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Anti-β_2M monoclonal antibodies kill myeloma cells via cell- and complement-mediated cytotoxicity

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Abstract

Our previous studies showed that anti-β_2M monoclonal antibodies (mAbs) at high doses have direct apoptotic effects on myeloma cells, suggesting that anti-β_2M mAbs might be developed as a novel therapeutic agent. In this study, we investigated the ability of the mAbs at much lower concentrations to indirectly kill myeloma cells by utilizing immune effector cells or molecules. Our results showed that anti-β_2M mAbs effectively lysed MM cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which were correlated with and dependent on the surface expression of β_2M on MM cells. The presence of MM bone marrow stromal cells or addition of IL-6 did not attenuate anti-β_2M mAb-induced ADCC and CDC activities against MM cells. Furthermore, anti-β_2M mAbs only showed limited cytotoxicity toward normal B cells and non-tumorous mesenchymal stem cells, indicating that the ADCC and CDC activities of the anti-β_2M mAbs were more prone to the tumor cells. Lenalidomide potentiated in vitro ADCC activity against MM cells and in vivo tumor inhibition capacity induced by the anti-β_2M mAbs by enhancing the activity of NK cells. These results support clinical development of anti-β_2M mAbs, both as a monotherapy and in combination with lenalidomide, to improve MM patient outcome.

Keywords

β_2-microglubulin; monoclonal antibody; antibody-dependent cell-mediated cytotoxicity; complement-dependent cytotoxicity; multiple myeloma

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Authorship: Contribution: MZ and QY initiated the work, designed the experiments, and wrote the paper. JQ, YLan, YLu, and HL performed the experiments and statistical analyses, BH, YZ, JH, and JY provided samples and critical suggestion to this study.

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Introduction

Multiple myeloma (MM) is a clonal plasma cell neoplasm that utilizes the bone marrow (BM) microenvironment for survival and proliferation[1-3]. Current myeloma therapies such as hematopoietic cell transplantation and combinatorial chemotherapies are rarely curative and relapse is common. This implies that therapy-resistant myeloma-initiating cells exist and that new therapeutics must be developed to target and eradicate these chemoresistant myeloma cells.

Lenalidomide is a potent novel thalidomide analog which has demonstrated remarkable clinical activity in the treatment of MM[4]. The strong evidence-based clinical success of lenalidomide in MM patients has led to its approval by US-FDA under the trade name of Revlimid® capsules by Celgene Corporation. However, adverse effects and drug resistance have been observed in MM patients, which are great challenges for the extended application of lenalidomide[5].

Targeted immunotherapy with monoclonal antibodies (mAbs) is an effective and safe method for the treatment of cancers. However, there is still no mAb-based cancer therapy approved to treat patients with MM. Early clinical trials of mAbs targeting CD20 and CD38 have conveyed only very limited benefit to the treatment of MM[6-8]. In recent years, efforts have been made to identify potential therapeutic mAbs by defining alternative or novel MM target antigens, i.e., CD40[9, 10], IL6R[11], HM1.24[12, 13], CD74[14], CD47[15], TRAIL-R1[16], CS1[17], as well as to conjugate mAbs with classic or novel drugs to specifically kill MM cells, i.e., CD56-maytansinoid (DM1)[18], CD138-DM1/DM4[19]. Development of mAbs with improved cytotoxicity, targeting new and known myeloma-associated antigens, continues to be an active research area.

β_2-microglubulin (β_2M) is a part of the major histocompatibility complex (MHC) class I molecule on the cell surface of nucleated cells[20]. We have recently demonstrated that human β_2M is a potential target for MM treatment[21]. Our previous studies showed that anti-β_2M mAbs have strong direct apoptotic effects on myeloma and other hematological malignancies with less toxicity on normal tissues and cells in vitro and in mouse models[21, 22], suggesting that anti-β_2M mAbs might be a novel therapeutic agent for MM. Furthermore, others have reported similar results by using anti-MHC class-1 single-chain Fv diabody or anti-β_2M antibodies to induce apoptosis in human myeloma[23], renal cell carcinoma[24], and prostate cancer[25].

Natural killer (NK) cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) is a critical mechanism for many approved therapeutic mAbs[26-28]. FcγRIIIa, a member of the leukocyte receptor family FcγRs, is known to be a major triggering receptor of ADCC in NK cells. FcγRIIIa polymorphism status of NK cells from cancer patients plays a key role in the clinical outcome of patients receiving rituximab[27], trastuzumab[29], or cetuximab[28]. Complement-dependent cytotoxicity (CDC) is a cytolytic cascade mechanism by which complement proteins present in serum are activated by antigen-specific antibodies. CDC is triggered by the binding of C1q, a subunit of C1, to the CH2 domain of a cell-bound IgG antibody, leading to the formation of the membrane attack complex (MAC) and ultimately

lysis of target cells[30]. Human IgG1 and IgG3 efficiently mediate effector function activities, while IgG2 and IgG4 are generally ineffective[31, 32].

In this study, we evaluated anti-β_2M mAb-mediated ADCC and CDC activities against established human MM cell lines and primary MM cells from patients. The ADCC and CDC activities of anti-β_2M mAbs were more against tumor cells, and BM microenvironment could not protect MM cells from anti-β_2M mAb-mediated ADCC and CDC activities. Lenalidomide enhanced in vitro and in vivo anti-β_2M mAb-mediated ADCC activities.

Materials and Methods

Cell lines and primary cells

Human myeloma cell line ARP-1 and CAG were established at the University of Arkansas for Medical Sciences from BM aspirates of patients with MM[33]. MM.1S was kindly provided by Dr. Steven Rosen of Northwestern University (Chicago, IL). U266 WT and U266/R10R were generously provided by Dr. Robert Z. Orlowski of MD Anderson Cancer Center[34] (Houston, TX). RPMI-8226 was purchased from ATCC. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors by Ficoll-Hypaque density centrifugation. B cells were separated from fresh PBMCs using EasySep™ Human B Cell Enrichment Kit (Stem cell Technologies). $CD138^+$ myeloma cells were purified from BM aspirates of MM patients using RoboSep™ Human Whole Blood and Bone Marrow CD138 Positive Selection Kit (Stem cell Technologies). Human bone marrow-derived mesenchymal stem cells (MSCs) were established from BM aspirates of patients with MM as previously described[35]. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and were maintained in 37°C with 5% CO_2.

ADCC and CDC assays

β_2M-specific D1 mAbs were generated as previously described[21], and mouse IgG1 (mIgG1; BioLegend) was used as isotype control. ADCC and CDC were measured by ^{51}Chromium (^{51}Cr)-release assays. In ADCC assay, PBMCs from normal volunteers were used as effector cells, and in CDC assay guinea pig serum (Sigma-Aldrich) was used as the source of complements. Target cells (1×10^6) including myeloma cells, normal B cells or MSCs were incubated with 200 μCi of ^{51}Cr for 1 hour at 37°C with gentle resuspension of pellet at 15 minute intervals. After washing, cells were plated at 10,000 cells/well in 96-well U-bottom plate with PBMCs or guinea pig serum. This was followed by the addition of anti-β_2M mAbs at final concentrations ranging from 5 to 20 μg/ml. In some experiments, PBMCs were pretreated with lenalidomide (2 μM; Selleck Chem) or human IL-2 (10 U/ml; R&D Systems) for 48 hours before assay, or target cells were co cultured with human IL-6 (1 ng/ml; R&D Systems), or the plates were precoated with MSCs overnight before adding target cells. Cells were then incubated for 4 hours at 37°C, and cell-released ^{51}Cr was measured using a gamma-Counter. Spontaneous release was determined from target cells without the addition of anti-β_2M mAbs, PBMCs or guinea pig serum, and maximum release was determined from target cells with 6% Triton X-100 without the addition of the mAbs, PBMCs or guinea pig serum. Percent cytotoxicity was calculated as [(counts in sample −

spontaneous release)/(maximum counts − spontaneous release)] ×100%. All experiments were performed in triplicate.

β_2M short-hairpin RNA transfection of myeloma cells by lentivirus

Myeloma cells were transfected using human β_2M short-hairpin RNA (shRNA) lentiviral particles (Genecopoeia) according to the manufacturer's protocol to knockdown β_2M expression.

Western blotting

Western blotting was conducted as previously described[21]. Mouse anti-β_2M mAb was obtained from Santa Cruz Biotech. Rabbit anti-β-actin polyclonal antibody was obtained from Sigma-Aldrich. The experiments were carried out in triplicate.

Quantitative real-time PCR

The primers for amplification were as follows: β_2M-F 5′-AAT TGA AAA AGT GGA GCA TTC AGA-3′; β_2M-R 5′-GGC TGT GAC AAA GTC ACA TGG TT-3′; GAPDH-F 5′-CAC TCC TCC ACC TTT GAC G-3′; GAPDH-R 5′-ACC ACC CTG TTG CTG TAG C-3′. Gene expression levels in each cDNA sample were normalized to the internal *GAPDH* levels. The experiments were carried out in triplicate for each data point.

Cell proliferation

Cells were plated at a density of 1,000 cells/well in triplicate in 96-well culture plates. After two-day culture, cell proliferation was monitored by detecting absorbance at 490 nm with an automatic microplate reader using MTS assay (Promega). The experiments were carried out in triplicate.

Flow cytometry

APC-conjugated mAbs against human β_2M, HLA-ABC, CD138, and isotype controls were obtained from BioLegend. FITC-labeled Annex in V antibody and PI were purchased from Life Technologies. Data were acquired with a flow cytometer (FACS Calibur; BD Biosciences). The experiments were carried out in triplicate.

Enzyme-linked immunosorbent assay

Cell culture supernatants were collected, and the amount of secreted β_2M in the supernatants was quantified using human β_2M Quantikine IVD ELISA Kit (R&D Systems). The experiments were carried out in triplicate.

In vivo tumor xenograft models

Six week old male SCID mice (Jackson Laboratory) were injected subcutaneously in the right flank with 1×10^6 APR-1 cells. Three to four weeks later when palpable tumors (5 mm in diameter) developed, mice (5 per group) were intraperitoneally injected with lenalidomide (25 mg/kg), anti-β_2M mAbs (5 mg/kg) subcutaneously (around tumors) or in combination of both every 3 days. Control mice received equal amounts of mIgG1 or DMSO. Tumors were measured every 3 days with calipers and tumor volumes (mm^3) were calculated as (width^2 ×

length)/2. Mice were humanely sacrificed when moribund or when subcutaneous tumors reached 15 mm in diameter. All mice were maintained in American Association of Laboratory Animal Care-accredited facilities, and studies were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and Cleveland Clinic.

In situ apoptosis assay

In situ tumor cell apoptosis was determined using the TdT-mediated dUTP nick-end labeling (TUNEL) assay (Boehringer-Mannheim). Sectioned tumor tissue was embedded in paraffin. Three slides from each group were evaluated for the apoptotic cells. Six slide fields were randomly examined using a defined rectangular field area with × 200 magnification, and apoptotic cells were counted in each field.

Statistical Analysis

The Student t test was used to compare various experimental groups. A P value < 0.05 was considered statistically significant. Unless otherwise indicated, the values provided are means and standard deviations (SDs).

Results

Anti-β_2M mAbs mediate ADCC activities against myeloma cells

The ADCC activity of anti-β_2M mAbs was evaluated using PBMCs isolated from healthy donors as effector cells. As shown in Figure 1A, anti-β_2M mAbs at low concentrations (5-20 μg/ml) were able to, in a dose-dependent manner, mediate significant ADCC activities against myeloma ARP-1 cells ($P < 0.05$ to $P < 0.01$, compared with mIgG1 control). Significant cell lysis could be observed at an E:T ratio of 40:1 (one myeloma cells: 40 PBMCs); about 40% of myeloma cells were lysed in the culture with the mAbs and only fewer than 10% in those with mIgG1 ($P < 0.01$). Next, the ADCC activity of anti-β_2M mAbs was evaluated against a panel of MM cell lines including ARP-1, MM.1S, U266, CAG and RPMI-8226. Compared to mIgG1, anti-β_2M mAbs induced effective lysis of MM cells (Figure 1B; $P < 0.05$ to $P < 0.01$). Maximal lysis induced by anti-β_2M mAbs ranged from 30% to 50%, which were 2-fold higher over controls for all MM cell lines assayed. Furthermore, B cells and human bone marrow-derived MSCs were used to evaluate the side effects of mAbs treatment on normal cells. At 20 μg/ml of antibody concentration and an E:T ratio of 40:1, the lysis of normal B cells and MSCs was observed in 8.1% and 14.4% cells, respectively, compared with 45.4% of ARP-1 cells (Figure 1C; $P < 0.05$). These results indicated that anti-β_2M mAb-mediated ADCC activity was more towards myeloma cells. In line with these results, purified $CD138^+$ primary myeloma cells but not $CD138^-$ non-myeloma cells from the same patients were sensitive to anti-β_2M mAb-mediated ADCC (Figure 1D; $P < 0.01$). Taken together, these results indicated that anti-β_2M mAbs at low concentrations were effective at mediating ADCC activities against myeloma cells.

Anti-β_2M mAb-mediated ADCC activities correlate with the expression of β_2M on cell surface

To evaluate the significance of MM cell surface β_2M expression in anti-β_2M mAb-mediated ADCC effects, lentiviral systems were utilized to knockdown myeloma cell expression of β_2M. The knockdown efficiency was evaluated by flow cytometry, western blotting, quantitative real-time PCR, and ELISA. The results showed that β_2M shRNA reduced about 70% of surface expression of β_2M (Figure 2A; $P < 0.01$, compared with controls) and HLA-ABC (Figure 2B; $P < 0.01$, compared with controls). Significant reduction in the total β_2M protein (Figure 2C) and β_2M mRNA (Figure 2D) was observed ($P < 0.05$, compared with controls). In addition, β_2M-knockdown cells secreted significantly lower amount of soluble β_2M as compared to control cells (Figure 2E; $P < 0.05$). Next, the ADCC activities mediated by anti-β_2M mAbs in β_2M-knockdown cells were examined. Compared with control myeloma cells in which about 40% of cells were lysed, only fewer than 20% of β_2M-knockdown cells was killed (Figure 2F; $P < 0.05$), indicating that anti-β_2M mAb-mediated ADCC activity depended on the expression of surface β_2M on myeloma cells.

BM microenvironment factors do not protect myeloma cells from anti-β_2M mAb-mediated ADCC

Increasing evidence has shown that BMSCs in the myeloma tumor bed provide a tumor promoting microenvironment and protect MM cells from chemotherapy drug-induced apoptosis[36]. IL-6 is an important survival cytokine for MM[37], and promotes MM cell survival under chemotherapy agent dexamethasone treatment[38]. Therefore, we investigated whether BMSCs and IL-6 were able to protect MM cells from anti-β_2M mAb-induced ADCC. Our results showed that equally strong anti-β_2M mAb-induced ADCC activities were seen against ARP-1 (Figure 3A) and U266 (Figure 3B) cells in cultures with or without IL-6 or MSCs. These findings suggested that the ADCC activity overcomes the protective effects of IL-6 and BMSCs on MM cells and that the mAbs may be effective at mediating ADCC activity against MM cells in their microenvironment.

IL-2 and lenalidomide enhance anti-β_2M mAb-mediated ADCC

IL-2 is an essential factor for the differentiation and activity of NK cell, and is involved in the adaptive immune responses[39]. Lenalidomide is an immunomodulatory drug that has been used effectively for the treatment of MM[40], and is also known to increase the activity of NK cells[9, 10]. To investigate whether these agents may enhance anti-β_2M mAb-mediated ADCC activity, PBMCs from healthy donors were preincubated with IL-2 or lenalidomide for 48 hours before assay. The results showed that pretreatment of effector cells with IL-2 or lenalidomide enhanced anti-β_2M mAb-mediated ADCC against ARP-1 (Figure 3C) and U266 cells (Figure 3D), and the effect of lenalidomide was stronger than IL-2 ($P < 0.05$). Next we combined lenalidomide and IL-2 to determine whether there was synergistic effect of the two. The results (Figure 3E, 3F) showed that there was no further enhancing effect of combining lenalidomide and IL-2 compared with lenalidomide alone. These results indicated that NK cell activators lenalidomide and IL-2 could enhance anti-β_2M mAb-mediated ADCC, and lenalidomide was more efficient than IL-2, whereas the two NK cells activators had no additive or synergistic effect on the ADCC activity.

Combination treatment of anti-β_2M mAbs and lenalidomide in vitro and in vivo

To investigate whether there was a synergistic effect of lenalidomide and anti-β_2M mAb, we analyzed ADCC activities of anti-β_2M mAbs on lenalidomide-sensitive U266 WT and lenalidomide-resistant U266/R10R cell lines[34]. MTS assay was used and confirmed the drug sensitivity of the cell lines to lenalidomide (Figure 4A). As shown in Figure 4B, lenalidomide alone was only effective on U266 WT cells, while anti-β_2M mAbs alone could induce ADCC activities on both lenalidomide-sensitive U266 WT and lenalidomide-resistant U266/R10R cell lines. Combination of both induced similar cell apoptosis in U266 WT and U266/R10R cells, which was more efficacious than either of the treatments alone ($P < 0.05$ to $P < 0.01$). These results indicated that anti-β_2M mAbs was effective on both lenalidomide-resistant and -sensitive cells and could enhance the anti-tumor effects of lenalidomide.

We next examined the therapeutic activities of anti-β_2M mAbs in combination with lenalidomide in vivo in a xenograft myeloma model. SCID mice bearing ARP-1 subcutaneous tumors (n = 5 per group) were treated with lenalidomide, anti-β_2M mAbs, or a combination of both every 3 days for a total of 3 weeks. DMSO and mIgG1 were used as controls. The doses of the treatments were chosen based on our preliminary studies (data not shown). As shown in Figure 4C, although treatment with anti-β_2M mAbs or lenalidomide alone significantly reduced the tumor burdens in the mice ($P < 0.05$ to $P < 0.01$, compared with DMSO or mIgG1 controls), combinational treatment with anti-β_2M mAbs and lenalidomide was more efficacious than either of the treatments alone ($P < 0.05$ and $P = 0.104$, respectively, compared with mAbs or lenalidomide alone; and $P < 0.01$, compared with DMSO or mIgG1 controls). We used TUNEL assay to detect tumor cell apoptosis in treated mice. As shown in Figure 4D and 4E, significantly higher numbers of apoptotic tumor cells were detected in mice treated with lenalidomide ($P < 0.05$) and with anti-β_2M mAbs and lenalidomide ($P < 0.01$) compared with DMSO or mIgG1 controls. These data indicated that anti-β_2M mAbs and lenalidomide displayed enhanced in vitro and in vivo therapeutic effects against MM.

Anti-β_2M mAbs mediate CDC activities against myeloma cells

Next, we evaluated anti-β_2M mAb-mediated CDC activities using guinea pig serum as the source of complements. As shown in Figure 5A, anti-β_2M mAbs mediated, in a dose-dependent manner, significant CDC activities against myeloma cells as compared with mIgG1 control ($P < 0.05$ to $P < 0.01$). Heat-inactivated guinea pig serum was used as a negative control, and no CDC activities were detected (data not shown). Anti-β_2M mAb-induced CDC activity was evaluated in a panel of MM cell lines including ARP-1, MM.1S, U266, CAG and RPMI-8226. Compared to mIgG1, anti-β_2M mAbs effectively lysed all MM cells via mediating CDC (Figure 5B; $P < 0.05$ to $P < 0.01$). We examined anti-β_2M mAb-mediated CDC activities against normal B cells, MSCs and ARP-1 cells. The maximal lysis of B cells was 12.8%, MSC cells was 11.1%, and ARP-1 cells was 42.7% at mAb concentration of 20 μg/ml (Figure 5C), which indicated that the CDC activity of anti-β_2M mAbs was more towards myeloma cells. Similarly, anti-β_2M mAbs induced strong CDC lysis of MM patient-derived $CD138^+$ primary tumor cells (Figure 5D; $P < 0.05$). These

results indicated that anti-β_2M mAbs induced CDC activities against human MM cell lines and primary tumor cells from MM patients.

We examined whether myeloma-supporting stromal cells and IL-6 could protect MM cells against CDC-mediated lysis. In the studies, IL-6 or MSCs were cocultured with myeloma cells during CDC assay. Our results showed that strong anti-β_2M mAb-induced CDC activity was seen against ARP-1 (Figure 6A) and U266 (Figure 6B) cells in the absence or presence of MSCs or IL-6, suggesting that anti-β_2M mAbs still triggered CDC lysis of MM cells in the presence of BMSCs and IL-6. Anti-β_2M mAb-mediated CDC activity also depended on the expression of β_2M on the cell surface, because fewer β_2M-knockdown myeloma cells were killed as compared with control myeloma cells (Figure 6C; $P < 0.05$). Finally, we analyzed anti-β_2M mAb-mediated CDC activity on lenalidomide-sensitive cells U266 WT and lenalidomide-resistant cells U266/R10R, and the results showed that both cell lines were equally sensitive to the killing (Figure 6D).

Discussion

A large number of antibody therapeutics target surface antigens on tumor cells while simultaneously recruiting immune effector cells to specifically destroy the malignant cells. mAbs are emerging as a major new class of drugs that confer great benefits to cancer patients. Enhancing ADCC and CDC activities is one of the most promising ways to improve the clinical efficacy of already-approved antibodies, and this concept is actively being examined in the clinic, especially in the field of hematological malignancy treatment[41, 42]. In this study, we observed ADCC and CDC activities of anti-β_2M mAbs against established human MM cell lines and primary MM cells from patients. More importantly, lenalidomide enhanced anti-β_2M mAb-mediated ADCC activities through increasing the activity of NK cells.

The potent ADCC and CDC activities of anti-β_2M mAbs were more prone to the tumor cells and had lower effects on normal B cells and BMSCs. These findings indicated the potential of an immunotherapeutic strategy against MM by anti-β_2M mAbs with low side effects. The ADCC and CDC activities of anti-β_2M mAbs were dependent on the level of surface expression of β_2M. Anti-β_2M mAbs were also able to induce significant ADCC and CDC activities against MM cells with low β_2M surface expression. Therefore, anti-β_2M mAb may impact a larger and heterogeneous β_2M-expressing cancer patient population.

Previous research has shown that BMSCs in MM BM play a crucial role in MM drug resistance[43]. IL-6 promotes myeloma cell proliferation and drug resistance by activating PI3K-Akt pathway[44]. Our results showed strong ADCC and CDC activities of anti-β_2M mAbs on MM cells in the presence of BMSCs or IL-6, indicating its ability to overcome the MM growth and survival advantages conferred by the BM microenvironment. These findings suggested that anti-β_2M mAb has potent anti-MM activity and may be used to treat MM patients who have become resistant to conventional chemotherapy drugs.

IL-2 and lenalidomide treatment of NK cells could augment the activities of NK cells[10, 45]. IL-2 has been assessed, alone or in combination with IL-2-activated killer cells (“adoptive

immunotherapy"), for its anticancer potential in several animal models and in patients with various forms of advanced cancers[46]. Lenalidomide has been shown to modulate the activity of NK cells and macrophages in vitro and in vivo, providing the scientific rationale to combine it with mAb-based cancer therapies[10, 17, 47]. Anti-β_2M mAb, with its enhanced effector cell interaction capability, is expected to have superior anti-MM activity in combination with IL-2 and lenalidomide. Our results showed that IL-2 and lenalidomide pretreatment of effector cells significantly augmented anti-β_2M mAb-induced ADCC against ARP-1 and U266 MM cells. Synergy between anti-β_2M mAb and lenalidomide could also be found in vivo, underscoring a potential clinical development strategy for combining anti-β_2M mAb with lenalidomide to treat patients.

In our ADCC assays, the MM cell lines were allogeneic to the effector PBMCs or T cells. However, no significant lyse was observed in MM cells in cultures of allogeneic PBMCs without the mAbs, indicating that the alloreactivity did not affect the evaluation of anti-β_2M mAb-induced ADCC in the target cells. As the ADCC assay is a 4-hour assay, this is too short for alloreactivity of allogeneic PBMCs/T cells to be activated and observed.

ADCC is dependent on the interaction of the IgG Fc domain with FcγRs on effector cells. In this study, we used mouse anti-human β_2M-specific IgG1 mAbs to generate ADCC activities with human FcγRs on human NK cells. It is known that there is a cross-reaction between mouse IgGs and human FcγRs on human effector cells[48, 49]. However, for the therapeutic application, humanized mAbs will be developed and used to reduce the risk for immunogenicity of the mAbs in patients.

In conclusion, this study has demonstrated the significantly enhanced ADCC and CDC activities of anti-β_2M mAbs on myeloma but not normal cells, suggesting that anti-β_2M mAbs may be a more promising next-generation immunotherapeutic for the treatment of MM. Moreover, lenalidomide potentiated anti-β_2M mAb-induced MM cell killing via NK-mediated ADCC, which provides a rationale to combine these drugs to improve patient outcome in MM.

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Abbreviations

β_2M	β_2-microglubulin
MM	multiple myeloma
mAb	monoclonal antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
CDC	complement-dependent cytotoxicity
BMSCs	bone marrow stromal cells

MSCs	mesenchymal stem cells
IL-6	Interleukin-6
NK cell	Natural killer cell
MHC	major histocompatibility complex
MAC	membrane attack complex
PBMCs	peripheral blood mononuclear cells
TUNEL	TdT-mediated dUTP nick-end labeling
^{51}Cr	^{51}Chromium
shRNA	short-hairpin RNA
SDs	standard deviations

What's new?

anti-β_2M mAb-mediated ADCC and CDC activities were correlated with the expression of β_2M on the cell surface. anti-β_2M mAbs only showed limited cytotoxicity toward normal B cells or non-tumoric MSCs, indicating that the ADCC and CDC activities of anti-β_2M mAbs were more prone to the tumor cells. Lenalidomide synergistically enhanced in vitro ADCC against MM cells and in vivo tumor inhibition induced by anti-β_2M mAbs through increasing the activity of NK cells. These results support clinical development of anti-β_2M mAbs, both as a monotherapy and in combination with lenalidomide, to improve patient outcome of MM.

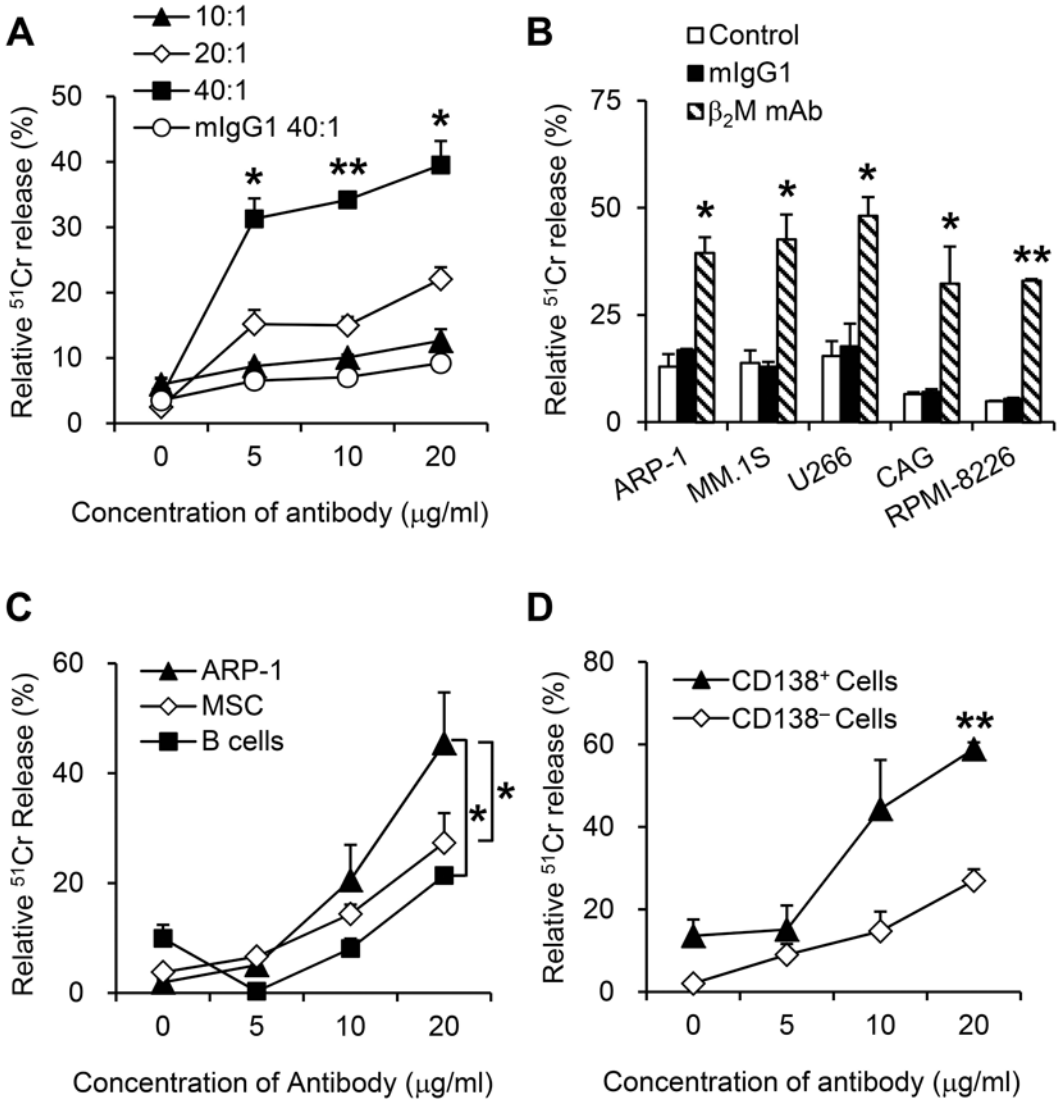

Fig. 1. ADCC activities of anti-β_2M mAbs against MM cells

Myeloma cells were incubated with ^{51}Cr for 1 hour, washed, and incubated with different numbers of PBMCs and anti-β_2M mAbs D1 or mIgG1 for 4 hours. Shown is ADCC killing of (**A**) ARP-1, (**B**) different MM cell lines, (**C**) MSCs, B cells and ARP-1, and (**D**) CD138$^+$ primary MM cells and CD138$^-$ nonmyeloma cells from MM patients, mediated by anti-β_2M mAbs at different concentrations (A, C, and D) and at different E:T ratios (A). In B, C and D, an E: T ratio of 40:1 was used. Summarized data from three performed independent experiments are shown. *$P < 0.05$, **$P < 0.01$.

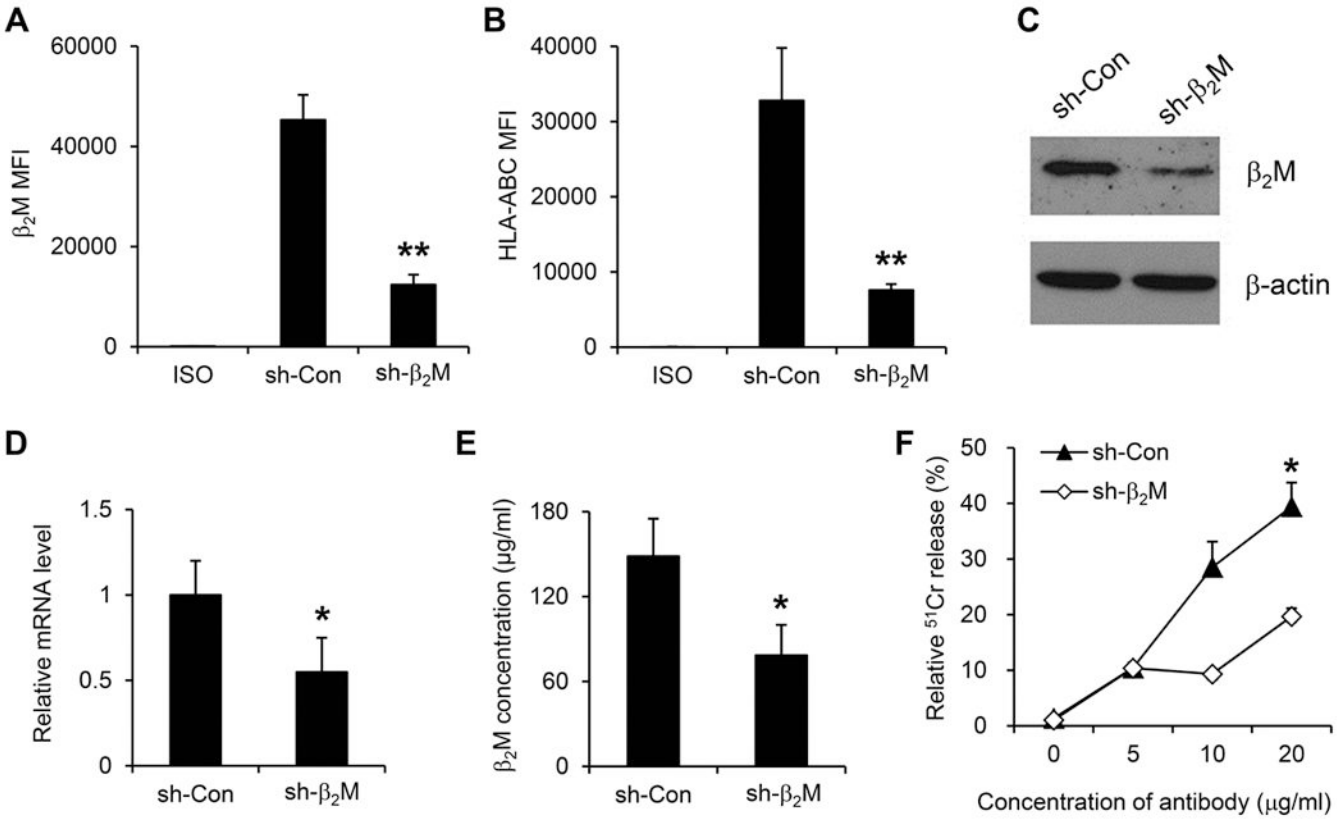

Fig. 2. ADCC activities of anti-β_2M mAbs correlated with cell surface expression level of β_2M

Cell surface expression of (**A**) β_2M and (**B**) HLA-ABC in β_2M-knockdown stably transfected cell line ARP-1. Numbers represent MFI (mean fluorescence intensity). (**C**) Western blotting analysis of the protein levels of β_2M in the MM cells. β-actin was used as an internal control. (**D**) Quantitative real-time RT-PCR analysis of the relative mRNA levels of *β_2M* in the MM cells. *GAPDH* was used as an internal control. (**E**) Secreted β_2M concentration by the MM cells. (**F**) ADCC activities of anti-β_2M mAbs against the MM cells. An E:T ratio of 40:1 was used. Summarized data from three performed independent experiments are shown. *$P < 0.05$, **$P < 0.01$.

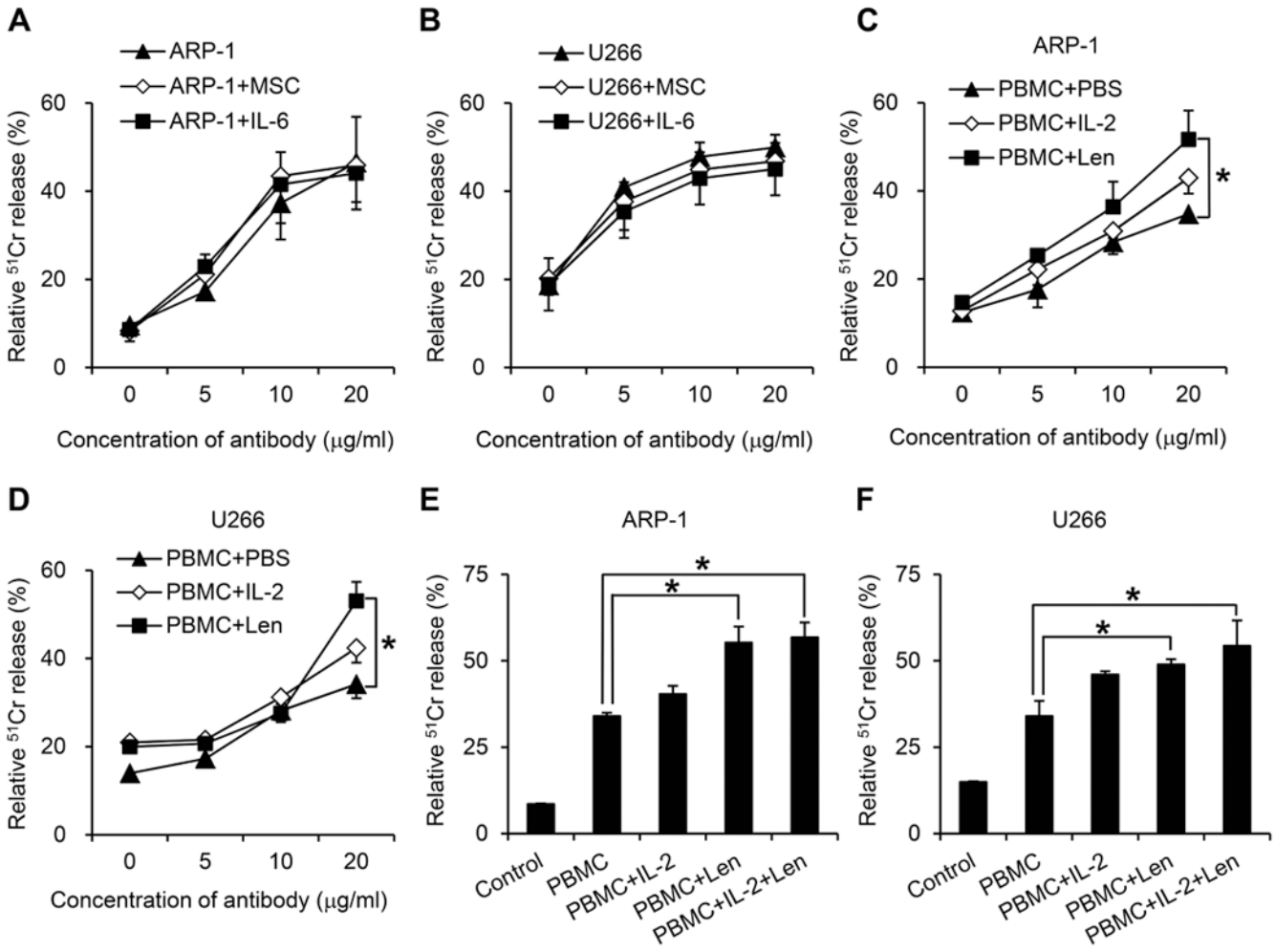

Fig. 3. The effects of BM microenvironment or NK cell activators on anti-β_2M mAb-mediated ADCC activities

ADCC activities against (**A**) ARP-1 or (**B**) U266 cells mediated by anti-β_2M mAbs in the presence or absence of MSCs and IL-6. IL-2 and lenalidomide enhance the ADCC activities of anti-β_2M mAbs against (**C**) ARP-1 and (**D**) U266 cells. Combination of IL-2 and lenalidomide had no further additive or synergistic effect to enhance ADCC activities of anti-β_2M mAbs against (**E**) ARP-1 and (**F**) U266 cells. An E:T ratio of 40:1 was used in these studies. Summarized data from three performed independent experiments are shown. *$P < 0.05$.

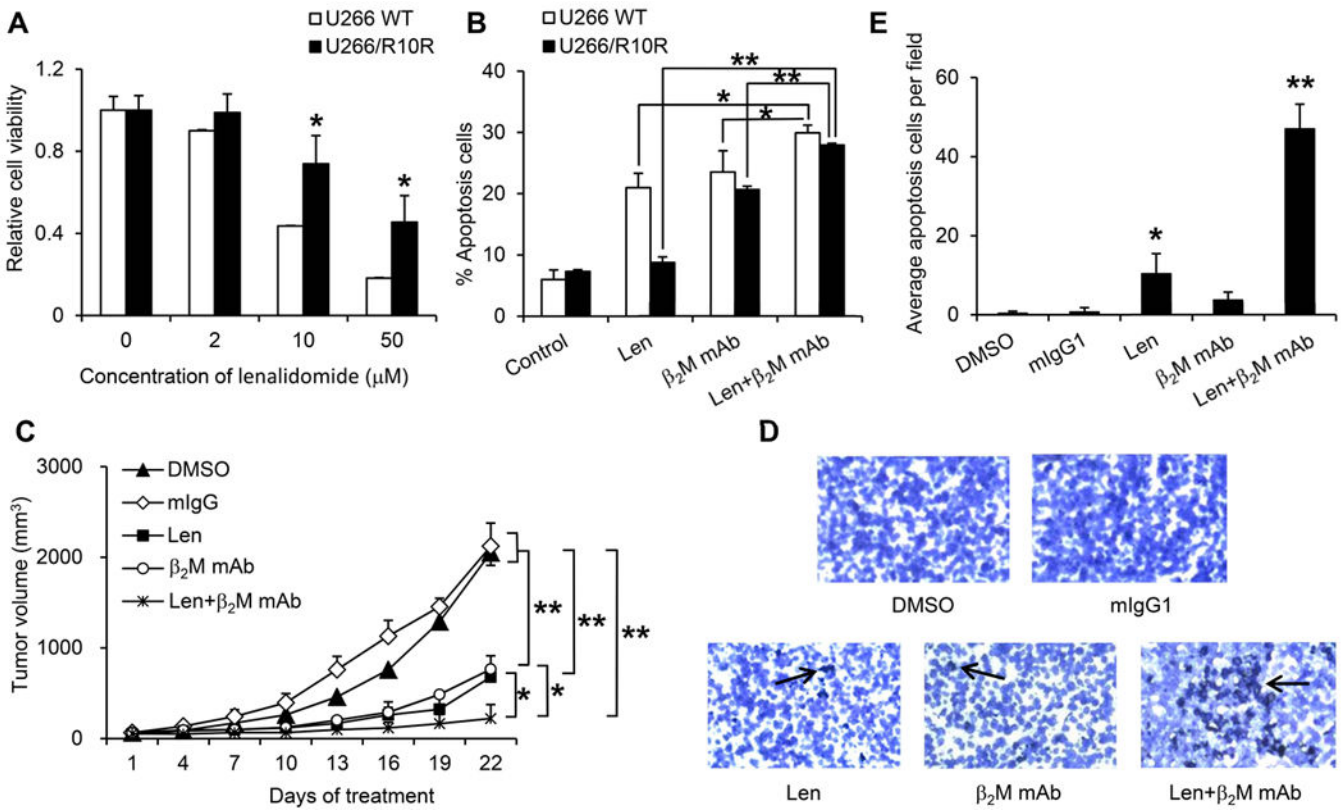

Fig. 4. Lenalidomide enhances the ADCC activities of anti-β_2M mAbs both in vitro and in vivo

(**A**) Viability of lenalidomide-resistant U266/R10R and -sensitive U266 WT MM cells in culture with different concentrations of lenalidomide for 48 hours. (**B**) ADCC activity detected as apoptosis of lenalidomide-resistant U266/R10R and -sensitive U266 WT MM cells. The MM cells and normal PBMCs were first incubated separately with lenalidomide for 48 hours, followed by washing and incubating together with anti-β_2M mAbs for 4 hours. Controls included medium alone (control), lenalidomide alone, and ADCC without lenalidomide. An E:T ratio of 40:1 was used. $CD138^+$ MM cell apoptosis was detected using Annexin V and PI staining. (**C**) Tumor burden of ARP-1 tumor-bearing SCID mice (n=5) treated with lenalidomide alone, anti-β_2M mAbs alone, or in combination. (**D, E**) In situ TUNEL assay was performed to detect cell apoptosis in the tumors of treated mice. Representative images were given in (**D**). Average numbers of apoptotic tumor cells from 6 randomly chosen fields were given in (**E**). Summarized data from three performed independent experiments are shown. *$P < 0.05$, **$P < 0.01$.

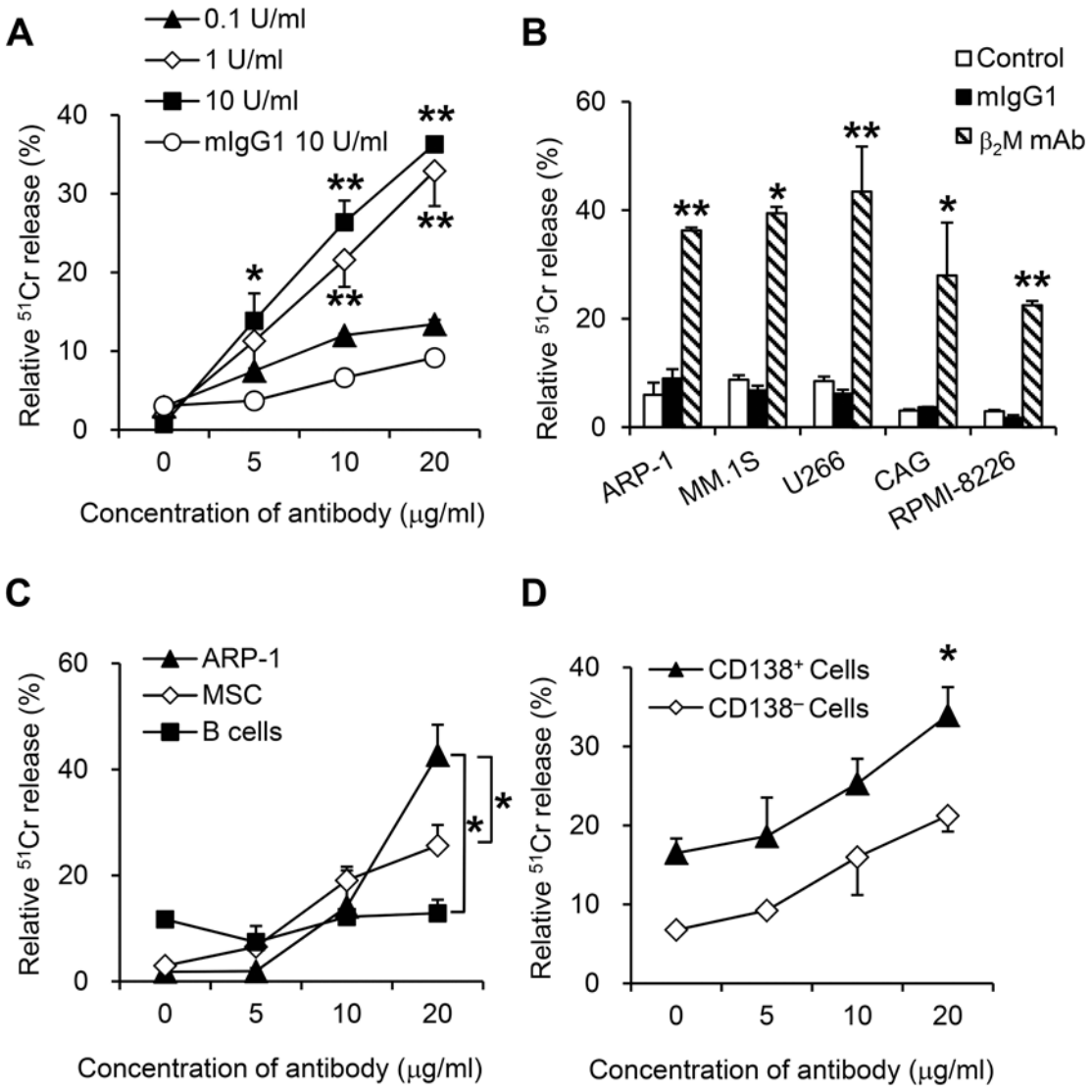

Fig. 5. CDC activities of anti-β_2M mAbs in MM cells

Myeloma cells were incubated with ^{51}Cr for 1 hour, followed by washing, addition of different concentrations of guinea pig serum and anti-β_2M mAbs or mIgG1, and incubation for 4 hours. Shown is CDC killing of (**A**) ARP-1, (**B**) different MM cell lines, (**C**) MSCs, B cells and ARP-1, and (**D**) CD138$^+$ primary MM cells and CD138$^-$ nonmyeloma cells from MM patients, mediated by different concentrations of anti-β_2M mAbs (A, C, and D) and different concentrations of guinea pig serum (A). In B, C and D, a concentration of guinea pig serum at 10 U/ml was used. Summarized data from three performed independent experiments are shown. *$P < 0.05$, **$P < 0.01$.

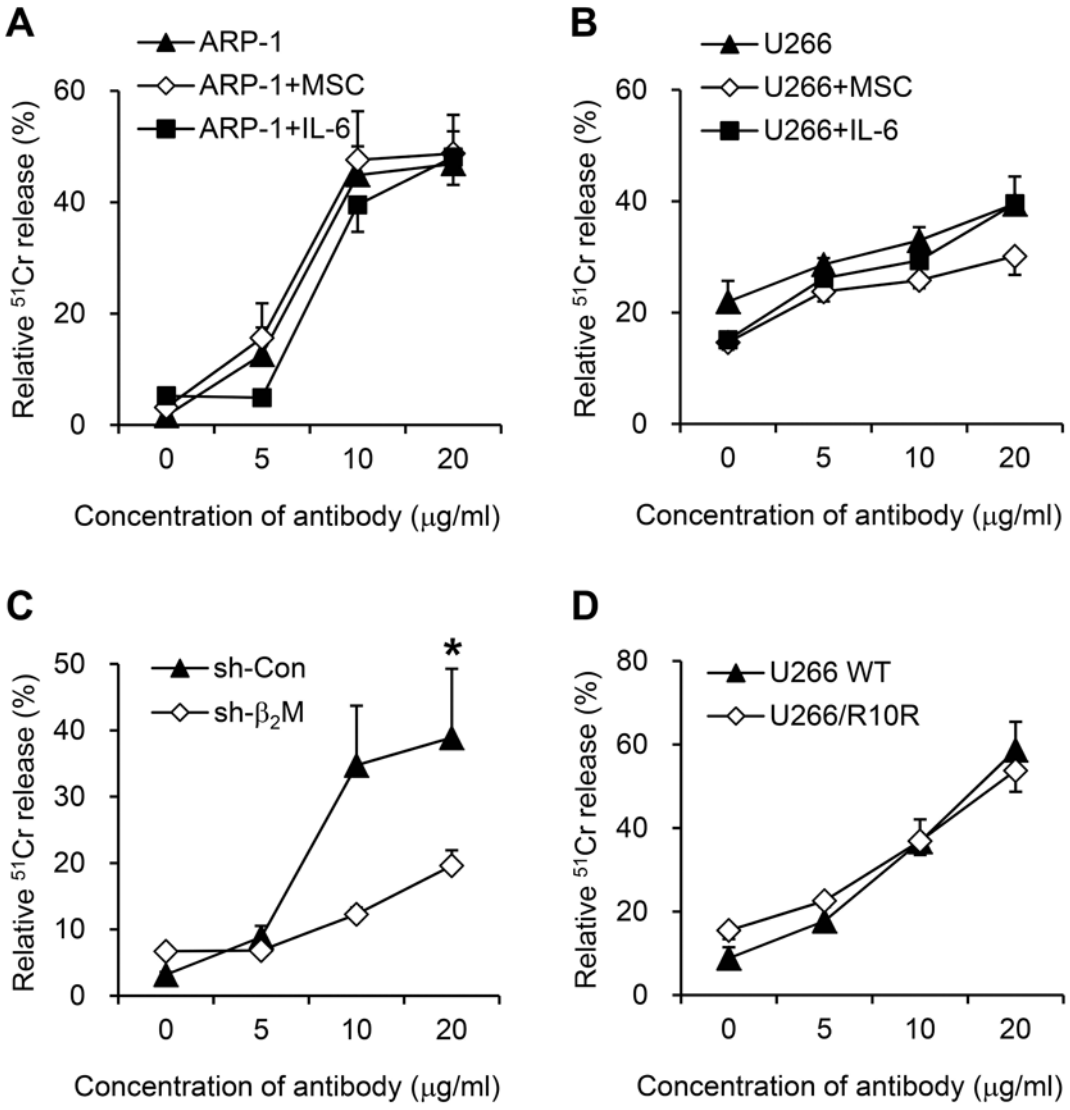

Fig. 6. CDC activities of anti-β_2M mAbs

CDC activities of anti-β_2M mAbs against (**A**) ARP-1 and (**B**) U266 cells in the presence or absence of MSCs and IL-6. (**C**) CDC activities of anti-β_2M mAbs against β_2M-knockdown stably transfected cell line ARP-1. (**D**) CDC activities of anti-β_2M mAbs against both lenalidomide-resistant U266/R10R and -sensitive U266 WT cells. A concentration of guinea pig serum at 10 U/ml was used in these studies. Summarized data from three performed independent experiments are shown. **P* < 0.05.