transporters that promote detoxification of tumor cells in response to treatment contribute to the failure of many therapeutic regimens. The data presented here demonstrates the importance of testing novel therapeutic agents under the conditions of EM-DR and suggests the need to incorporate such testing into standard preclinical protocols to reduce the rate of failure of new drug candidates in clinical trials.

Materials and methods

Cell culture

RPMI-8226 and U266 cell lines (ATCC) were cultured in RPMI-1640 with L-glutamine (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin/streptomycin (pen/strep) (Sigma). NCI-H929 cells (ATCC) were grown in RPMI-1640 with L-glutamine supplemented with 10% FBS, 1% pen/strep, and 5×10^{-5} M β-mercaptoethanol (Sigma). The immortalized BM mesenchymal stem cell line (hTERT-MSC) was a kind gift from Dr. Carlotta Glackin (Beckman Research Institute, City of Hope National Medical Center) and was grown in αMEM (Sigma) supplemented with 10% FBS, 1% L-glutamine (Sigma), and 1% pen/strep [8].

All cells were cultured at 37°C in a 5% CO_2 tissue culture incubator.

Drug treatments

Cells were set-up as described below and treated with various concentrations of huN901, IMGN901, dexamethasone, and lenalidomide alone or in combinations and were incubated for 96 hours at 37°C in a 5% CO_2 tissue culture incubator. All drugs were provided by Im-munoGen, Inc. (Waltham, MA). HuN901 antibody and IMGN901 were dissolved in PBS to a stock concentration of 1 mg/ml. Unconjugated DM1 (DM1-SMe, a mixed disulfide of DM1 with thiomethane to block the free thiol of DM1, avoiding thiol-disulfide exchange with culture medium components) was supplied in ethanol as a stock solution at 1.73 nM. Stock solutions of huN901, IMGN901, and DM1-SMe were aliquoted and stored at -20°C. The stock solution of dexamethasone (American Regent, Inc.) was supplied at 4 mg/ml in sodium phosphate and was stored at room temperature protected from light. To prepare the stock solution, lenalidomide was dissolved in DMSO at 27 mg/ml, aliquoted and stored at -80°C.

Drugs were diluted in BMGM or BMCM to final concentrations described in 'Results'. HuN901 antibody was used as an isotype control in all experiments and vehicle controls set-up as appropriate for each compound. Different culture conditions were set-up as described below and were incubated with the anti-myeloma agents for 96 hours in a 37°C in 5% CO_2 incubator. The effects of the drugs were measured using an MTS assay.

Culture conditions (**Figure 1B**)

2-D culture: RPMI-8226 or U266 cells were seeded at 35,000 cells/well and NCI-H929 cells at 50,000 cells/well in 96-well plates in 200 µl of the bone marrow growth medium (BMGM) [RPMI-1640 with L-glutamine, 6.2×10^{-4} M $CaCl_2$ (Sigma), 1×10^{-6} M sodium succinate (Sigma), 1×10^{-6} M hydrocortisone (Sigma), 20% FBS, and 1% pen/strep] containing anti-myeloma drugs.

rBM: To mimic the microenvironment of the bone, the reconstructed BM (rBM) culture was set-up as previously described [9]. Briefly, wells of a 96-well plate were coated with 30 µl of

reconstructed endosteum (rEnd) [77 µg/ml of human fibronectin, 29 µg/ml of rat tail collagen I (Millipore) in 1X PBS] for 30 minutes at room temperature. Subsequently, the remaining liquid rEnd was removed and the surface was overlaid with cells/rBM mixture. MM cells were resuspended in 10 µl PBS/well at 35,000 cells/well for RPMI-8226 and U266 or 50,000 for NCI-H929 cells, and mixed with 40 µl of rBM matrix [2:1 vol/vol mixture of Matrigel (BD Biosciences) and human fibronectin (Millipore)]. The cell/rBM mixture was allowed to gel for 30 minutes at 37°C in a 5% CO_2 incubator. After the rBM was set, 200 µl of BMGM or BM conditioned medium (BMCM) [BMGM conditioned by a confluent monolayer of hTERT-MSC cells for 3 days] with anti-myeloma drugs or the appropriate vehicle controls was added to each well.

Clusters (tumor-tumor): The rBM culture was set-up as described above with the following modifications. Once the rBM matrix solidified 200 µl of BMGM was added to each well and the cells were allowed to grow and form clusters for 4 days. Subsequently, media was removed and replaced with BMGM containing the drugs.

Co-culture (tumor-stroma): To set-up MM/stromal cell co-cultures, individual wells of a 96-well plate were coated with rEnd as described above. Next, hTERT-MSC cells were added to the coated surface at 6,000 cells/well in 200 µl of hTERT-MSC growth medium and allowed to adhere overnight. To establish direct contact between stromal and MM cells, the growth medium was removed and the MM cells at densities described above were placed on top of the stromal layer in 10 µl of PBS/well. MM cells were subsequently overlaid with rBM matrix at 40 µl/well and the co-culture was allowed to gel for 30 minutes in a tissue culture incubator. Once set, 200 µl of BMGM (with or without drugs) was added to each well.

MTS assay

MTS assay was performed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) assay kit (Promega) per manufacturer instructions. Briefly, 96 hours after treatment 20 µL of the MTS/PMS solution was added per well and the plates were incubated for 4 hours at 37°C in a 5% CO_2 incubator. The

OD was read at 492 nm on a Multiscan Asent microplate reader (Thermo Scientific).

Flow cytometry

Cells were incubated with 0.08 nM huN901-Alexa488 or CD56-FITC (Beckman Coulter) at a 1:100 dilution for 1 hour at room temperature, and washed with PBS. Viability of CD56-positive and CD56-negative populations was assessed using the Annexin V-FITC Apoptosis Kit (Beckman Coulter). Cells were treated with IMGN901, isolated from rBM as described below, and stained with CD56-PE at 1:100 dilution for 1 hour at room temperature (without fixation). Subsequently, cells were washed with cold PBS and stained with Annexin V-FITC per manufacturer's instructions. Samples were processed on a Quanta flow cytometer (Beckman Coulter) and the data was analyzed using FlowJo software.

Microscopy

Cells were cultured in the presence of huN901-Alexa488 or an isotype control in BMGM for 96 hours in a 37°C, 5% CO_2 incubator. Cultures were washed with PBS and imaged on a Zeiss AxioObserver inverted microscope equipped with 488 nm filter set. Images were processed using Axiovision software 4.7.3 (Zeiss) and Photoshop CS6 software packages.

RT-PCR

MM cells were co-cultured with hTERT-MSC as described above. To isolate MM cells from the rBM matrix, the mixture of cells and matrix was removed from the culture plates and incubated in 2-3 volumes of ice-cold PBS-EDTA recovery solution (5 mM EDTA, 1 mM $NaVO_4$, 1.5 mM NaF in PBS) for 1 hour at 4°C. Once matrix was dissolved, cells were centrifuged at 1,000 rpm for 10 minutes, washed with PBS, and resuspended in 500 µl of TRI Regent (Sigma). MM cells were loosely attached to the stromal cells and were easily removed from the plate together with the ECM, while TERT-MSCs remained attached to the surface of the plate.

RNA was isolated using TRI Reagent (Sigma) per manufacturer's instructions. RNA concentration was determined on a NanoDrop ND-1000 spectrophotometer, aliquoted, and
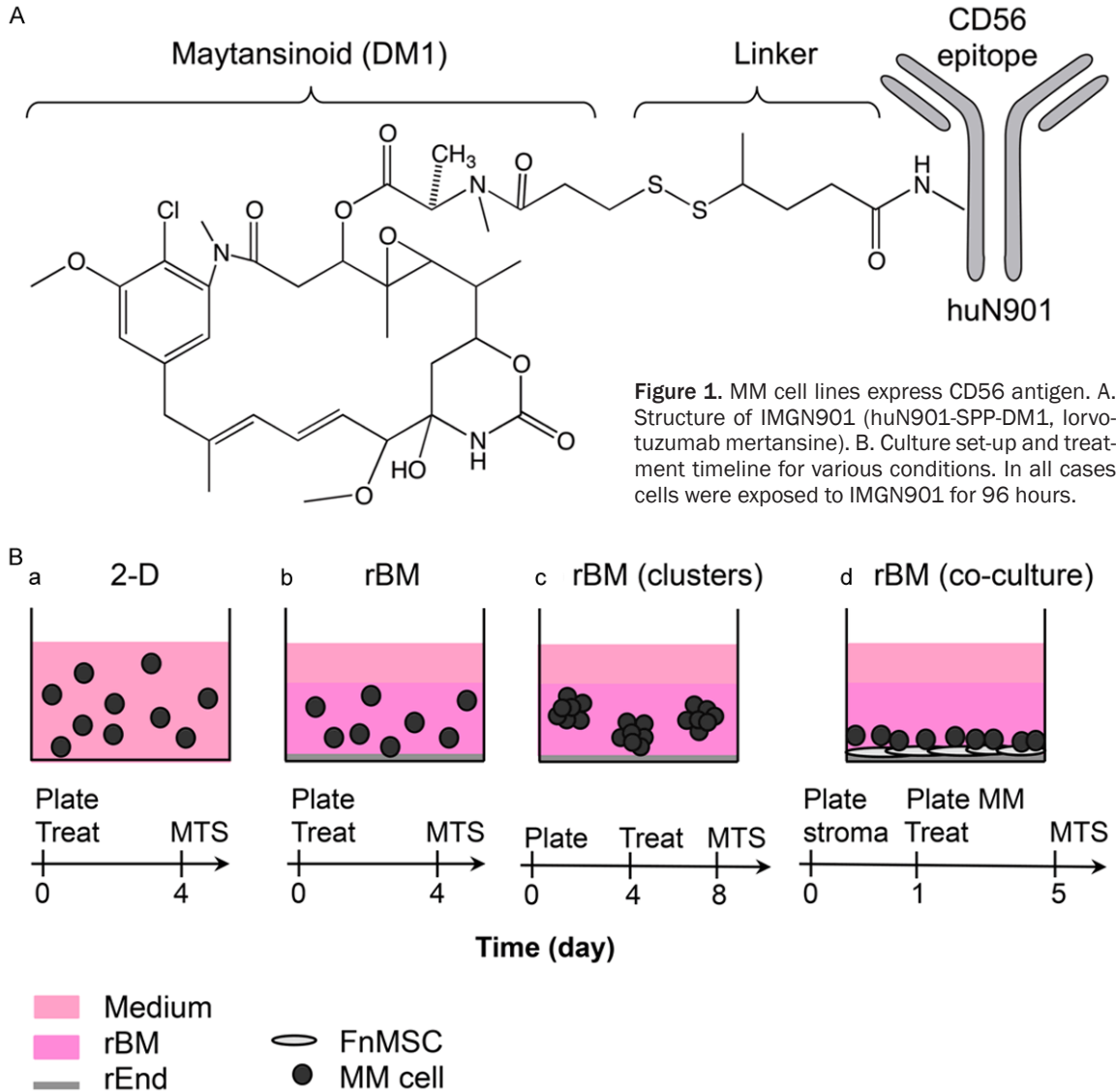
Figure 1. MM cell lines express CD56 antigen. A. Structure of IMGN901 (huN901-SPP-DM1, lorvotuzumab mertansine). B. Culture set-up and treatment timeline for various conditions. In all cases cells were exposed to IMGN901 for 96 hours.

stored at -80°C. RT-PCR was performed using OneStep RT-PCR kit (Qiagen) per manufacturer's instructions. The primers for human MDR1 and GAPDH were as follows: forward MDR1 primer 5'-TCACCAAGCGGCTCCGATACAT-3' and reverse MDR1 primer 5'-CCCGGCTGTTGTCTCC-ATAGGC-3' [25]; forward GAPDH primer GA-GTCAACGGATTTGGTCGT and reverse GAPDH primer GACAAGCTTCCCGTTCTCAG. RT-PCR was performed using 1 μg of total RNA.

PCR was performed on a Mastercycler thermo-cycler (Eppendorf) using the following program: 1) cDNA synthesis step: 50°C for 30 minutes, 95°C for 15 minutes; 2) PCR step (35 cycles): 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute; 3) final extension step: 72°C for 10 minutes. Amplified DNA fragments were re-solved on a 1% agarose gel with the expected product size of 1043 base pairs.

Statistical analysis

Data were presented as mean ± s.e.m of at least 3 independent experiments performed in triplicate. To calculate LC_{50} values, four-parameter logistic regression was applied to log-transformed percent viability measurements. Significance was analyzed by a one-way analysis of variance (ANOVA) with Tukey's post-test or two-way ANOVA and Bonferroni's correction as post-test to compare experiments with multiple parameters with *p*-values <0.05 considered statistically significant. All analysis was per-
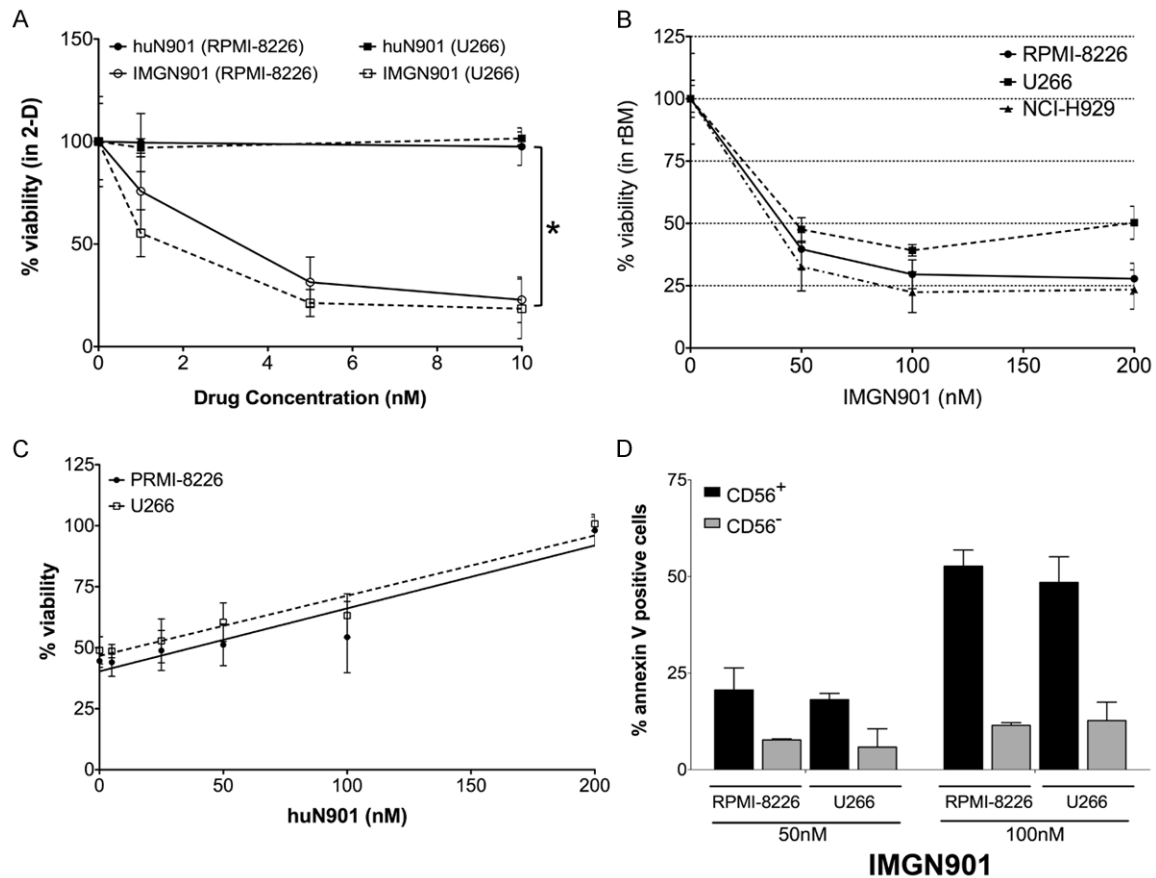
Figure 2. IMGN901 is cytotoxic in rBM cultures in a dose dependent manner. A. RPMI-8226 and U266 cells were grown in 2-D cultures with increased concentrations of unconjugated huN901 or IMGN901. Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. A significant reduction in viability was observed in cells treated with IMGN901 compared to those treated with huN901 as measured by one-way ANOVA (*p-value =0.002). B. RPMI-8226, U266, and NCI-H929 cells were grown in rBM with BMGM. Increased concentrations of IMGN901 were added to each culture as shown in **Figure 1A**. Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. Based on the two-way ANOVA analysis, there was a significant drug-concentration effect for each cell line under all growth conditions (p-value <0.0001). C. RPMI-8226 and U266 cells grown in rBM cultures were co-treated with increasing doses of unconjugated huN901 (0 nM, 5 nM, 25 nM, 50 nM, 100 nM, 200 nM) and a constant dose (100 nM) of IMGN901. Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. Unconjugated huN901 reduced the cytotoxic effects of IMGN901 in a linear fashion, with increased amount of huN901 corresponding to increased cell viability. D. RPMI-8226 and U266 cells were treated with 50 nM and 100 nM IMGN901 in rBM cultures. Cells were isolated from rBM and stained for annexin V and CD56 expression. The proportion of CD56$^+$ and CD56$^-$ cells undergoing apoptosis, i.e. positive for annexin V, were measured by flow cytometry.

formed using Prism 5.0 software (GraphPad Software).

Results

IMGN901 is cytotoxic in reconstructed bone marrow (rBM) MM cell culture in a dose dependent manner

IMGN901 (huN901-SPP-DM1, lorvotuzumab mertansine) is an antibody-drug conjugate composed of the humanized anti-CD56 anti-

body, huN901 (lorvotuzumab), linked to the cytotoxic maytansinoid effector molecule, DM1, via a disulfide linkage. (**Figure 1A**). Efficacy of IMGN901 to eliminate the MM cells under the conditions of EM-DR was evaluated in MM cell lines RPMI-8226, U266, and NCI-H929 treated with IMGN901 alone or in combination with lenalidomide or dexamethasone. Since IMGN901 specifically targets CD56-expressing cells, we determined the CD56 status of each cell line. RPMI-8226, U266, and NCI-H929 cells were stained with a commercially avail-
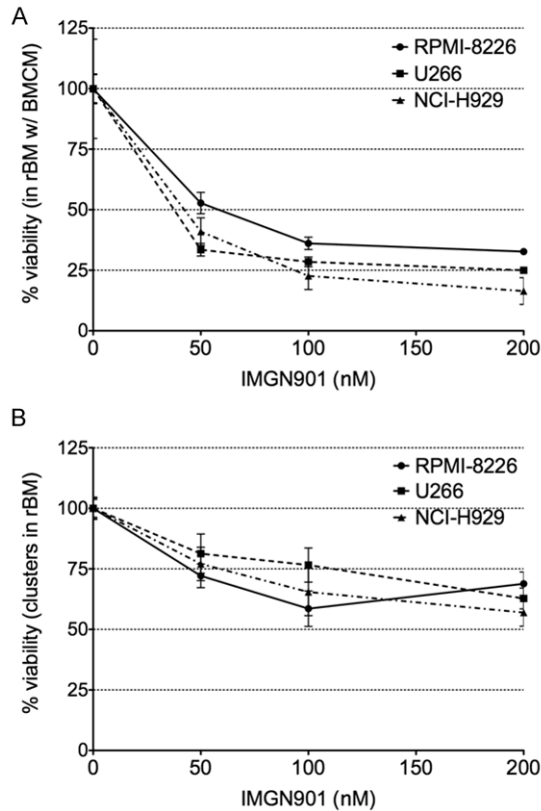
Figure 3. Cell-cell adhesion blocks IMGN901-induced cytotoxicity. RPMI-8226, U266, and NCI-H929 cells were grown (A) in rBM with BMCM, (B) as clusters formed in rBM, or (C) as MM/stromal co-cultures in rBM. Increased concentrations of IMGN901 were added to each culture as shown in **Figure 1B**. Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. Based on the two-way ANOVA analysis, there was a significant drug-concentration effect for each cell line under all growth conditions (p-value <0.0001).

able mouse-anti-human CD56 antibody and its binding was compared to the interaction of huN901 with these cell lines. RPMI-8226, U266, and NCI-H929 cells exhibited 95%, 59%, and 85% positive staining with huN901, closely matching the staining pattern observed using the commercial CD56 antibody (Figure S1). Next, we established that unconjugated huN901 antibody was not toxic at 200 nM, the highest dose to be used through this study (Figure S2). Compared to MM cells treated with huN901 in 2-D cultures (**Figure 1Ba**), IMGN901 was cytotoxic with LC_{50} values of 3.4 nM and 1.7 nM for RPMI-8226 and U266 cells respectively (**Figure 2A**).

Next, we tested the cytotoxicity of IMGN901 in an rBM model, a 3-dimensional culture where the cells are grown in ECM set-up to mimic the BM microenvironment *in vivo* (**Figure 1Bb**) [9]. To confirm that IMGN901 is able to reach the cells at the bottom of the rBM culture, Alexa-488-conjugated huN901 was added to the rBM and its capacity to penetrate and diffuse through the rBM was evaluated by fluorescent microscopy. As shown in the Figure S3, RPMI-8226 cells at the bottom of rBM culture stained

positive with huN901-Alexa488 after a 4-day exposure to the unconjugated lorvotuzumab. When cells grown in rBM were treated with IMGN901, dose-dependent cytotoxicity was observed with LC_{50} values of 42.2 nM, 48.6 nM, 37.5 nM for RPMI-8226, U266, and NCI-H929 respectively (**Figure 2B**). To determine the specificity of IMGN901 for its target (i.e. $CD56^+$ cells), we performed a competition assay. Cells grown in rBM cultures treated with 100 nM of IMGN901 were co-treated with increased doses of unconjugated huN901. HuN901 was able to compete with IMGN901 for the binding to MM cells and its addition reduced the cytotoxic effects of IMGN901 in a linear fashion, where increasing doses of huN901 resulted in increased cell survival (**Figure 2C**). Since the MTS assay is a surrogate measure of cell viability [8], we wanted to determine whether IMGN901 induces apoptosis in MM cells. RPMI-8226 and U266 cells grown in rBM and treated with IMGN901 were stained with Annexin-V-FITC to measure the extent of apoptotic cell death. At 50 nM, IMGN901 induced apoptosis in 20% of MM $CD56^+$ cells, while at 100 nM, programmed cell death was evident in 50% of $CD56^+$ cells (**Figure 2D**).

Interestingly, IMGN901 also induced low levels (7% and 12% respectively for cell treated with 50 nM and 100 nM of IMGN901) of apoptosis in CD56⁻ cells (**Figure 2D**).

IMGN901 is cytotoxic in the context of the physiological ECM, but fails to overcome drug-resistance mediated by cell-cell interactions

Consistent with the studies that demonstrated induction of drug-resistance when cells were exposed to the ECM, the LC_{50} values for cells treated with IMGN901 in rBM were 10-30X higher than for cells treated in 2-D (**Figure 2B** vs. **2A**). Next, we wanted to assess the effects of the soluble factors derived from the bone marrow stromal cells on the efficacy of IMGN901. MM cells were cultured in rBM with BMCM (see Materials and Methods) as a source of stroma-derived soluble factors. Under these conditions, IMGN901 was as effective in eliminating MM cells as in rBM cultures with the standard growth medium (BMGM) with LC_{50} values of 59.8 nM, 38.2 nM, 43 nM for RPMI-8226, U266, or NCI-H929 cells respectively (**Figure 3A**).

Finally, we ascertained how the potency of IMGN901 is affected by the cell-cell interactions in the form of tumor-tumor adhesion, where MM cells formed clusters of malignant cells (**Figure 1Bc** and Figure S4), and tumor-stromal binding, where MM cells were grown in direct contact with BM stromal cells (**Figure 1Bd**). To establish tumor-tumor interactions, MM cells were seeded in rBM cultures and grown for 4 days prior to the addition of IMGN901. This set-up allowed for MM cells to form clusters of tightly interacting tumor cells (Figure S4). Upon formation of clusters, MM cells became insensitive to IMGN901 with LC_{50} values exceeding 200 nM (**Figure 3B**). The same results were obtained when MM cells were cultured in direct contact with hTERT-MSCs as a source of BM stroma (LC_{50}>200 nM) (**Figure 3C**).
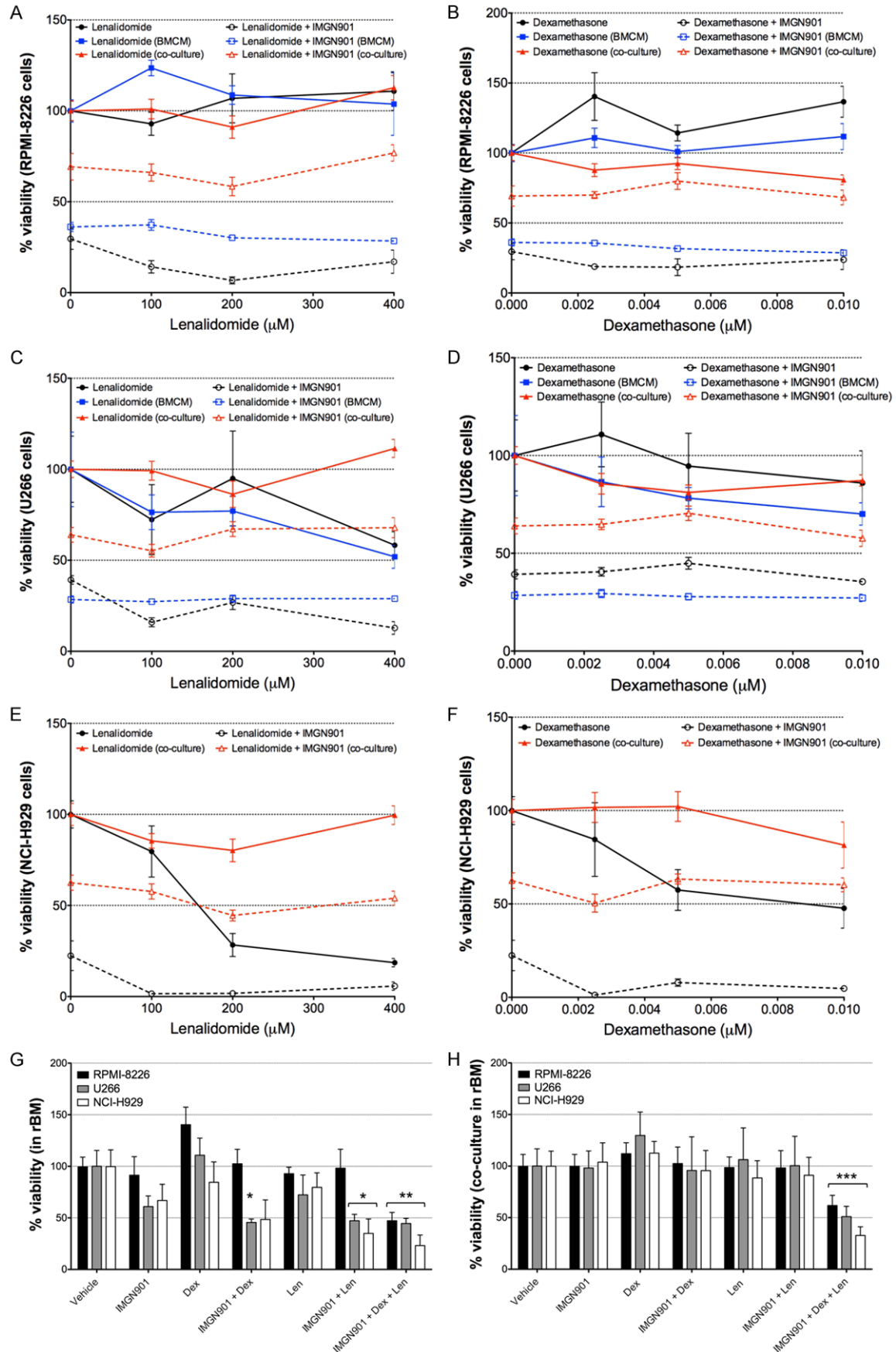
Triple combination of IMGN901, lenalidomide, and dexamethasone overcomes stromal-mediated drug resistance

To determine the benefits of combining IMGN901 with currently used therapeutics to reduce adhesion-mediated drug-resistance, we set-up co-treatment experiments. MM cells grown in rBM were treated with 100 nM IMGN901 and increased concentrations of lenalidomide or dexamethasone. When grown in the context of BM ECM, RPMI-8226 and U266 cells were insensitive to either lenalidomide or dexamethasone alone at concentrations tested (**Figure 4A-D**, black lines; *p*-value >0.05), while NCI-H929 cells responded to both drugs (**Figure 4E**, **4F**, black lines; *p*-value <0.0001 and 0.03 for lenalidomide and dexamethasone respectively). Furthermore, when tested in rBM cultures in the presence of BMCM or BM stromal cells, lenalidomide or dexamethasone alone had no effect on cell viability (**Figure 4**; *p*-value >0.05). A significant increase in cytotoxicity was observed when IMGN901 was combined with lenalidomide in RPMI-8826 and NCI-H929 cells, but not U266 cells (**Figure 4**; *p*-value =0.015 and 0.004 for RPMI-8226 and NCI-H929 respectively). However, IMGN901/dexamethasone combination was effective only in NCI-H929 cells (*p*-value =0.007). Addition of BMCM to the rBM culture blocked the cytotoxicity of IMGN901/lenalidomide (**Figure 4**). Furthermore, significant resistance was observed when co-cultures of RPMI-8226, U266, or NCI-H929 cells with hTERT-MSC were treated with IMGN901 in combination with lenalidomide or dexamethasone (**Figure 4**).

Since stromal-mediated drug resistance remained a problem even for combination treatments, we wanted to determine whether a co-treatment with all 3 drugs could overcome the effects of tumor-stroma contacts. Thus, RPMI-8226, U266, and NCI-H929 cells grown in rBM in co-culture with hTERT-MSC were co-treated with IMGN901, lenalidomide, and dexamethasone. Lower doses of each drug compared to those used in previous experiments (IMGN901 (25 nM vs. 100 nM), lenalidomide (100 µM vs. 400 µM), and dexamethasone (2.5 nM vs. 10 nM)) induced significant reduction in MM cells in both dual and triple combinations (**Figure 4G**). However, as predicted by previous experiments, when co-cultured with hTERT-MSCs, MM cells were resistant to dual treatments. When co-cultures where treated with all 3 drugs, a statistically significant, near 50% reduction in cell viability was obtained (**Figure 4H**). These results were highly encouraging and suggested that identifying proper combination treatment regimens may overcome cell-adhesion mediated drug-resistance.

Figure 4. IMGN901/lenalidomide/dexamethasone combination overcomes tumor-stromal drug-resistance. MM cells were grown in rBM with BMGM or BMCM alone or in co-culture with hTERT-MSCs. Cultures were treated with 100 nM IMGN901 in combination with increasing concentrations of lenalidomide (A, C, E) or dexamethasone (B, D, F). Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. (A, B) RPMI-8226 cells; (C, D) U266 cells; (E, F) NCI-H929 cells. Two-way ANOVA analysis was performed to evaluate significance of all treatment combinations (see text for details). RPMI-8226, U266, and NCI-H929 cells were grown in rBM (G) or co-culture in rBM (H) and treated with a combination of IMGN901 (25 nM), dexamethasone (2.5 nM), and lenalidomide (100 mM). Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. Based on the one-way ANOVA analysis comparing drug-treated samples with vehicle controls for each cell line, there was a significant effect of the triple-treatment for each cell line (*p-value =0.044; **p-value <0.0001; ***p-value =0.0028).
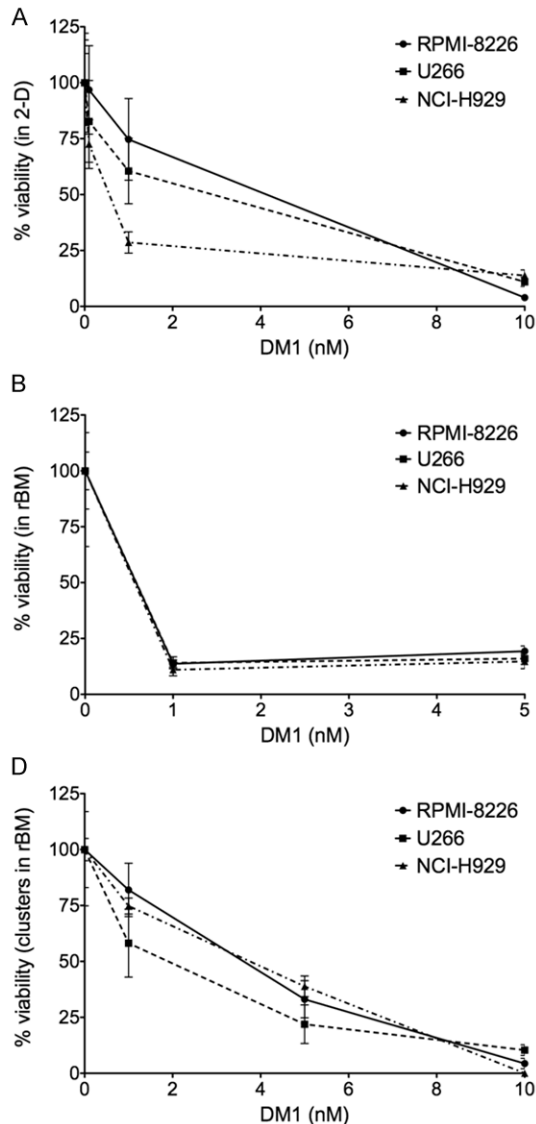

Figure 5. Free DM1 fails to completely overcome drug-resistance mediated by MM cell adhesion to BM stroma. RPMI-8226, U266, and NCI-H929 cells were grown with increased concentrations of free DM1. Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. Cells were grown (A) in 2-D culture, (B) in rBM with BMGM, (C) in rBM with BMCM, (D) as clusters formed in rBM, or (E) as MM/stromal co-cultures in rBM. Cell viability was measured using an MTS assay and data was normalized to the viability observed in vehicle-treated controls. Based on the two-way ANOVA analysis, there was a significant drug-concentration effect for each cell line under all growth conditions (p-value <0.0001).

Unconjugated DM1 is able to overcome tumor-tumor, but not tumor-stroma induced drug-resistance

To better understand the dynamics of drug-resistance induced by cell-cell adhesion we wanted to test how DM1 alone performs under conditions of tumor-tumor and tumor-stroma interactions. We hypothesized that cell-cell interactions may block lorvotuzumab binding sites, and thus, prevent DM1 from reaching the target cells. MM cells were treated with DM1-
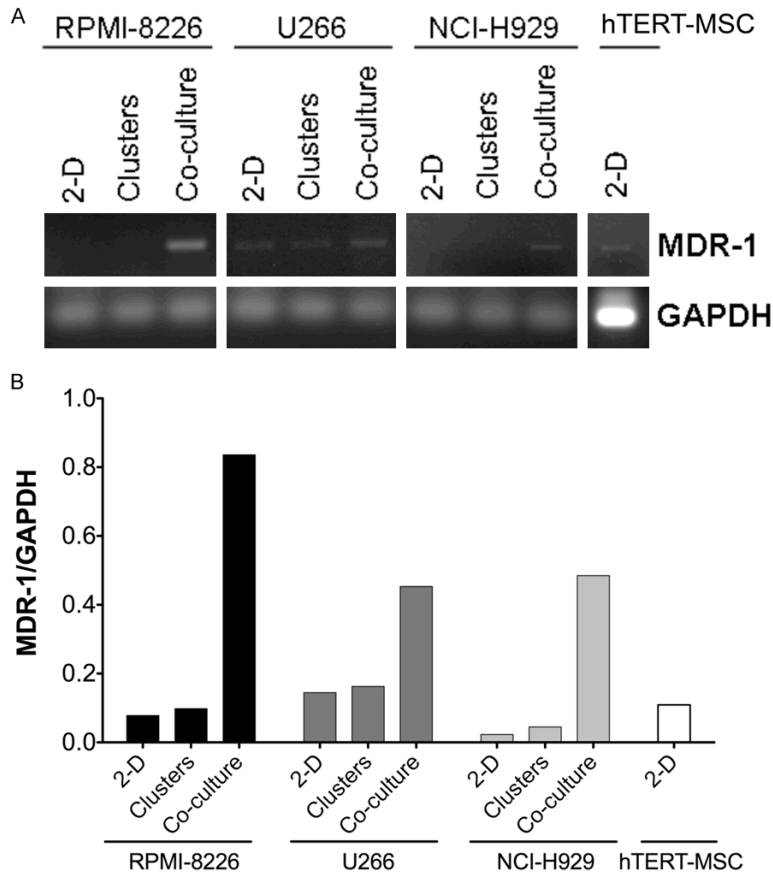
Figure 6. Direct contact between BM stromal cells and MM cells induces MDR-1 expression in MM cells. A. RNA was isolated from RPMI-8226, U266, and NCI-H929 cells cultured under various conditions for 4 days in the absence of drug treatement. MDR-1 expression was determined by RT-PCR. B. MDR-1 expression was quantified as a ratio of MDR-1/GAPDH signal.

RPMI-8226 and NCI-H929 >100 nM and U266 at 86.8 nM (**Figure 5E**).

MDR-1 is elevated in MM-stromal co-cultures

Since unconjugated DM1 failed to overcome stromal-mediated drug resistance we set out to determine the mechanism driving the survival of MM cells when grown in contact with the BM stroma. One of the most extensively studied pathways leading to drug-resistance is the activation of p-glycoprotein, also know as multi-drug resistance protein 1 (MDR-1), an ABC transporter responsible for efflux of cytotoxic compounds from tumor cells [26, 27]. To determine whether MDR-1 is responsible for stromal-mediated drug resistance in our system, MM cells were cultured in 2-D or in rBM as clusters or in co-culture with the BM stromal cells at a ratio of 6:1 or 8:1 for RPMI-8226/U266 and NCI-H929 cells to hTERT-MSCs respectively. Subsequently, expression of MDR-1 in MM cells was measured by RT-PCR and compared to the expression of the housekeeping gene GAPDH. Compared to other culture conditions, MM cells grown in co-culture with BM stroma expressed elevated levels of MDR-1 (**Figure 6A, 6B**).

Discussion

To evaluate the clinical potential of investigational therapeutics it is imperative to test new drugs under physiological conditions taking into account tissue microenvironment and EM-DR. Here we present preclinical evaluation of IMGN901 (huN901-SPP-DM1, lorvotuzumab mertansine), an antibody-drug conjugate composed of the humanized anti-CD56 antibody lorvotuzumab (huN901) linked to the cytotoxic maytansinoid effector molecule DM1 via a disulfide linkage. IMGN901 was tested in standard 2-D cultures where cells were grown on

SMe in 2-D cultures or grown in contact with ECM in rBM or rBM with BMCM. Unconjugated DM1-SMe induced cell death in 2-D cultures with LC$_{50}$ values of 3.7 nM, 1.9 nM, 0.63 nM, in rBM with LC$_{50}$ values of 0.59 nM, 0.58 nM, 0.57 nM, and in rBM with BMCM with LC$_{50}$ values of 0.66 nM, 1.36 nM, 0.59 nM for RPMI-8226, U266, and NCI-H929 cells respectively (**Figure 5A-C**). Interestingly, DM1 was not susceptible to the drug-resistance observed when rBM cultures were treated with IMGN901. Mild resistance to DM1 treatment was observed in tumor-tumor clusters with LC$_{50}$ values at 4.13 nM, 2 nM, and 3.8 nM for RPMI-8226, U266, and NCI-H929 cells respectively, suggesting that the observed resistance to IMGN901 could be due to the inability of the bulky lorvotuzumab to reach malignant cells inside the clusters (**Figure 5D**). However, tumor-stromal cultures were resistant to DM1 with LC$_{50}$ values for

the surface of tissue culture plastic or in reconstructed BM, rBM, cultures set-up to reconstruct the ECM and stromal microenvironment of the BM [9]. IMGN901 was also tested in modified rBM culture where MM cells were exposed to the soluble factors produced by BM stromal cells since a number of factors secreted by the BM stroma have been shown to induce drug-resistance in tumor cells [6, 13, 14]. Additionally, we ascertained the effects of cell-cell interactions on the efficacy of IMGN901 in the context of tumor-tumor and tumor-stroma contacts (**Figure 1B**).

Compared to the unconjugated huN901 antibody that did not induce cell death in MM cells, IMGN901 was cytotoxic in a dose-dependent manner in 2-D cultures. However, culturing tumor cells on a solid plastic surface is not representative of the tissue architecture and has been shown to affect response to therapy [8-12]; therefore, we tested the potency of IMGN901 under the conditions of EM-DR where MM cells were cultured in the rBM model suspended in the ECM protein mixture recapitulating the microenvironment of the human BM [9]. On average, we observed a 15-fold increase in the LC_{50} values when comparing 2-D and rBM cultures confirming previous observations that the interaction with ECM induces drug-resistance in tumor cells. Soluble factors produced by stroma found in the blood, lymph, or interstitial tissues have also been shown to reduce the efficacy of therapeutic agents [6], thus, we evaluated IMGN901 in rBM cultures in the presence of the secreted factors produced by a human BM mesenchymal stem cells. There was no increase in drug-resistance when stromal-derived soluble factors were added to the rBM culture, suggesting that such factors do not hinder the binding of IMGN901 to MM cells. However, when we took into account cell-cell interactions between tumor cells and between tumor and stromal cells present in the BM, the potency of IMGN901 was greatly diminished, with LC_{50} values not reached at the doses tested (up to 200 nM). This suggested two possibilities. First, cell-cell interactions prevented IMGN901 from penetrating into the tissue, and thus, the drug could not reach a fraction of tumor cells. Second, cell-cell interactions may have activated drug-resistance mechanisms in the tumor cells.

In an attempt to overcome the observed cell-cell adhesion mediated drug-resistance we tested IMGN901 in combinations with lenalidomide and dexamethasone. Under the conditions and concentrations tested, neither lenalidomide nor dexamethasone alone was able to induce cytotoxicity in MM cells grown in rBM. When combined with IMGN901, lenalidomide induced significantly higher levels of cytotoxicity than IMGN901 alone. In contrast with previously published studies [28-30], the efficacy of the combination therapy was obliterated tumor-stromal co-cultures. The discrepancy in our findings compared to the other studies is likely explained by the differences in experimental set-up. Our rBM culture is designed for long-term maintenance and propagation of the entire myeloma clone and the supporting bone marrow stroma. In contrast the experiments set-up by Ikeda, *et al.* and Tassone, *et al.,* are designed for short-term evaluation of isolated cell populations. Due to the failure of the dual therapy to overcome cell-adhesion mediated drug-resistance, we evaluated the efficacy of the triple combination: IMGN901, lenalidomide, and dexamethasone in rBM and in co-cultures. Using lower doses of each agent compared to the dual combinations, we demonstrated that this triple combination is effective in eliminating MM cells in rBM. Moreover, the IMGN901/lenalidomide/dexamethasone combination was able to overcome cell-adhesion mediated drug-resistance in MM cells. Additional *in vitro* studies and *in vivo* evaluation will be necessary to identify the optimal concentrations to achieve maximum efficiency in eliminating MM cells from the BM.

To understand the source of cell-cell adhesion induced drug-resistance we repeated 2-D, rBM, and co-culture experiments using unconjugated DM1 as treatment. Grown in clusters, tumor cells exhibited a 5-fold increase in drug-resistance, with average LC_{50} values of 3.32 nM compared to 0.58 nM for single cells grown in rBM. Although a degree of resistance was clearly present under conditions of tumor-tumor cell contacts, a modest elevation in LC_{50} values suggested that DM1 was able to penetrate the MM cell clusters. Therefore, we hypothesize that the reason for the observed loss of efficacy of IMGN901 in the context of tumor-tumor interactions was the inability of the antibody-drug conjugate to reach all the cells within

the cluster. However, as was observed with IMGN901, DM1 failed to overcome resistance generated by the interactions between MM and BM stromal cells. To better understand the mechanism of this resistance, we evaluated the status of MDR-1 under various culture conditions. MM cells were grown in 2-D culture or as clusters or in co-culture with stromal cells in rBM. A 3- to 20-fold increase in the expression of MDR-1 was detected in MM cells grown in direct contact with the stroma, thus supporting our hypothesis that MM-stromal adhesion induces drug-resistance in tumor cells. Interestingly, low levels of MDR-1 were also detected in the BM stromal cells contributing to their survival post treatment. Further studies will need to focus on better understanding the stromal-mediated drug-resistance to create compounds robust enough to eliminate MM cells in the presence of stromal elements.

Acknowledgements

Abbreviations

ANOVA, analysis of variance; BM, bone marrow; BMCM, bone marrow conditioned medium; BMGM, bone marrow growth medium; ECM, extracellular matrix; EM-DR, environmental-mediated drug-resistance; hTERT-MSC, human telomerase immortalized mesenchymal stem cells; MDR-1, multidrug resistance protein 1; MM, multiple myeloma; MSC, mesenchymal stem cells; PC, plasma cell.

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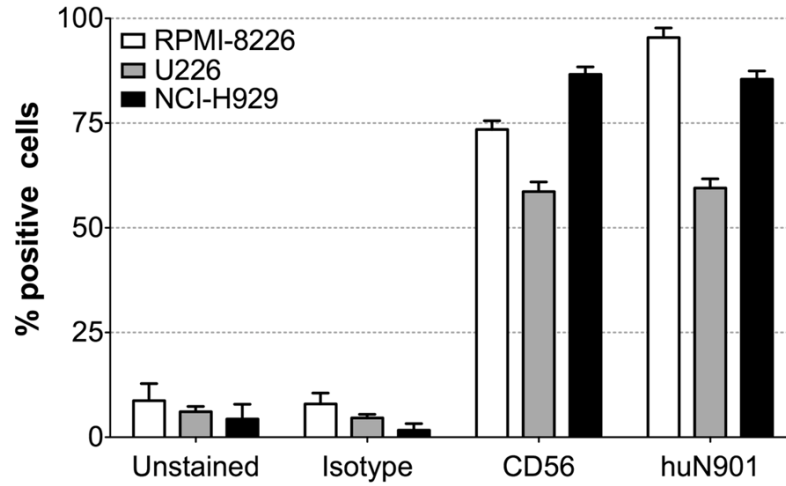
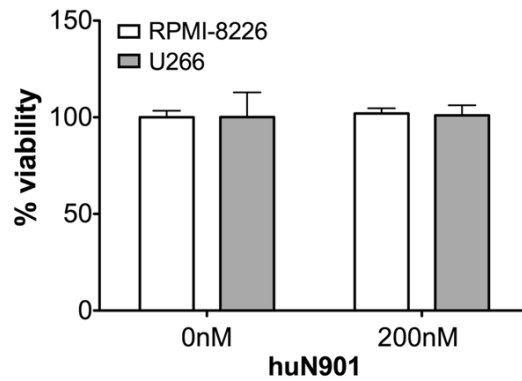**Figure S1**. MM cell lines express CD56 agtigen blind huN901.

Figure S2. huN901 dose not affect proliferation and viability of MM cell. RPMI-8226 and U266 cell were cultured in rBM in the presence vehicle control (0 nM huN901) or 200 nM huN901 for 96 hours. Cell proliferation and viability were assessed using an MTS assay.
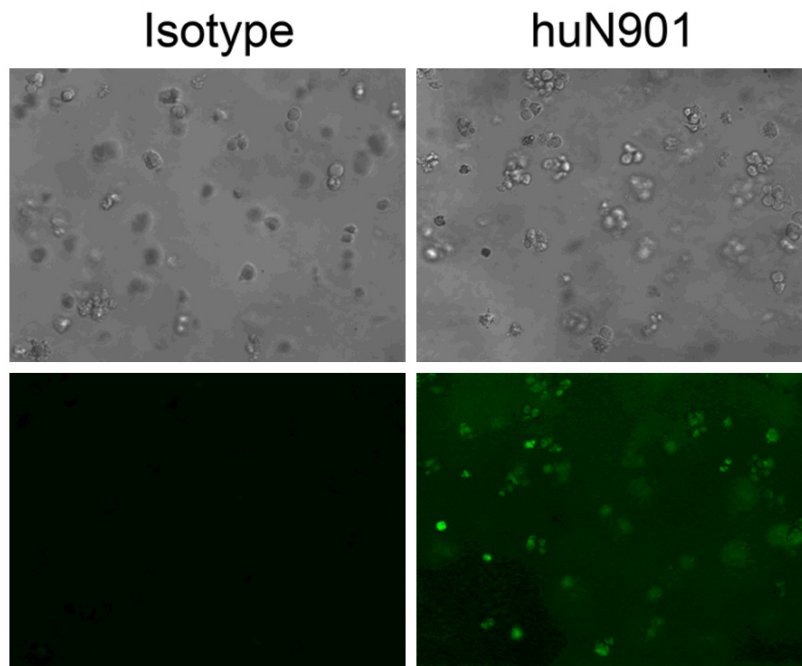
Figure S3. huN901 penetrates rBM matrix. RPMI-8226 cell were cultured in rBM in the presence of istype control or 0.08 nM huN901 antibodies conjugated to Alcxa488 for 96 hours.
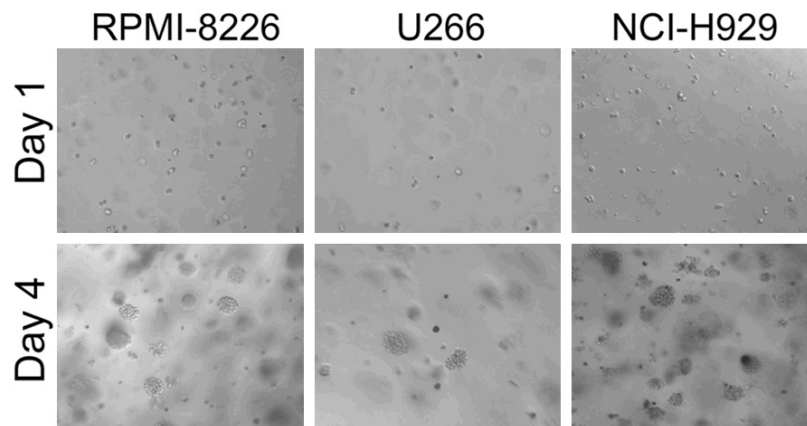

Figure S4. MM cell form clusters when grown in rBM. RPMI-8226, U266, NCI-H929 cell were cultured in rBM and cluster formation was evaluated by brightfield microscopy.