Short title: Anti-β2M antibodies exclude cytokine receptors from lipid rafts

Anti-β2-microglobulin monoclonal antibodies induce apoptosis in myeloma cells by recruiting MHC class I to and excluding growth and survival cytokine receptors from lipid rafts

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ABSTRACT

We recently showed that monoclonal antibodies (mAbs) against β2M have a remarkably strong apoptotic effect on myeloma cells. The mAbs induced apoptosis by recruiting MHC class I to lipid rafts, activated JNK, and inhibited PI3K/Akt and ERK pathways. Growth and survival cytokines such as IL-6 and IGF-I, which could protect myeloma cells from dexamethasone-induced apoptosis, did not affect mAb-mediated cell death. This study was undertaken to elucidate the mechanisms underlying anti-β2M mAb-induced PI3K/Akt and ERK inhibition and the inability of IL-6 and IGF-I to protect myeloma cells from mAb-induced apoptosis. We focused on lipid rafts and confirmed that these membrane microdomains are required for IL-6 and IGF-I signaling. By recruiting MHC class I into lipid rafts, anti-β2M mAbs excluded IL-6 and IGF-I receptors and their substrates from the rafts. The mAbs were not only redistributed the receptors in cell membrane, but also abrogated IL-6- or IGF-I-mediated JAK/STAT3, PI3K/Akt, and Ras/Raf/ERK pathway signaling, which are otherwise constitutively activated in myeloma cells. Thus, this study further defines the tumoricidal mechanism of the mAbs and provides strong evidence to support the potential of these mAbs as therapeutic agents for myeloma.
Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of monoclonal plasma cells in the bone marrow. Binding of myeloma cells to bone marrow stromal cells triggers transcription and secretion of cytokines from stromal cells, which not only promote growth, survival and migration of myeloma cells but also confer resistance to conventional chemotherapy. Previous studies have shown that cytokines such interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-I) are the major growth and survival factors for myeloma cells, and play a crucial role in the onset of plasma cell tumors in mice. Specifically, IL-6 binds to gp80 (CD80, IL-6 receptor; IL6R), which is expressed on most myeloma cell lines and patient tumors, and induces phosphorylation and dimerization of gp130. Phosphorylation of gp130 in turn activates multiple downstream signaling pathways, such as JAK/STAT3, Ras/Raf/MAP kinases (MAPK), and PI3K/Akt, and triggers myeloma cell growth, survival, and drug-resistance. Likewise, IGF-I binds to IGF-I receptor (IGF-IR) and exerts its anti-apoptotic effects on myeloma cells via activating anti-apoptotic signaling pathways, such as Ras/Raf/MAPK and PI3K/Akt. Therefore, it may be useful to disrupt growth factor-mediated anti-apoptotic signaling pathways for myeloma therapy, which might provide the framework to develop and validate novel antimyeloma agents to overcome drug-resistance and improve patient outcome.

Lipid rafts, cholesterol- and glycosphingolipid-enriched dynamic patches in the plasma membrane, organize the plasma membrane into functional units. These raft domains act as platforms for conducting different signals into cells for various functions, including cytokine-mediated growth signaling. Integral proteins in the cellular membrane, such as caveolins and flotillins, can modify lipid rafts structurally and functionally, and may therefore affect subsequent cellular functions. Some reports have shown that growth factors, such as IL-6, induce translocation of their receptors to lipid rafts and confer protection against dexamethasone treatment. Remacle-Bonnet and coworkers observed that lipid rafts segregated pro-apoptotic from anti-apoptotic IGF-IR-mediated signaling in tumor cells, suggesting that the localization of growth factor receptors outside lipid rafts might be involved in the transduction of apoptotic signals.
Furthermore, we and others demonstrated that lipid rafts might be involved in anti-β2-microglobulin (β2M), major histocompatibility complex (MHC) class II, and CD20 mAb-induced apoptosis in tumor cells, indicating that lipid rafts might also be an important platform for the mAb-mediated tumoricidal effects on myeloma cells.

We have recently shown that anti-β2M mAbs have remarkable tumoricidal activity on myeloma cells both in vitro and in xenograft myeloma SCID mouse models. We demonstrated that anti-β2M mAbs induced myeloma cell apoptosis by recruiting MHC class I molecules to lipid rafts, activated JNK and inhibited PI3K/Akt and ERK, compromised mitochondrial integrity, and activated the caspase-9-dependent cascade. To further elucidate the mechanisms of mAb-induced inhibition of PI3K/Akt and ERK signaling pathways and the inability of IL-6 and IGF-I to protect myeloma cells from apoptosis, we examined the localization of cytokine receptors and their signaling pathways in myeloma cells with or without treatment with anti-β2M mAbs. We confirmed that IL-6 and IGF-I signaling pathways depend on lipid rafts and showed that anti-β2M mAbs recruit MHC class I to and exclude cytokine receptors from lipid rafts.

**Materials and methods**

**Myeloma cell lines, primary myeloma cells, and reagents**

Human myeloma cell line ARP-1 was established at the Arkansas Cancer Research Center from bone marrow aspirates of patients with MM, and MM.1S was kindly provided by Dr. Steven Rosen of Northwestern University, Chicago, IL. Other cell lines were purchased from American Type Culture Collection (Rockville, MD). All cell lines were cultured in RPMI-1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in humidified 95% air and 5% CO2. Primary myeloma cells were isolated from bone marrow aspirates obtained from patients during a routine clinic visit. CD138++ myeloma cells were isolated by magnetic-bead sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The study was approved by the Institutional Review Board at The
University of Texas M. D. Anderson Cancer Center. Informed consent was provided according to the Declaration of Helsinki.

Recombinant human IL-6 and IGF-I were purchased from R&D Systems (Minneapolis, MN). Mouse IgG1 and dexamethasone were purchased from Sigma (St. Louis, MO). Monoclonal antibodies against HLA-ABC (clone W6/32) were purchased from Serotec Ltd.

**Generation of β2M-specific monoclonal antibodies (mAbs)**

We generated a panel of β2M-specific monoclonal antibodies as previously described. Among of them, D1 and E6 (isotype IgG1) were chosen for this study because of their strong antimyeloma activities.

**Apoptosis assays**

Cells were incubated with 50 µg/mL of β2M-specific mAbs D1 or E6, or mouse IgG1 as IgG control, with or without the addition of IL-6 (10 ng/mL) or IGF-I (50 ng/mL) for 48 hours (h). In some experiments, cells were treated with dexamethasone (10 µM) in the presence of β2M-specific mAbs and IL-6 (10 ng/mL) for 48 h. The fraction of apoptotic cells was determined by staining cells suspended in Annexin-V binding buffer (PharMingen) with FITC-conjugated Annexin-V and propidium iodide (PI), according to manufacturer’s instructions. After 15 min of incubation at room temperature, samples were analyzed by flow cytometry. Apoptotic cells were determined as Annexin V-positive cells.

**RNA interference**

Double-stranded, 21-mer siRNA corresponding to β2M was designed with the following sense and antisense sequences: sense: 5'-GAUUCAGGUUUACUCAC GUdTdT-3'; and antisense: 5'-ACGUGAGUAAACCUGAAUCdTdT-3', starting from nucleotide 91 of the β2M sequence (accession number AB021288). The siRNA was synthesized by Dharmacon (Lafayette, CO). Cells were harvested, plated on a 24-well plate with 2 × 10^5 cells per well, and 24 h later, transiently transfected with specific β2M siRNA or nonspecific/control siRNA using the Oligofectamine transfection reagent (Mirus, Madison, WI) according to manufacturer’s instructions. Seventy-two h after the
transfection, cells were harvested to examine surface β2M and HLA-ABC protein expression or used for experiments.

**Isolation of lipid rafts by sucrose density gradient ultracentrifugation**

The Caveolae/Raft Isolation Kit (Sigma) was used to isolate lipid rafts as low density, detergent-resistant membrane fractions by sucrose density gradient centrifugation using 1% Triton-X-100. Briefly, 5 × 10⁷ cells were lysed for 30 min in ice-cold lysis buffer. Cell lysates were mixed with OptiPrep to 35%, placed at the bottom of the ultracentrifuge tube, overlaid with 4 layers of 30 to 0% OptiPrep, and centrifuged at 200,000 × g using a TFT 65.13 rotor (Kontron Instruments) for 4 h at 4°C. Nine fractions (1 mL each) were collected from the top to the bottom of the gradients. The lipid rafts determined with CTB binding for GM1 gangliosides and with a caveolin-1 specific antibody were found in fractions 2 to 5. Non-lipid raft fractions were present in fractions 7 to 9, which were negatively stained by GM1 gangliosides and caveolin-1.

**Cholesterol depletion**

For cholesterol depletion, myeloma cells were preincubated with 5 mM of methyl-β-cyclodextrin (MCD, purchased from Sigma) for 30 min, washed, and incubated with or without β2M-specific mAbs (50 µg/mL) for 48 h, followed by cell apoptosis analysis.

**Immunoprecipitation assay**

Myeloma cells were incubated with IL-6 (10 ng/mL) or IGF-I (50 ng/mL) with or without 50 µg/mL of β2M-specific mAbs or mouse IgG1 on ice for 30 min, washed, and lysed in 1 mL RIPA buffer (10 mM Tris-HCL buffer, pH 7.5, 1% NP-40, 0.25% deoxycholate wt/vol, 2 mM EDTA, 10 mM orthovanadate). Cell lysates were incubated with antibodies specific to caveolin-1, gp130, or IGF-IRβ, followed by precipitating with protein G-Sepharose in a 50% wt/vol slurry. Immunoprecipitated proteins were washed in RIPA buffer, subjected to SDS-PAGE, and immunoblotted with specific antibodies against caveolin-1, gp130, IGF-IRβ, or MHC class I molecules.

**Western blotting analysis**
Cells were cultured with IL-6 (10 ng/mL) or IGF-I (50 ng/mL) with or without 50 µg/mL of β2M-specific mAbs, harvested, washed, and lysed with lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN₃, 1% Triton-X-100, 1% NP-40, 1 × protease inhibitor cocktail). Cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with antibodies against phosphorylated or non-phosphorylated IL-6R, JAK, STAT3, Akt, Raf, ERK1/2, IGF-IRβ, IRS-1, ASK1, MLK3, MEKK-1, MKK4, MKK7, and JNK (Cell Signaling Technology, Inc., Beverly, MA, and Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibodies conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography. For protein quantification, blots were scanned and analyzed by spot densitometry, and results were expressed as average value of pixels enclosed (AVG), calculated as the sum of all the pixel values after background correction divided by area.

**Statistical analysis**

All data are shown as means ± standard deviation. The Student t test was used to compare various experimental groups. Significance was set at P < .05.

**Results**

**Growth and survival factors do not abrogate β2M-specific mAb-induced myeloma cell apoptosis**

Previous studies showed that addition of IL-6 protected myeloma cells from dexamethasone-induced apoptosis. As we previously reported that anti-β2M mAbs induced apoptosis in myeloma cells, we asked whether IL-6 would also protect myeloma cells from the mAb-induced apoptosis. In our experiments, β2M-specific mAbs (50 µg/mL) were added to cultures of myeloma cell lines ARP-1 and MM.1S, with or without addition of 10 ng/mL of human IL-6. Cell apoptosis was examined 48 h later by Annexin-V staining assay. As shown in **Figure 1A**, β2M-specific mAb D1, but not mouse IgG1 (data not shown), and dexamethasone effectively induced apoptosis in myeloma...
cells. The addition of IL-6 did not affect mAb-induced apoptosis but significantly undermined dexamethasone-induced cell death ($P < .05$; Fig 1A). Increasing IL-6 concentration to 100 ng/mL still failed to protect myeloma cells from $\beta_2$M-specific mAb-induced apoptosis (data not shown). Likewise, $\beta_2$M-specific mAbs ($P < .01$, compared with medium controls) but not dexamethasone killed two IL-6-dependent cell lines XG1 and ANBL-6 in their culture with the addition of exogenous IL-6 (Figure 1B; 2 ng/mL for normal culture, or 20 ng/mL for high concentration; data not shown). However, in the absence of IL-6, both the mAb and dexamethasone induced apoptosis in these two cell lines ($P < .05$, compared with medium controls).

IGF-I is another important growth and survival factor for myeloma cells. Therefore, we examined the effect of IGF-I on $\beta_2$M-specific mAb-induced apoptosis. Myeloma cells were cultured with the $\beta_2$M-specific mAbs with or without the addition of IGF-I (50 ng/mL). Again, results showed that IGF-I did not affect $\beta_2$M-specific mAb-induced cell death but reduced dexamethasone-mediated apoptosis in myeloma cells (Figure 1A).

We next investigated the activity of IL-6 and IGF-I on freshly isolated primary tumor cells from patients with MM. Purified myeloma cells obtained from bone marrow aspirates of four newly diagnosed, previously untreated patients with MM were examined and showed sensitivity to $\beta_2$M-specific mAb-mediated killing (data not shown). As shown by the representative results depicted in Figure 1C, addition of 10 ng/mL IL-6 or 50 ng/mL IGF-I to the cultures had no effect on $\beta_2$M-specific mAb-induced apoptosis in the primary myeloma cells, whereas both IL-6 and IGF-I significantly reduced the percentages of apoptotic cells induced by dexamethasone ($P < 0.05$). Similar results were obtained with primary myeloma cells from all four patients (Figure 1D). Taken together, these results indicate that although IL-6 and IGF-I are potent protectors of myeloma cells against dexamethasone, they could not abrogate mAb-mediated apoptotic activities in myeloma cells.

To confirm the importance of surface $\beta_2$M as the target for $\beta_2$M-specific mAb-induced but not for dexamethasone-induced apoptosis and to exclude the involvement of surface $\beta_2$M in IL-6-mediated protection of apoptosis in myeloma cells, small interfering RNA
(siRNA) specific for human \( \beta_2M \) gene was synthesized and used to knockdown the \( \beta_2M \) expression in myeloma cells. We were able to achieve 70 to 80% reduction of surface \( \beta_2M \) and HLA-ABC protein expression on myeloma cells on day 3 after transfection with \( \beta_2M \)-specific siRNA but not with control siRNA or mock transfection (Fig. 2A). Detection of \( \beta_2M \) mRNA by RT-PCR confirmed these results (data not shown). This treatment was specific for \( \beta_2M \), as surface expression of HLA-DR (data not shown) and CD38 remained unchanged. Knockdown of surface \( \beta_2M \)/MHC class I on myeloma cells rendered cells resistant to \( \beta_2M \)-specific mAb-induced apoptosis but did not affect dexamethasone-induced or IL-6-mediated protection of myeloma cell apoptosis (Fig. 2B).

**\( \beta_2M \)-specific mAbs exclude growth and survival factor receptors from lipid rafts**

Our previous study showed that \( \beta_2M \)-specific mAbs inhibited MEK/ERK and PI3K/Akt pathways by binding to \( \beta_2M \) and recruiting MHC class I to the lipid rafts. As these pathways are downstream of IL-6 and IGF-I receptor activation, we hypothesized that MHC class I relocation to lipid rafts may disrupt IL-6 and IGF-I receptor signaling, as lipid rafts are considered to function in part as platforms for signaling from the receptors. Therefore, we examined the localization of IL-6 and IGF-I receptors on myeloma cells before and after \( \beta_2M \)-specific mAb treatment. Myeloma cells were incubated with IL-6 (10 ng/mL) or IGF-I (50 ng/mL), with or without \( \beta_2M \)-specific mAbs (50 \( \mu \)g/mL). Mouse IgG1 was used as control for the mAbs. Cells without treatment were used as control. After 30 min of treatment, cell lysates were prepared and separated using a discontinuous sucrose gradient ultracentrifugation followed by immunoblotting with specific antibodies. In the light buoyant density fractions, fractions 2 to 5 were positive for GM1 gangliosides, identified by CTB binding, and contain lipid rafts, which stained positive for caveolin-1, a raft-associated protein absent in the non-raft fractions 7 to 9 (Figure 3).

As shown in Figure 3A, IL-6R gp130, IGF-IR\( \beta \), IRS-1 (an IGF-IR substrate), and MHC class-I were detected in the non-raft fractions in control myeloma cells. Upon IL-6 or IGF-I stimulation, the majority of gp130, IGF-IR\( \beta \), and IRS-1 molecules were detected in the raft fractions, whereas MHC class-I were located in the non-raft fractions, indicating
that the cytokine receptors and substrates were relocalized to lipid rafts for signaling (Figure 3B). Surprisingly, in cells treated with both anti-β2M mAb (D1) and IL-6 or IGF-I (Figure 3D), but not mouse IgG1 and IL-6 or IGF-I (Figure 3C), most gp130 and IGF-IRβ were detected in the non-lipid raft fractions and fewer IRS-1 molecules were present in the raft fractions, while MHC class I were recruited to the lipid raft fractions as we showed previously. These findings suggest that the cytokine receptors and their substrate were excluded from the lipid rafts as a result of β2M-specific mAb-mediated recruitment of MHC class I to the rafts.

To confirm the results above, an immunoprecipitation assay was used to analyze the interactions of MHC class I, gp130, or IGF-IRβ with caveolin-1, a raft-associated protein able to directly bind cholesterol. Cell lysates prepared from myeloma cells treated with IL-6 or IGF-I, with or without β2M-specific mAbs, were precipitated by a specific antibody against caveolin-1, followed by Western blotting analysis to detect protein expression of gp130, IGF-IRβ, and MHC class I by specific antibodies. As shown in Figure 4A, MHC class I and caveolin-1 were coprecipitated from cells treated with cytokine and β2M-specific mAbs but not from cells treated with cytokine alone, confirming that MHC class I were localized within the lipid rafts upon β2M-specific mAb treatment. In contrast, both gp130 and IGF-IRβ were strongly associated with caveolin-1 in cells treated with the cytokine (IL-6 or IGF-I), and the addition of β2M-specific mAbs significantly reduced the amounts of gp130 and IGF-IRβ coprecipitated with caveolin-1. Likewise, immunoprecipitation using antibodies specific to gp130 (Figure 4B) or IGF-IRβ (Figure 4C) yielded similar amounts of gp130 or IGF-IRβ from cells treated with the cytokine (IL-6 or IGF-I) without or with β2M-specific mAbs, whereas the amounts of coprecipitated caveolin-1 were significantly lower in cells treated with the cytokine and the mAbs than cells treated with the cytokine alone. These results indicate that gp130 and IGF-IRβ were physically associated with caveolin-1 in cytokine-treated cells but not or much less in cytokine- and β2M-specific mAb-treated myeloma cells.

**Disruption of lipid rafts abrogates both IL-6 signaling and β2M-specific mAb apoptotic effects in myeloma cells**
To confirm the importance of the lipid rafts in IL-6 signaling and protection of myeloma cell apoptosis, we treated myeloma cells with MCD, an agent that disrupts the structure of lipid rafts in cell membrane. As shown in Figure 4D, MCD treatment abrogated IL-6-mediated protection of myeloma cell apoptosis induced by dexamethasone ($P < 0.05$ to $P < 0.01$), indicating that lipid rafts are crucial for IL-6R signaling to activate anti-apoptotic pathways in myeloma cells. As expected, MCD also abrogated $\beta_2$M-specific mAb-induced apoptosis in myeloma cells. Taken together, these results demonstrate that the $\beta_2$M-specific mAbs induced dominant changes in the distribution of MHC-I molecules and cytokine receptors in lipid rafts.

$\beta_2$M-specific mAbs inhibit growth and survival factor-mediated anti-apoptotic signaling pathways

To further elucidate the molecular mechanisms underlying apoptosis protection, we next examined the impact of $\beta_2$M-specific mAbs on the downstream signaling pathways of cytokine receptors. First, we focused on IL-6-activated JAK/STAT3, PI3K/Akt, and Ras/Raf/ERK pathways, which are essential to myeloma cell growth and survival. Myeloma cells were treated with IL-6 without or with $\beta_2$M-specific mAbs or mouse IgG1. Western blotting analysis was performed using specific antibodies against phosphorylated (p) or nonphosphorylated IL-6R, JAK1/2, STAT3, Akt, Raf, and ERK1/2. As shown in Figure 5A, treatment of cells with IL-6 or IL-6 plus mouse IgG1 (data not shown) induced phosphorylation of IL-6R (pIL-6R), and upregulated the levels of pJAK1/2, pSTAT3, pAkt, pRaf, and pERK, indicating that IL-6 activated JAK/STAT3, PI3K/Akt, and Ras/Raf/ERK signaling pathways. In contrast, $\beta_2$M-specific mAb treatment inhibited IL-6-induced phosphorylation of IL-6R and the kinases. These results are supported by protein quantification data of phosphorylated IL-6R and kinases ($P < 0.05$ and $P < 0.01$; Figure 5B). The levels of nonphosphorylated IL-6R and kinases remained unchanged. After 60 min of treatment, the kinase activities were undetectable in $\beta_2$M-specific mAb-treated myeloma cells.

Second, we examined the impact of $\beta_2$M-specific mAbs on IGF-I-mediated signaling pathways, including PI3K/Akt and Ras/Raf/ERK. As shown in Figure 5C, IGF-I or IGF-
I plus mouse IgG1 (data not shown) stimulated the phosphorylation of IGF-IRβ and its substrate IRS-1, and as a consequence, upregulated the expression of pAkt and pERK in myeloma cells. Treatment of cells with β2M-specific mAbs significantly downregulated the levels of pIGF-IRβ, pIRS-1, pAkt, and pERK induced by IGF-I (protein quantification data shown in Figure 5D; \( P < .05 \) and \( P < .01 \)). These results indicated that β2M-specific mAbs abrogate growth factor-mediated anti-apoptotic signaling pathways in myeloma cells.

**β2M-specific mAbs activate the upstream kinases of JNK signaling pathway**

Since our previous studies demonstrated that β2M-specific mAbs induced myeloma cell apoptosis via JNK activation, we examined the activities of kinases upstream of JNK signaling pathway, including ASK1, MLK3, MEKK1, and MKK4/7. As shown in Figure 6A-B, treatment of myeloma cells with β2M-specific mAbs significantly increased the level of phosphorylated ASK1, MLK3, MEKK1, MKK4, and MKK7 (\( P < .01 \)). The phosphorylation of these kinases was observed as early as 15 min and lasted for 2 h after the treatment, although their kinetics were slightly different. As a consequence, protein levels of phosphorylated JNK (pJNK) were increased in β2M-specific mAb-treated myeloma cells. These results further confirm that β2M-specific mAb activate the JNK signaling pathway.

**DISCUSSION**

The importance of IL-6 and IGF-I in the pathogenesis of MM is well documented\(^5-8\). Recent studies have also shown that these cytokines play an important role in myeloma cell survival and protect the tumor cells from chemotherapy drugs, such as dexamethasone-induced apoptosis\(^9\). IL-6 binds to its receptor gp80, induces phosphorylation and dimerization of gp130, and activates JAK/STAT3, PI3K/Akt, and Ras/Raf/ERK pathways in myeloma cells\(^10-12\). STAT3 regulates downstream protein expression of Bcl-2 family members Bcl-XL and Mcl-1, which inhibits mitochondria-dependent caspase cascade activation\(^26,27\). Other studies showed that IL-6 protects
myeloma cells against dexamethasone-induced apoptosis via activating PI3K/Akt signaling pathway. Likewise, IGF-I is another important growth and survival factor for myeloma cells. The assembly of signaling complex at the cytoplasmic domain of IGF-IR results in the activation of PI3K/Akt and Ras-dependent MAPK cascades. The strong anti-apoptotic activity of IGF-I in myeloma cells is mediated through Akt-mediated inactivation of the pro-apoptotic Bcl-2 family member Bad. In this study, we showed that, although IL-6 and IGF-I abrogate dexamethasone-mediated apoptosis, high levels of the cytokines could not reduce the apoptotic effects of β2M-specific mAbs on myeloma cells.

In our previous study, we demonstrated that by relocating to lipid rafts, MHC class I recruit and activate Lyn and PLCγ2, which in turn activate JNK. In this study, we examined the activation and phosphorylation of the kinases upstream of JNK and showed that ASK1, MLK3, MEKK-1, MKK4, and MKK7 are indeed phosphorylated in myeloma cells following anti-β2M mAb treatment. Thus, these results confirm our previous observations and conclusions. As shown in Figure 7, Lyn and PLCγ2 activation led to phosphorylation of ASK1, MLK3, and MEKK1, which in turn phosphorylate MKK4/7. As a consequence, MKK4/7 phosphorylates and activates JNK, leading to myeloma cell apoptosis. However, these findings do not explain why PI3K/Akt and ERK pathways were inhibited by β2M-specific mAbs.

To further define the mechanisms underlying β2M-specific mAb-induced apoptosis and the inability of IL-6 and IGF-I to counteract the effects of the mAbs on myeloma cells, we focused on lipid rafts because they are involved in cell growth and apoptosis signaling. IL-6 binds to IL-6R, recruits the receptors to lipid rafts, and stimulates downstream anti-apoptotic signaling pathways to resist dexamethasone treatment. In this study, we confirmed that stimulation of myeloma cells by IL-6 or IGF-I led to relocation of gp130, or IGF-IRβ, and its substrate IRS-1, to lipid rafts and increased affinity of receptor binding to caveolin-1, which regulates the structure and function of lipid rafts. Disruption of the integrity of lipid rafts interrupted IL-6 and IGF-I signaling and abrogated cytokine-mediated protection of myeloma cells against dexamethasone-induced apoptosis. In addition, we recently showed that MHC class I molecules are not present in lipid rafts.
under physiological conditions in myeloma cells. However, β2M-specific mAbs bound to surface β2M/MHC class I molecules and recruited them to lipid rafts, leading to MHC class I binding to caveolin-1 and consequently activating the upstream kinases of JNK. Moreover, we observed that MHC class I molecules replaces gp130, IGF-IRβ, and IRS-1 in lipid rafts, because these receptors and the substrates were found in the non-raft fractions after β2M-specific mAb treatment. These findings indicate that β2M-specific mAbs exclude growth factor receptors from lipid rafts and abrogate IL-6- or IGF-I-mediated JAK/STAT3, PI3K/Akt, and Ras/Raf/ERK pathway signaling, which are otherwise constitutively activated in myeloma cells. Thus, these results provide a plausible explanation for anti-β2M mAb-induced inhibition of PI3K/Akt and ERK pathways in myeloma cells. Our results also showed that disruption of lipid rafts by MCD abrogates both β2M-specific mAb-induced apoptotic effects on myeloma cells and IL-6-mediated protection against dexamethasone treatment, indicating that the integrity of lipid rafts is important for both pro-apoptotic and anti-apoptotic signaling.

In conclusion, this study demonstrated that anti-β2M mAbs induce cell death via recruiting MHC class I molecules to lipid rafts, which not only activate JNK via Lyn and PLCγ2, but also inhibit PI3K/Akt and ERK pathways by excluding IL-6 and IGF-I receptors from lipid rafts and disrupting their signaling pathways (Figure 7). These findings explain why the cytokines protect myeloma cells from dexamethasone-induced apoptosis but had no effect on cell death induced by the mAbs. Thus, this study further defines the tumoricidal mechanism of the mAbs and provides strong evidence to support the potential and implication of these mAbs as therapeutic agents for myeloma.
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The authors declare no competing financial interests.

AUTHORSHIP

Contribution: JY and QY initiated the work, designed the experiments, and wrote the paper. JY, XZ, JW, JQ, and LZ performed the experiments and statistical analyses, and MW and LWK provided patients’ samples and critical suggestions to this study.
References


LEGENDS TO FIGURES

**Figure 1.** Effects of IL-6 and IGF-I on β²M-specific mAb- or dexamethasone-induced apoptosis in myeloma cells. (A) Apoptosis of myeloma cells ARP-1 and MM.1S in 48-h cultures with β²M-specific mAb D1 (50 µg/mL) or dexamethasone (10 µM) in the presence or absence of IL-6 (10 ng/mL) or IGF-I (50 ng/mL). Similar results were obtained with other myeloma cell lines; (B) Apoptosis of two IL-6-dependent cell lines XG1 and ANBL-6 in 24-h cultures with β²M-specific mAb D1 (50 µg/mL) or dexamethasone (10 µM) in the presence or absence of IL-6 (10 ng/mL); Flow cytometry analysis from (C) a representative myeloma patient and (D) pooled data from all patients showing apoptosis of primary myeloma cells in 24-h cultures with β²M-specific mAb D1 (50 µg/mL) or dexamethasone (10 µM) in the presence or absence of IL-6 (10 ng/mL) or IGF-I (50 ng/mL). Similar results were obtained with primary myeloma cells with anti-β²M mAb E6. Apoptotic cells were determined by Annexin V-binding assay. Results of three experiments are shown. *P < .05, and **P < .01.

**Figure 2.** Knockdown of surface β²M/MHC class I abrogates β²M-specific mAb- but not dexamethasone-induced apoptosis in myeloma cells. (A) Levels (mean fluorescence intensity) of surface β²M, HLA-ABC, and CD38 on untreated myeloma cells and on cells treated with mock transfection or transfected with control (ctr) siRNA or β²M-specific siRNA. Shown are the results of myeloma cell line MM.1S. Similar results were obtained from ARP-1. Analysis was performed 72 h after transfection. (B) Apoptosis of myeloma cells (ARP-1 and MM.1S) transfected with control (ctr) siRNA or β²M-siRNA in cultures with β²M-specific mAb D1 (50 µg/mL) or dexamethasone (10 µM) in the presence or absence of IL-6 (10 ng/mL). In these experiments, cells were transfected with 400 nM siRNA, and 72 h later, washed and incubated with β²M-specific mAbs or dexamethasone for another 48 h. Apoptosis was detected by Annexin V-binding assay. Results of four experiments performed are shown. Similar results were obtained from other myeloma cell lines and anti-β²M mAb E6.

**Figure 3.** β²M-specific mAbs exclude growth factor receptors and their substrates from lipid rafts. Shown is the localization of IL-6R gp130, IGF-IRβ, IRS-1, MHC class I,
caveolin-1 (Cav-1), and GM1 gangliosides in lipid rafts (fractions 2–5) or nonraft fractions (fractions 7–9) in: (A) untreated myeloma cells; (B) IL-6 or IGF-I activated myeloma cells; (C) IL-6 or IGF-I activated myeloma cells in the presence of mouse IgG1; and (D) IL-6 or IGF-I activated myeloma cells in the presence of anti-β2M mAb D1. The concentrations of IL-6 (10 ng/mL), IGF-I (50 ng/mL), mouse IgG1 (50 µg/mL), and anti-β2M mAb D1 (50 µg/mL) were used. Lipid rafts were isolated from myeloma cells after treatment. The raft-fractions were confirmed by positive staining for GM1 gangliosides, identified by CTB binding, and by antibody specific to caveolin-1, a raft-associated protein. Results obtained with D1 mAb on MM.1S myeloma cells from one representative experiment out of four performed are shown. Similar results are obtained with other tumor cell lines, and with anti-β2M mAb E6.

**Figure 4.** Association of growth factor receptors with and integrity of lipid rafts in myeloma cell apoptosis. Immunoprecipitation (IP) using antibody specific to (A) caveolin-1 (Cav-1); (B) IL-6R gp130; and (C) IGF-IRβ, in myeloma cells treated with cytokines (Cyto; IL-6 or IGF-I) or cytokines together with mAb D1 (Cyto+D1), followed by Western blotting analysis (WB) using antibodies against gp130, IGF-IRβ, MHC class I (W6/32), or caveolin-1. In panels A, B, and C, expression of caveolin-1, gp130, and IGF-IRβ, respectively, serve as loading controls. Results obtained with D1 mAb on MM.1S from one representative experiment out of four performed are shown. Similar results were obtained with other tumor cell lines. (D) To deplete cholesterol and disrupt lipid rafts, cells were preincubated with MCD (5 mM, titrated in preliminary experiments) for 30 min, washed, and incubated further with β2M-specific mAb D1 (50 µg/mL) or dexamethasone (10 µM) in the presence or absence of IL-6 (10 ng/mL). Percentage of apoptotic cells was measured at 48 h by Annexin-V binding assay. Results from four experiments performed are shown. Similar results were obtained with anti-β2M mAb E6.

**Figure 5.** β2M-specific mAbs abrogate IL-6- and IGF-I-induced signaling pathways. (A) Western blot analysis and (B) Densitometric data (AVG) showing protein levels of phosphorylated (p) and nonphosphorylated IL-6R, JAK1, JAK2, STAT3, Akt, Raf, and ERK1/2 in myeloma cells treated with IL-6 (10 ng/mL) or IL-6 together with β2M-
specific mAbs (50 µg/mL). (C) Western blot analysis and (D) Densitometric data (AVG) showing protein levels of phosphorylated (p) and nonphosphorylated IGF-IRβ, IRS-1, Akt, Raf, and ERK1/2 in myeloma cells treated with IGF-I (50 ng/mL) or IGF-I together with β2M-specific mAbs (50 µg/mL). Results obtained with D1 mAb on MM.1S from one representative experiment out of three performed are shown. Similar results were obtained with other tumor cell lines.

**Figure 6.** β2M-specific mAbs activate the JNK signaling pathway. (A) Western blot analysis and (B) Densitometric data (AVG) showing protein levels of phosphorylated (p) and nonphosphorylated ASK1, MLK3, MEKK-1, MKK4, MKK7, and JNK in β2M-specific mAb (D1)-treated myeloma cells. Results obtained with D1 mAb on MM.1S myeloma cells from one representative experiment of three performed are shown. Similar results were obtained with β2M-specific mAb E6 with this and other myeloma cell lines.

**Figure 7.** Schematic presentation of IL-6, IGF-I, and β2M-specific mAb-induced anti-apoptotic or apoptotic signaling pathways in myeloma cells. Raft: lipid rafts.
Figure 1

Figure 2
Figure 3

Figure 4
Figure 5

Figure 6
Figure 7
Anti-β2-microglobulin monoclonal antibodies induce apoptosis in myeloma cells by recruiting MHC class I to and excluding growth and survival cytokine receptors from lipid rafts

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