Targeting β_2-microglobulin for induction of tumor apoptosis in human hematological malignancies

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Summary

We discovered that monoclonal antibodies (mAbs) specific to human β_2-microglobulin (β_2M) induce apoptosis in vitro and were therapeutic in mouse models of myeloma and other hematological tumor cells. Cell death occurred rapidly, without the need for exogenous immunological effector mechanisms. The mAbs induced cell death via recruiting MHC class I molecules to lipid rafts and activating Lyn and PLCγ2, leading to activated JNK and inhibited PI3K/Akt and ERK, compromised mitochondrial integrity, and caspase-9-dependent cascade activation. Although the expression of β_2M on normal hematopoietic cells is a potential safety concern, the mAbs were selective to tumor-transformed cells and did not induce apoptosis of normal cells. Therefore, such mAbs offer the potential for a therapeutic approach to hematological malignancies.

Introduction

Within the past decade, monoclonal antibodies (mAbs) have broadened the therapeutic armamentarium in oncology (Lin et al., 2005). Hematological malignancies are recognized as particularly promising targets, reflected by the current list of FDA-approved mAbs that are used to treat patients (Owaidah and Aljurf, 2002; Reff et al., 2002). The mAbs exert their in vivo effect largely through the immunological effector mechanisms of complement-mediated lysis and/or antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, their efficacy is dependent on intact immunological mechanisms in the treated patients. Although the molecules targeted by these mAbs are usually widely expressed on normal lymphoid and myeloid cells in addition to malignant cells, the therapeutic efficacy of these mAbs has been promising (Lin et al., 2005; Owaidah and Aljurf, 2002; Reff et al., 2002). Nevertheless, it would be useful to develop mAbs with the capability to kill tumor cells, but not normal cells, without complement and ADCC.

β_2M is an 11.6 kDa nonglycosylated polypeptide composed of 100 amino acids. It is part of the major histocompatibility complex (MHC) class I molecule on the cell surface of nucleated cells. Its best characterized function is to interact with and stabilize the tertiary structure of the MHC class I α chain (Bjorkman and Burmeister, 1994). Because it is noncovalently associated with the α chain and has no direct attachment to the cell membrane, β_2M on the cell surface can exchange with free β_2M present in serum-containing medium (Strominger, 2002). Free β_2M is found in body fluids under physiological conditions as a result of intracellular release. Elevated levels of serum β_2M are present in hematological malignancies, including lymphomas (Cooper and Plesner, 1980), leukemias (Molica et al., 1999; Shvidel et al., 1996), and multiple myeloma (Barlogie et al., 1999; Bataille et al., 1983), and correlate with a poor prognosis regardless of a patient's renal function (Alexanian et al., 1985; Bataille et al., 1983). This observation suggests an important, yet unidentified, role of this protein in these malignancies. While examining the effects of β_2M on myeloma cells, we made an exciting discovery; namely, that mAbs against β_2M have a remarkably strong apoptotic effect on myeloma cells and on other hematological tumor cells. In this report, we demonstrate potent tumoricidal activity of β_2M-specific mAbs both in vitro and in animal models and elucidate the apoptotic and signaling pathways induced by β_2M-specific mAbs in tumor cells.

SIGNIFICANCE

Monoclonal antibodies (mAbs) are currently and have been successfully used to treat cancers. This report describes findings that mAbs specific to human β_2-microglobulin (β_2M) exhibited potent in vitro tumoricidal activity on several myeloma, lymphoma, and leukemia cell lines and primary myeloma cells from patients, and were therapeutic in vivo in xenograft mouse models of myeloma and other tumors. We present preclinical evidence of their low toxicity on the human hematopoietic system, demonstrate the efficacy of the mAbs as a single agent to treat established tumors in animal models, and elucidate apoptotic and signaling pathways induced by the mAbs. These mAbs offer the potential for a therapeutic approach to hematological malignancies.

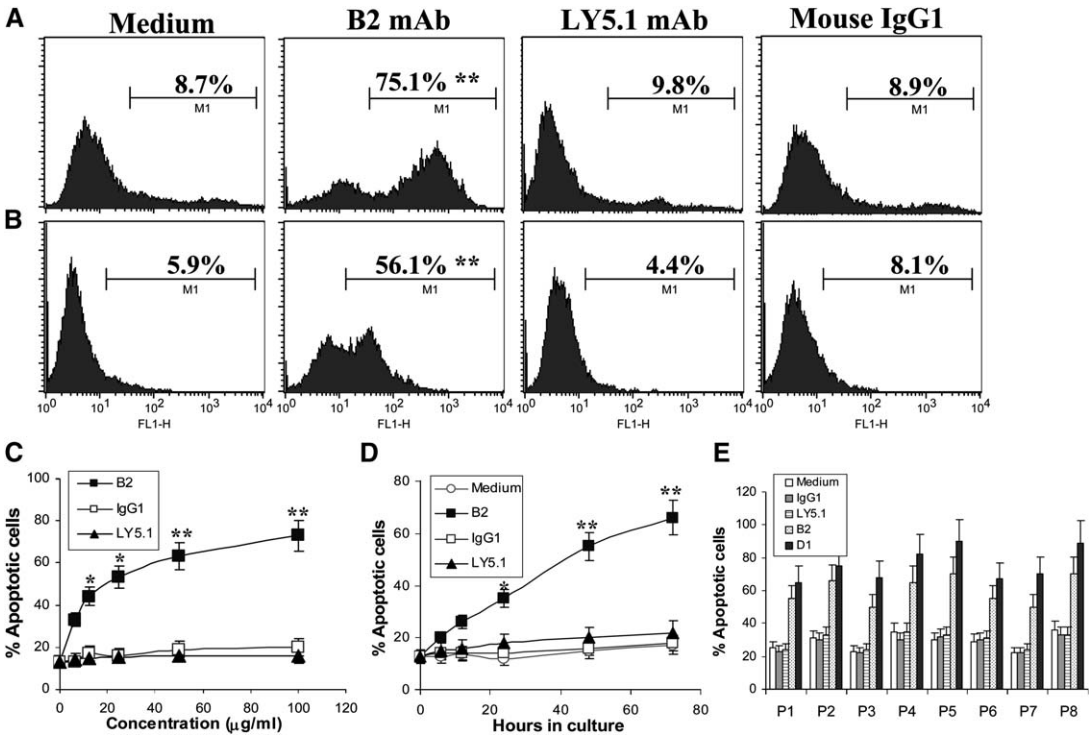

Figure 1. In vitro tumoricidal activity of β_2M-specific mAbs

A: Annexin V-staining, and **B**, TUNEL assay for detection of apoptosis in myeloma cells in cultures for 48 hr with medium or with the addition of 100 μg/ml β_2M-specific mAb B2, HLA-ABC-specific mAb LY5.1, or mouse IgG1. **C**: Dose-dependent (in a 48 hr culture), and **D**, time-dependent response of β_2M-specific mAb-induced apoptosis (50 μg/ml mAbs) in myeloma cells induced by B2, LY5.1, and mouse IgG1. Results of four independent experiments using myeloma cell line RPMI-8226 are shown. Similar results were obtained with other myeloma cell lines. **E**: Induction of apoptosis, detected by Annexin V binding assay, in primary myeloma cells from eight patients (P1–P8) with multiple myeloma in medium or induced by 50 μg/ml B2 or D1 β_2M-specific mAbs in 24 hr cultures. Results of three experiments are shown. Similar results were obtained with TUNEL assay. *$p < 0.05$; **$p < 0.01$. Error bars = SEM.

Results

Tumoricidal activity of β_2M-specific antibodies

In our study examining the effects of β_2M on myeloma cells, a commercially available mAb against human β_2M (clone B2, mouse IgG1; supplied in PBS without sodium azide from Serotec Ltd., Oxford, UK) (Liabeuf et al., 1981) was used in the blocking experiments. To our surprise, B2 mAb induced apoptosis in a large percentage of myeloma cells ($p < 0.01$, compared with medium control), evident by both Annexin V binding (Figure 1A) and TUNEL assays (Figure 1B). Control mAbs LY5.1 (IgG1) or W6/32 (IgG2a; data not shown) specific for human leukocyte antigen (HLA-ABC) molecule, purified mouse IgG (data not shown), or IgG1 had no effect. The apoptotic response was dose- (Figure 1C) and time-dependent (Figure 1D); significant induction of apoptosis in myeloma cells was already seen at a low mAb concentration as 12.5 μg/ml in a 24 hr culture ($p < 0.05$, compared with medium control). The apoptotic effect of the mAb was not counteracted by myeloma cell-survival cytokines such as IL-6 or insulin-like growth factor-I (data not shown). The tumoricidal activity was not restricted to this mAb; similar results were obtained with other commercial antibodies, such as mAb BBM.1 (ATCC, Rockville, MD) and a polyclonal antibody (MP Biomedicals, Inc., St. Louis, MO), although their apoptotic effects differed (data not shown).

We generated a panel of mouse mAbs against human β_2M by immunizing Balb/c mice with purified β_2M, fusing spleen lymphocytes with SP2/0 myeloma cells, screening, and expanding hybridomas. After several rounds of fusion and screening, more than 20 hybridomas producing significant amounts of mAbs against human β_2M were obtained. Four of these clones were selected because they secreted IgG1-isotype mAbs; this is the same isotype as B2 mAb. Two of the mAbs, designated D1 and E6, were strong apoptosis inducers (Table 1), whereas the other two had weak tumoricidal activity (data not shown). Therefore, D1 and E6, together with B2, were used to test for induction of tumor cell apoptosis with a panel of 16 surface β_2M/HLA-ABC$^+$ and 2 β_2M/HLA-ABC$^{-/low}$ cell lines of hematological malignancies, including Burkitt's lymphoma, non-Hodgkin's lymphoma, mantle cell lymphoma, T cell leukemia, acute and chronic myelogenous leukemias, and multiple myeloma. The mAbs killed between 44% to 90% of cells from 16 β_2M/HLA-ABC$^+$ tumor cell lines. No or weak effects were observed on β_2M/HLA-ABC$^{-/low}$ cell lines Daudi and K562 cells. However, statistical analysis revealed no linear correlation between killing efficiency and the level of β_2M/HLA-ABC expression in β_2M/HLA-ABC$^+$ tumor cell lines. In these experiments, the control mAbs LY5.1 and W6/32 and purified mouse IgG1 were tested in parallel, and no significant apoptosis was observed (data not shown).

Melphalan and dexamethasone are two commonly used chemotherapy drugs for myeloma. Myeloma cell lines ARP-1 and MM.1S are sensitive to dexamethasone, and RPMI-8226 and U266 are sensitive to melphalan. We compared the capacity

Table 1. Killing of hematological tumor cells by anti-β_2M mAbs

Cell lines		Surface β_2M expression[a]	Percentage killing by mAbs[b] (50 μg/ml)		
Name	Tumor type	MFI	B2	D1	E6
ARK	multiple myeloma	2037	76	84	80
APR-1	multiple myeloma	1872	61	70	64
CAG	multiple myeloma	1120	65	70	65
MM.1R	multiple myeloma	1276	70	90	83
MM.1S	multiple myeloma	1590	73	86	80
RPMI-8226	multiple myeloma	1584	64	66	62
U266	multiple myeloma	2166	63	80	75
XG-1	multiple myeloma	1560	56	64	60
BJAB	Burkitt's lymphoma	2319	53	56	54
CA46	Burkitt's lymphoma	2289	67	76	76
Raji	Burkitt's lymphoma	1245	45	53	50
Mino	mantle cell lymphoma	1936	60	66	56
Granta 519	mantle cell lymphoma	1860	52	49	44
SP53	mantle cell lymphoma	2383	50	44	39
Jurkat	T cell leukemia	873	44	53	48
KG1	myelogenous leukemia	1002	55	57	60
K562	myelogenous leukemia	60	15	20	19
Daudi	B cell lymphoma	7	8	7	9

[a]Expressed as mean fluorescence intensity (MFI).
[b]Shown are percentages of Annexin V-positive apoptotic cells at 48 hr cultures, which were confirmed by TUNEL assay. The percentages of apoptotic cells in medium alone were 3% to 20% (median: 10%). Each number represents the average of three to five independent experiments.

of the mAbs with these chemotherapy agents to kill myeloma cells. As potent as melphalan, the mAbs, especially D1 ($p < 0.05$, compared with dexamethasone), had stronger tumoricidal activity than dexamethasone on sensitive cell lines (see Figure S1 in the Supplemental Data), even though higher drug concentrations were used (10–20 μM) (Georgii-Hemming et al., 1996; Hardin et al., 1994). These mAbs efficiently killed dexamethasone-sensitive MM.1S and dexamethasone-resistant MM.1R, suggesting that chemotherapy-refractory myeloma is potentially sensitive to these mAbs.

We investigated whether the β_2M-specific mAbs would also be active on freshly isolated primary myeloma cells from patients. Purified myeloma plasma cells obtained from bone marrow aspirates of eight multiple myeloma patients were efficiently and uniformly killed by the mAbs ($p < 0.05$ to $p < 0.01$; Figure 1E). Reduced cell viability was already observed after 4 hr of treatment. After 24 hr only about 10% to 30% of cells remained viable in cultures with the addition of the mAbs, while the viability of the cells in control medium was 60% to 80%. The control mAb LY5.1 and mouse IgG1 had no effect on the primary myeloma cells. As noted before (Yi et al., 1997), all of these primary myeloma cells expressed surface β_2M/HLA-ABC molecules at levels equal to those found on myeloma cell lines (see Figure 3A).

Since we have shown that bone marrow stromal cells such as osteoclasts promote growth and protect myeloma cells from apoptosis in vitro (Yaccoby et al., 2004), we studied the effect of the mAbs on myeloma cell survival in a coculture with osteoclasts. Primary myeloma cells from patients were isolated, and after 48 hr culture in the presence of the mAbs, fewer viable (Figure 2A) and more apoptotic myeloma cells (Figure 2B) were recovered from the cocultures ($p < 0.05$, compared with controls). Interestingly, the mAbs did not affect the viability of osteoclasts measured by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Figure 2C), which suggested that β_2M-specific mAbs selectively killed malignant, but not normal, cells.

To confirm the findings that mAbs could selectively kill myeloma cells without damaging normal hematopoietic cells, a coculture of myeloma cells (0.2×10^6 cells/ml) with peripheral blood mononuclear cells (PBMCs) (1×10^6 cells/ml) from healthy donors was employed, which partially mimics the in vivo situation in which myeloma cells are surrounded and outnumbered by β_2M/MHC class I-expressing normal cells. To minimize T cell activation induced by myeloma cells, 0.4 μm pore size Transwell inserts were used to separate myeloma cells from PBMCs. Cultures of (1) PBMCs or myeloma cells alone and (2) cocultures with medium only or with the addition of the control mAb LY5.1 or mouse IgG1 served as controls. As shown in Figure 2D, β_2M-specific mAbs killed myeloma cells with the same efficacy irrespective of whether the (co)cultures contained PBMCs. No cell apoptosis was induced in PBMCs or in lymphocyte subsets, such as $CD3^+$ T cells, $CD19^+$ B cells, or $CD16^+$ NK cells (Figure 2E). As this is an important issue for future clinical application, we tested the mAbs on purified blood lymphocytes, both resting and phytohemagglutinin-activated (Figure 2F), and on purified bone marrow $CD34^+$ stem cells (Figure 2G) from healthy individuals. Both types of cells were resistant to β_2M-specific mAb-mediated apoptosis. Similar results were obtained with resting and lipopolysaccharide-activated B cells, purified from peripheral blood of healthy donors using CD19-specific antibody-coated magnetic beads (data not shown). Control mAb LY5.1 (data not shown) or mouse IgG1 (Figure 2) did not induce apoptosis in myeloma or normal cells. Taken together, these findings clearly demonstrated the ability of the mAbs to specifically target and kill myeloma cells in the presence of normal hematopoietic cells without damaging the normal cells, and suggested that the potential hematological side effects of the mAbs may be minimal.

Selectivity and mechanism of β_2M-specific mAb-induced cell death

As β_2M and HLA-ABC molecules are expressed on nucleated cells, we compared the expression levels of these molecules on myeloma tumor cells and normal blood cells. As shown in Figure 3A, myeloma cells expressed significantly higher levels of β_2M and HLA-ABC than normal peripheral blood monocytes and lymphocytes ($p < 0.05$). Furthermore, immunohistochemical staining of patients' bone marrow biopsies demonstrated that myeloma plasma cells, but not normal hematopoietic cells, were stained by antibody against β_2M (Figure 3B), which again indicated that myeloma cells express higher levels of the molecule than other normal marrow cells. Normal marrow cells also express low levels of these molecules that were not detected by the insensitive method. These results suggested that myeloma plasma cells may be a preferential target for the mAbs.

We next investigated whether the presence of soluble β_2M in medium would block the apoptotic effects of β_2M-specific mAbs on tumor cells. In these experiments, β_2M-specific mAbs (50 μg/ml; molecular weight of IgG: 150 kDa) were preincubated for 30 min with much higher molar concentrations of β_2M (50–100 μg/ml; molecular weight of β_2M: 11.6 kDa) before tumor cells were added to the cultures and incubated for another 48 hr. The apoptosis-inducing effects of the mAbs were reduced, but not blocked (Figure 3C). Further experiments

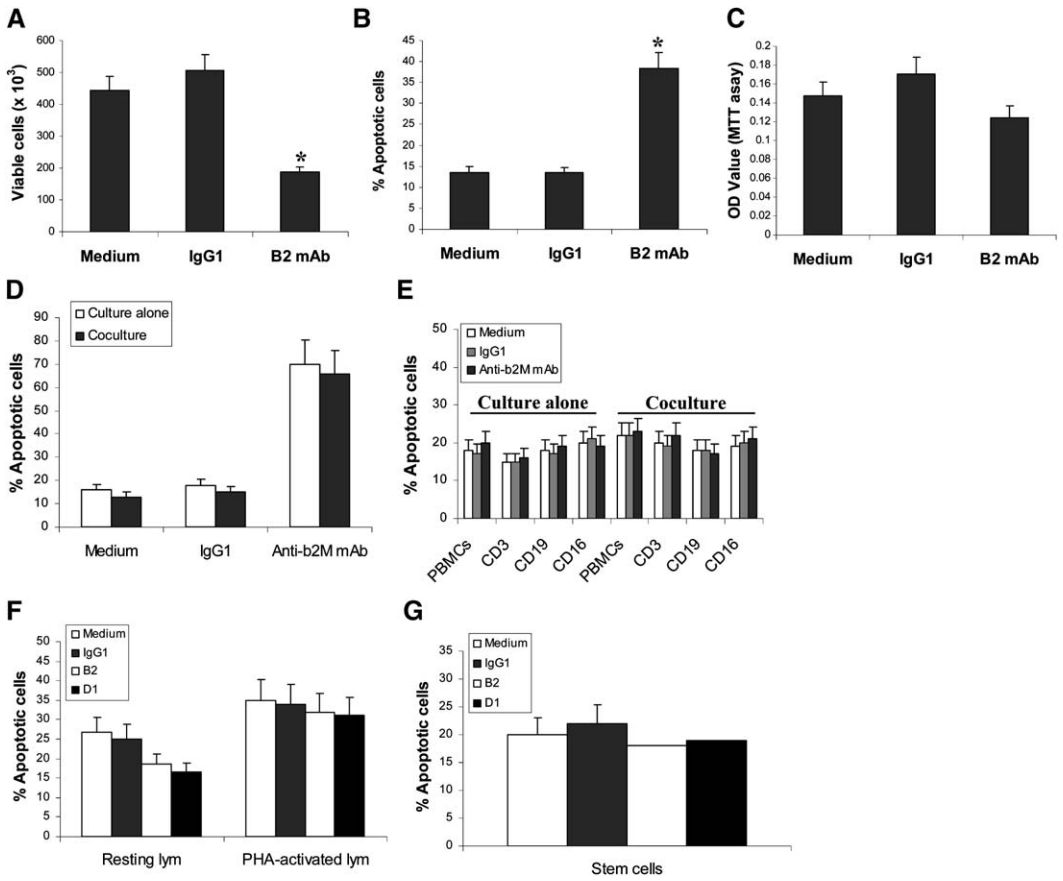

Figure 2. Killing of myeloma, but not normal hematopoietic cells, by β_2M-specific mAbs

Effect of β_2M-specific mAbs on primary myeloma cells and normal osteoclasts in their cocultures. **A:** Viable myeloma cells; **B**, percentage of apoptotic myeloma cells; and **C**, viability of osteoclasts in the cocultures with the addition of 50 μg/ml B2 mAb or mouse IgG1 for 48 hr. Cocultures with medium alone served as control. Details are described in Experimental Procedures. Effect of β_2M-specific mAbs on myeloma cell lines and normal PBMCs in their cocultures: **D**, Apoptotic myeloma cells, and **E**, apoptotic PBMCs and lymphocyte subsets in their cocultures, in which PBMCs (10^6/ml) were seeded on wells and myeloma cells (0.2×10^6/ml) were placed on inserts. β_2M-specific mAbs (50 μg/ml) were added to the wells and incubated for 48 hr. Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum. No lymphocyte growth factors were added. After culture, cells were recovered and apoptotic cells were detected using the Annexin V binding assay. To examine apoptotic lymphocyte subsets, PE-conjugated CD3-, CD19-, and CD16-specific antibodies were used to identify the subsets, while FITC-conjugated Annexin V was used to identify apoptotic cells. Shown are the results of three experiments using D1 mAb on MM.1S. Similar results were obtained with ARP-1 and B2 mAb. **F**: Detection of apoptosis in lymphocytes (Lym; both resting and PHA-activated), and **G**, bone marrow-derived stem cells in cultures with the mAbs. Experiments were performed with 50 μg/ml B2 or D1 mAbs in 48 hr cultures. Results of four independent experiments are shown. *$p < 0.05$. Error bars = SEM.

showed that, under such conditions (50 μg/ml β_2M-specific mAbs preincubated with 50–100 μg/ml β_2M for 30 min), the β_2M-specific mAbs could still bind to surface β_2M (Figure 3D), although to a lesser extent. These results indicated that the mAbs have a higher affinity for surface-bound rather than soluble β_2M and may explain why β_2M-specific mAbs are tumoricidal despite the presence of high concentrations of soluble β_2M.

To further verify that cell surface β_2M was the target, small interfering RNA (siRNA) for β_2M gene was synthesized and used to knockdown β_2M expression in myeloma cells. After extensive preliminary studies to optimize the dose of siRNA and transfection, we were able to achieve 70% to 80% reduction of surface β_2M (and HLA-ABC; data not shown) protein expression on myeloma cells on day 3 after transfection with β_2M-specific siRNA (Figure 3E), which remained at low levels for up to 7 days (data not shown). Detection of β_2M mRNA by RT-PCR confirmed this result (data not shown). This treatment was specific for β_2M, as surface expression of MHC class II (HLA-DR) and CD38 (data not shown) remained unchanged. It is evident that knockdown of surface β_2M/MHC class I on myeloma cells rendered the cells resistant to β_2M-specific mAb-induced apoptosis, whereas mock control and control siRNA had no effect (Figure 3E). As expected (Caplen and Mousses, 2003), all of these treatments or transfections killed a small percentage of the cells.

It is conceivable that β_2M-specific mAbs triggered intracellular signaling by binding to surface β_2M/MHC class I molecules, leading to tumor cell apoptosis. Therefore, we examined the localization and density of β_2M/MHC class I molecules and their complexes with β_2M-specific mAbs in tumor cells by immunofluorescence staining and flow cytometry analysis. As shown in Figure 3F, cells recovered from 0 hr of culture (incubating cells with β_2M-specific mAbs on ice for 20 min) displayed surface staining by β_2M-specific mAbs. Cells recovered from cultures for 6 to 24 hr at 37°C displayed a gradual intracellular staining, demonstrating internalization of β_2M-specific mAb-MHC class I complexes. By 48 hr, mainly apoptotic bodies were seen. In

parallel to these results, a downregulation of surface HLA-ABC molecules was seen on tumor cells during this time frame after treatment with β_2M-specific mAbs (Figure 3G; $p < 0.05$ to $p < 0.01$, compared with the starting level), while surface MHC class II (HLA-DR) expression was not affected (data not shown). These results indicate that binding of β_2M-specific mAbs to cell surface β_2M/MHC class I molecules led to internalization and downregulation of these molecules, which might be responsible for induction of apoptosis in treated cells.

Various types of apoptosis, such as receptor-mediated or stress-induced apoptosis, depend on the activation of caspases. To investigate whether and which caspase cascades participated in β_2M-specific mAb-induced apoptosis, activation and cleavage of caspase-9, -8, -3, -7, and subsequent cleavage of PARP were investigated by western blotting with specific antibodies. Treatment with β_2M-specific mAbs resulted in activation and cleavage of caspase-9, -3, -7, and PARP, but not caspase-8, in myeloma cells (Figure 4A). Caspase (-9, -3) and PARP activation (Figure 4B) and cell apoptosis (Figure 4C) were inhibited by the pan-caspase inhibitor Z-VAD and caspase-9 inhibitor Z-LEHD, but not by caspase-8 inhibitor Z-IETD (data not shown). Since these results indicated that the intrinsic apoptosis pathway was utilized, we next investigated the involvement of mitochondria and its associated proapoptosis and antiapoptosis proteins. β_2M-specific mAbs upregulated the expression of proapoptotic proteins Bad and Bax (Figure 4D). While the expression of antiapoptotic proteins Bcl-2 and Bcl-xL remained unchanged, the level of phosphorylated Bcl-2 (pBcl-2) was increased and that of phosphorylated Bad (pBad) was decreased; this favors the induction of apoptosis due to increased permeability of mitochondria (Srivastava et al., 1998; Zha et al., 1996), leading to release of cytochrome c into cytosol. Indeed, at 24 hr and onward, cytosolic cytochrome c (cCyto C) was detected in β_2M-specific mAb-treated tumor cells, whereas the amount of total cytochrome c (tCyto C) remained the same (Figure 4D).

In addition to caspase cascades, we also examined the signaling pathways induced by β_2M-specific mAbs. We focused on the mitogen-activated protein kinase and PI3K/Akt pathways, which are essential for cell survival and growth. As shown in Figure 4E, β_2M-specific mAb treatment increased the level of phosphorylated JNK (pJNK) and decreased those of pAkt and pERK. These effects were observed as early as 15 min after the treatment. By 2 hr, pAkt and pERK were undetectable. The levels of nonphosphorylated JNK, Akt, and ERK remained stable. Hence, β_2M-specific mAbs activated the JNK pathway and inhibited PI3K/Akt and ERK pathways in treated cells, all of which could lead to induction of apoptosis (Franke et al., 1997; Xia et al., 1995). On the other hand, normal cells, such as B cells, expressed higher levels of pJNK and lower levels of pAkt and pERK, compared with myeloma cells, which remained unchanged after the treatment with the mAbs (Figure S2). To verify the importance of JNK activation in the induction of apoptosis in myeloma cells, we used a specific JNK inhibitor (JNK inhibitor II or SP600125 [Bennett et al., 2001]; Calbiochem-Novabiochem, La Jolla, CA). As complete inhibition of JNK activity would lead to apoptosis, we titrated and used a low dose (5 μM) of the inhibitor to partially inhibit JNK activity (Figure 4F) without affecting cell viability (Figure 4G). Our results showed that JNK inhibitor II almost completely abrogated apoptosis induced by the mAbs.

Next, we attempted to find out how and through what linkers β_2M-specific mAbs induce signaling and apoptosis in myeloma cells. We identified lipid rafts because they are often involved in cell signaling and apoptosis (Cherukuri et al., 2001; Harder, 2004), and previous studies suggested that they may be responsible for MHC class II and CD20 mAb-induced apoptosis (Deans et al., 2002; Nagy and Mooney, 2003). As MHC class II, but not MHC class I, molecules were found in lipid rafts under physiological conditions (Vogt et al., 2002), we hypothesized that MHC class I relocation to lipid rafts and tyrosine kinases Lyn, Syk, and phospholipase-Cγ2 (PLCγ2) may be involved in apoptosis. These kinases have been previously shown to be involved in MHC class I signaling (Skov et al., 1995, 1997). As shown in Figure 5, MHC class I and Syk were detected in the nonraft fractions (fractions 7–9) in untreated (not shown) and mouse IgG1-treated myeloma cells (Figure 5A, left panels), whereas in β_2M-specific mAb-treated myeloma cells, MHC class I, but not Syk, were also detected in the lipid rafts (fractions 2–5; Figure 5A, right panels). Lyn kinase was detected in the raft fractions in both cells. Similar results were obtained with other tumor cell lines (data not shown). However, in normal B cells treated with or without the mAbs, MHC class I and the kinases were detected only in the nonraft fractions (Figure 5B). These results indicated that Lyn is associated with the rafts, and that MHC class I molecules were recruited to the lipid rafts in myeloma, but not in normal B cells, after treatment with the mAbs. To confirm the involvement of lipid rafts in β_2M-specific mAb-induced apoptosis, we treated myeloma cells with methyl-β-cyclodextrin (MCD), an agent that disrupts the structure of lipid rafts in cell membrane (Anderson et al., 2000), and showed that such a treatment abrogated apoptosis of the cells induced by the mAbs (Figure 5C). Thus, these results provided strong evidence for a crucial role of lipid rafts in β_2M mAb-induced apoptosis of tumor cells and a plausible explanation for the selectivity of the mAbs.

An immunoprecipitation assay was used to confirm the interactions of MHC class I with these kinases. Using antibodies against MHC class I or the kinases, we showed that Lyn and PLCγ2 were physically associated with MHC class I in myeloma cells after treatment with the mAbs, because immunoprecipitation of MHC class I by mAb W6/32 coprecipitated Lyn and PLCγ2 in β_2M-specific mAb-treated, but not in control (not shown) or mouse IgG1-treated, myeloma cells, whereas Syk was coprecipitated from both cells (Figure 5D). On the other hand, immunoprecipitation of Lyn (Figure 5E) or PLCγ2 (Figure 5F) kinases by their respective antibodies also coprecipitated MHC class I in β_2M-specific mAb-treated, but not in control (not shown) or mouse IgG1-treated, myeloma cells. In addition, western blot analysis showed that Lyn and PLCγ2 were also phosphorylated in myeloma cells treated with β_2M-specific mAbs (Figure 5G). Taken together, these results demonstrated that β_2M/MHC class I on myeloma cells, after binding to and being cross-linked by the mAbs, relocate to lipid rafts, recruit, and activate Lyn and PLCγ2 (but not Syk), which in turn activate JNK and inhibit PI3K/Akt and ERK, and induce apoptosis in myeloma cells (Figure S3).

In vivo therapeutic efficacy of β_2M-specific mAbs on established tumors

To test the in vivo efficacy of β_2M-specific mAbs, we inoculated severe combined immunodeficient (SCID) mice subcutaneously with myeloma cell lines ARP-1 or MM.1S, Burkitt's lymphoma CA46, mantle cell lymphoma Granta 519, or acute myelogenous

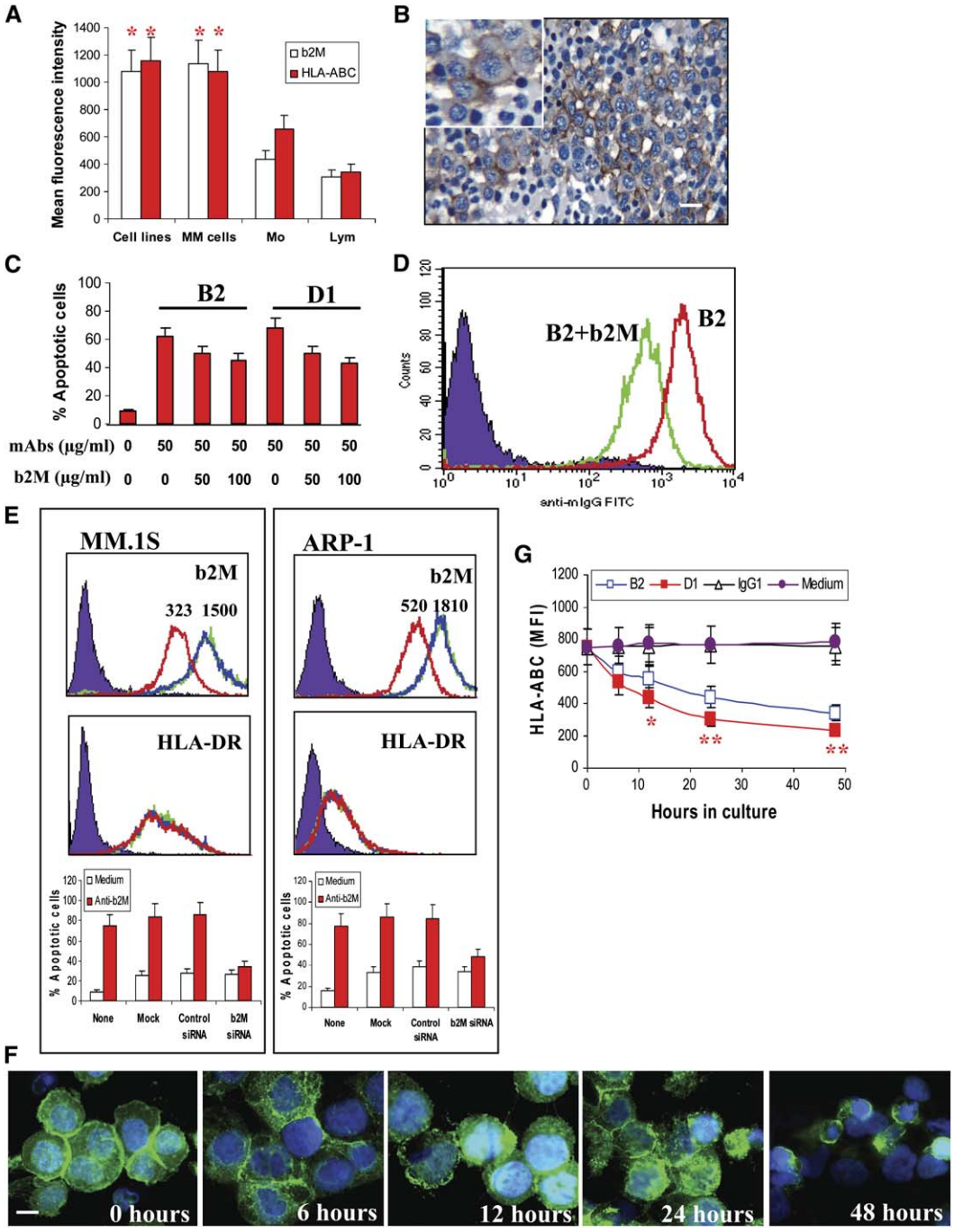

Figure 3. Selectivity and mechanism of β_2M-specific mAb-induced cell death

Expression and modulation of β_2M and HLA-ABC on myeloma cells by β_2M-specific mAbs. **A**: Flow cytometry examining the levels of surface β_2M and HLA-ABC molecules on myeloma cell lines (cell lines, n = 8), primary myeloma cells (MM cells, n = 6), and peripheral blood monocytes and lymphocytes from healthy donors (n = 6). **B**: Staining for β_2M in bone marrow biopsies of patients with myeloma by immunohistochemistry, which showed that only myeloma plasma cells, but not normal hematopoietic cells, were stained by β_2M-specific antibody. Scale bar, 20 μM. The insert shows a higher magnification (×1200). **C**: Effects of soluble β_2M on β_2M-specific mAb (B2 and D1)-induced apoptosis in myeloma cells, and **D**, effects of soluble β_2M on β_2M-specific mAb (B2) binding to surface β_2M. In these experiments, equal amounts, unless otherwise indicated, of β_2M-specific mAbs and soluble β_2M were preincubated to form immune complexes, followed by the addition of myeloma cells (RPMI-8226; similar results were obtained with other cells) to the culture. Apoptosis was detected after cells were cultured 24 hr at 37°C by Annexin V binding assay, and surface staining was performed after 30 min incubation (on ice) by washing and incubating the cells with FITC-conjugated antibody to mouse IgG. Cells preincubated with B2 mAb alone (B2) served as control for surface binding of the mAb. Representative results of five independent experiments are shown. **E**: knockdown of surface β_2M by specific siRNA abrogated β_2M-specific mAb-induced apoptosis in myeloma cells. Shown are the results of two myeloma cell lines, MM.1S and ARP-1. The upper panels show the reduction of surface β_2M, and middle panels show the level of HLA-DR on myeloma cells detected by flow cytometry. Numbers above the histograms represent mean fluorescence intensity. Filled blue histograms represent cells stained with control IgG, and open histograms show the levels of β_2M expression on cells treated with mock siRNA (green) or transfected with control siRNA (blue) or β_2M-specific siRNA (red). Assay was performed 72 hr after transfection. Representative results of three independent experiments are shown. The lower panels show percentages of apoptotic cells induced by β_2M-specific mAb D1. Similar results were obtained with B2 mAb. In these

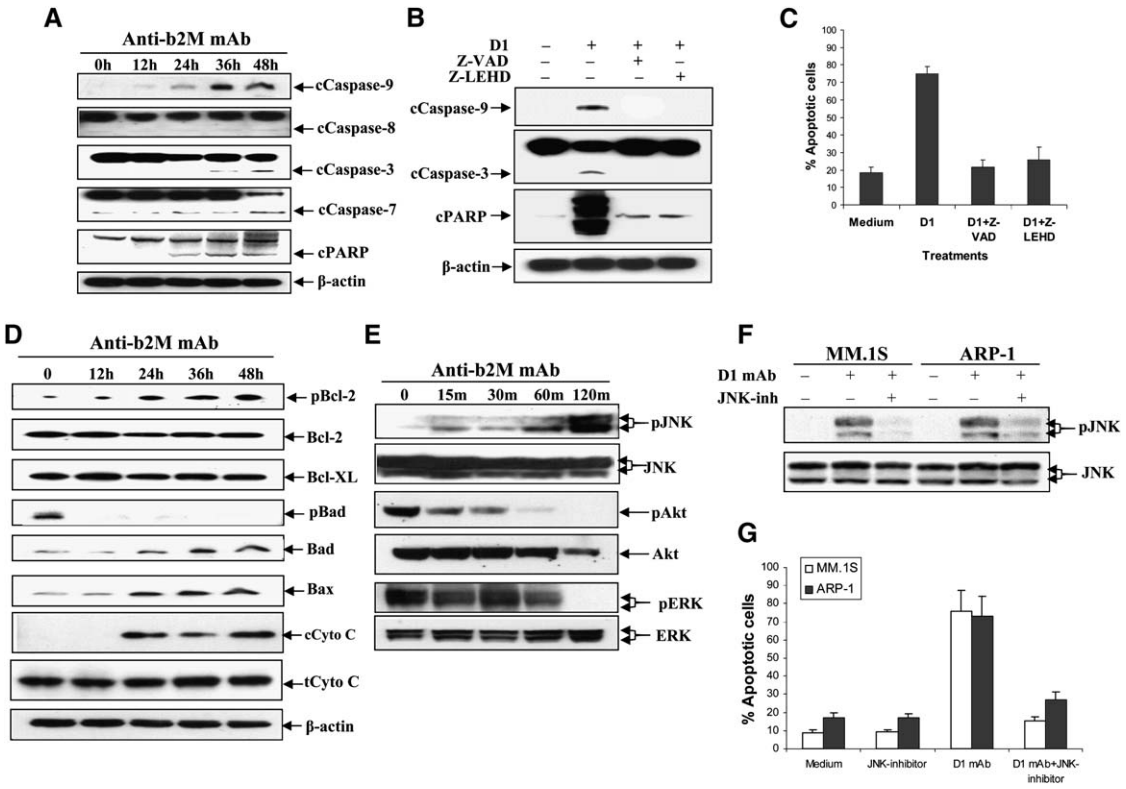

Figure 4. β_2M-specific mAb-induced apoptosis and signaling pathways

A: Western blot analysis showing protein levels of procaspase and cleaved caspases and PARP in β_2M-specific mAb (D1)-treated myeloma cells. Inhibition of caspase and PARP activation and processing (**B**) and apoptosis (**C**) is shown in β_2M-specific mAb (D1)-treated myeloma cells with the addition of the pan-caspase inhibitor Z-VAD or caspase-9 inhibitor Z-LEHD. Arrows indicate cleaved (c) caspases and PARP. **D**: Western blot analysis showing protein levels of mitochondria-related proapoptotic and antiapoptotic proteins (cCyto C: cytosolic cytochrome c; tCyto C: total cytochrome c), and **E**, phosphorylated (p) and nonphosphorylated JNK, Akt, and ERK in β_2M-specific mAb (D1)-treated myeloma cells. Results obtained with D1 mAb on MM.1S myeloma cells from one representative experiment of four performed are shown. Similar results are obtained with other β_2M-specific mAbs on other myeloma cell lines as well as primary myeloma cells. JNK inhibitor II abrogated (**F**) β_2M-specific mAb-induced JNK activation, and **G**, apoptosis in myeloma cells. Medium control contained DMSO. Results obtained with B2 or D1 mAb on MM.1S and/or ARP-1 myeloma cells from one representative experiment out of four performed are shown. Similar results are obtained with other β_2M-specific mAbs on this and other myeloma cell lines. *$p < 0.05$; **$p < 0.01$. Error bars = SEM.

leukemia KG1, and SCID-hu mice (Yaccoby et al., 1998) with primary myeloma cells freshly isolated from patients, and monitored tumor development. Since we wanted to examine the therapeutic potential of the mAbs as a single agent, treatment was initiated when palpable tumors ($\geq$5 mm in diameter for SCID mice or human M proteins reaching >50 μg/ml for SCID-hu mice) developed and after murine NK cells were depleted. Treatment with intraperitoneal injections of β_2M-specific mAbs significantly reduced tumor burdens and retarded tumor growth, measured as tumor volumes (upper panels; Figure 6A for myeloma and Figure 6B for other tumors, $p < 0.05$ to $p < 0.01$, compared with controls) and/or levels of circulating M-proteins or their light chains secreted by the myeloma cells in SCID mice (Figures S4A and S4B for ARP-1 and MM.1S) or in SCID-hu mice (primary MM, Figure 6A; $p < 0.05$ to $p < 0.01$, compared with controls). Within the observation period, all control SCID mice died, whereas 80% or more mAb-treated mice survived with minimal tumor burdens (lower panels; Figure 6A for myeloma and Figure 6B for other tumors, $p < 0.01$, compared with controls). Subcutaneous injections around the tumors of the β_2M-specific mAbs were even more effective in eradicating the established myeloma, including primary myeloma, and most of the treated mice were without detectable tumors at the end of the study (data not shown). Together, the efficacy data are promising and raise the possibility that such mAbs could become useful and potent therapeutic agents for treating hematological malignancies.

β_2M-specific mAbs caused no damage to human hematopoiesis or β_2M/HLA-A2-expressing murine tissues and organs

SCID-hu and human β_2M/HLA-A2-transgenic (A2-) mice were utilized to examine the potential impact and toxicity of β_2M-specific mAbs on human hematopoiesis and human-like tissues and organs. Tumor-free SCID-hu and A2-mice were treated in the same way as tumor-bearing mice, and were sacrificed two

experiments, cells were transfected with 400 nM siRNA, and 72 hr later, washed and incubated with 50 μg/ml β_2M-specific mAbs for another 48 hr. Apoptosis was detected by Annexin V binding assay. **F**: Immunofluorescence staining for extracellular and intracellular localization of β_2M-specific (B2) mAb/MHC class I molecules, visualized by FITC-conjugated antibody to mouse IgG1. Scale bar, 10 μM. **G**: Staining for surface HLA-ABC molecules on myeloma cells in cultures (at different time points) with the addition of B2 or D1 β_2M-specific mAbs. Medium or mouse IgG1 served as controls. Representative results of three independent experiments are shown. *$p < 0.05$; **$p < 0.01$. Error bars = SEM.

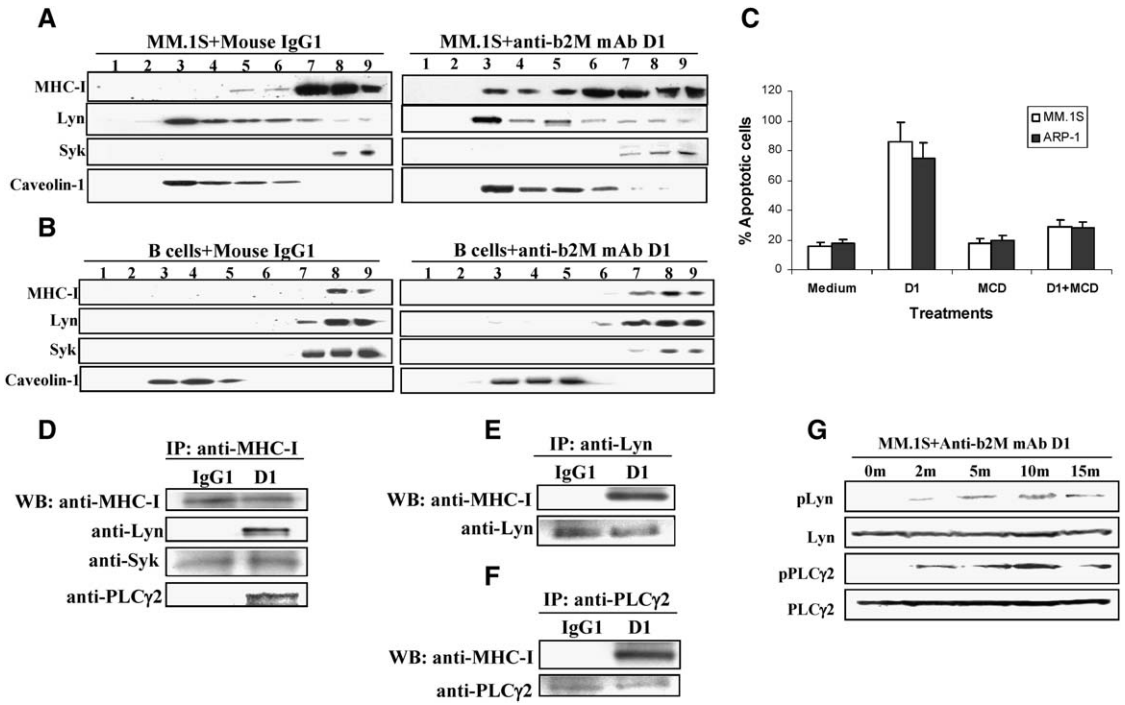

Figure 5. Involvement of lipid rafts and kinases in β_2M-specific mAb-induced apoptosis in tumor cells

Localization of MHC class I, Lyn, and Syk in lipid rafts (fractions 2–5) or nonraft fractions (fractions 7–9) in **A**, myeloma cells, or **B**, normal B cells after treatment with mouse IgG1 (left panels) or β_2M-specific mAbs (right panels). Lipid-raft fractions were confirmed by positive staining for caveolin-1, a raft-associated protein. **C**: Methyl-β-cyclodextrin (MCD) inhibits β_2M-specific mAb-induced apoptosis of myeloma cells. In these experiments, cells were preincubated with MCD (5 mM, titrated in preliminary experiments) for 30 min, washed, and incubated further with or without β_2M-specific mAbs D1 or B2 (50 μg/ml). Apoptosis was measured at 48 hr by the Annexin V binding assay. Immunoprecipitation using antibodies against **D**, MHC class I; **E**, Lyn; or **F**, PLCγ2 in myeloma cells treated with IgG1 or the mAbs (D1 or B2). Blotting antibodies used were against MHC class I, Lyn, Syk, and PLCγ2 (**D**), MHC class I and Lyn (**E**), or MHC class I and PLCγ2 (**F**). **G**: Western blot showing phosphorylated Lyn (pLyn) and pPLCγ2 in myeloma cells after treatment with β_2M-specific mAbs. Nonphosphorylated kinases were also shown. Results obtained with D1 mAb on MM.1S and/or ARP-1 myeloma cells from one representative experiment out of four performed are shown. Similar results are obtained with other tumor cell lines and primary myeloma cells. Error bars = SEM.

days after the final injection of β_2M-specific mAbs. Implanted human bones from SCID-hu mice and organs including heart, liver, kidney, lung, and spleen from A2-mice were removed for histological examination. Treatment with the mAbs or IgG1 did not change the body weight of the mice (Figure S5A) or cause cell or tissue damage in marrow cells of the implanted human bones, or in the murine organs that express human β_2M/HLA-A2 molecules (Figure S5B). We also examined myeloma-bearing SCID mice after completing mAb treatment and found no normal tissue damage (data not shown).

In vitro and in vivo experiments were conducted to examine the possibility that β_2M-specific mAbs, by binding to and blocking MHC class I on normal cells, trigger NK-mediated killing of the cells. PBMCs freshly isolated from healthy donors were labeled with chromium 51 (^{51}Cr), washed, and incubated with or without mAb D1 or IgG1 for 30 min, followed by incubation with purified, autologous NK cells for 4 hr. As shown in Figure S5C, NK cells did not lyse PBMCs pretreated with or without D1 mAb or IgG1. However, these NK cells efficiently killed K562 cells. Furthermore, no cell apoptosis or tissue damage was observed after immunohistochemical examination of the human marrow cells from SCID-hu mice, in which D1 mAb and subsequently purified human NK cells were injected directly into the implanted human bones (data not shown). These studies further confirmed our results that β_2M-specific mAbs may be less toxic or nontoxic to normal human cells and tissues in vivo.

In vivo validation of the mechanisms underlying apoptosis of tumor cells induced by β_2M-specific mAbs

To confirm whether β_2M-specific mAbs utilized the same mechanisms of apoptosis induction in tumor cells in vivo, tumor-bearing mice were sacrificed after treatment, and tumors were removed for immunohistochemical analysis. As shown in Figure 7, injections of β_2M-specific mAbs led to upregulation and activation of pJNK, cleavage of caspase-9 and caspase-3 (data not shown), and induction of apoptosis of myeloma cells detected by in situ TUNEL assay. Injection of the JNK inhibitor ($\geq$10 μM) around the tumors prior to each mAb treatment not only inhibited β_2M-specific mAb-induced JNK phosphorylation, caspase-9 cleavage, and induction of apoptosis (Figure 7A), but also abrogated the therapeutic efficacy of the mAbs on established myeloma in SCID mice (Figure 7B). The TUNEL assay was also used to confirm induction of apoptosis of other tumors in SCID mice (Figure S6). Hence, these results validated the mechanisms of β_2M-specific mAb-induced signaling and apoptosis in tumor cells.

Discussion

This study describes a finding that mAbs against β_2M induced apoptosis in all tested surface β_2M/MHC class I-positive tumor cells of human hematological malignancies, including established cell lines and primary tumor cells isolated from patients.

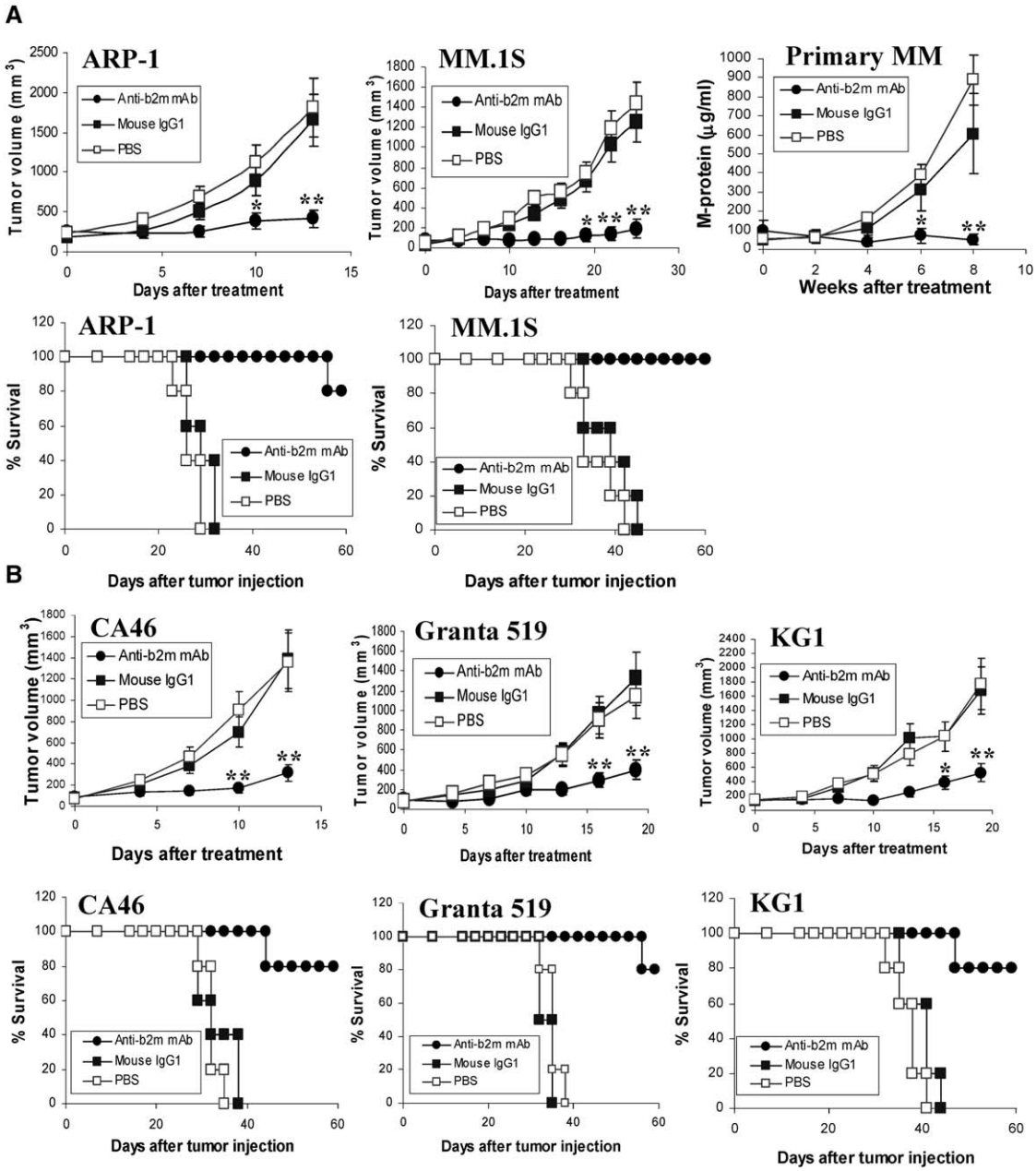

Figure 6. In vivo therapeutic effects of β_2M-specific mAbs on established human myeloma and other hematological tumors in SCID or SCID-hu mouse models

SCID mice were xenografted subcutaneously with ARP-1, MM.1S, CA-46, Granta 519, or KG1 cells, and tumor burdens were monitored as tumor volumes (upper panels; **A** for myeloma cells, and **B** for other tumor cells). Mice received intraperitoneal injections (every 3 days for a total of four injections) of 250 μl ascites containing about 500 μg D1 β_2M-specific mAb or 500 μg mouse IgG1, or an equal volume of PBS. Survival of SCID mice is shown in lower panels; **A** for myeloma cells, and **B** for other tumor cells (survival times: β_2M-specific mAb group versus PBS or IgG1 groups: $p < 0.01$ in all models). Results from one representative experiment with five mice per group of three performed using D1 mAb are shown. Similar results are obtained with another β_2M-specific mAb (E6) on these two myeloma cell lines. In SCID-hu mice (six mice per group), primary myeloma cells from patients (n = 3) were directly injected into implanted human bones, and tumor growth was monitored as levels of circulating human M-protein or its light chain (**A**, far right; Primary MM). Mice received the same treatments as SCID. Note: because primary myeloma cells only grow in implanted human bone and not in murine bone marrow or other organs, these mice usually do not develop systemic symptoms and become moribund; therefore, no death data were available. *$p < 0.05$; **$p < 0.01$. Error bars = SEM.

These mAbs also exhibited a strong tumoricidal and therapeutic activity in vivo in xenograft mouse models of myeloma (including primary myeloma cells in SCID-hu host), lymphomas, and leukemia, without damaging marrow hematopoietic cells of implanted human bone or murine organs that express human β_2M/HLA-A2 molecules. The mAbs induced apoptosis in a caspase-dependent manner, in the absence of secondary cross-linking and immunological effector mechanisms such as complement and ADCC. Therefore, β_2M-specific mAbs offer a potential therapeutic approach to hematological malignancies.

MHC class I and II molecules may be unique targets for induction of apoptosis. Both murine and fully human HLA-DR-specific mAbs have been shown to inhibit growth and induce programmed death of MHC class II-bearing tumor cells (Nagy

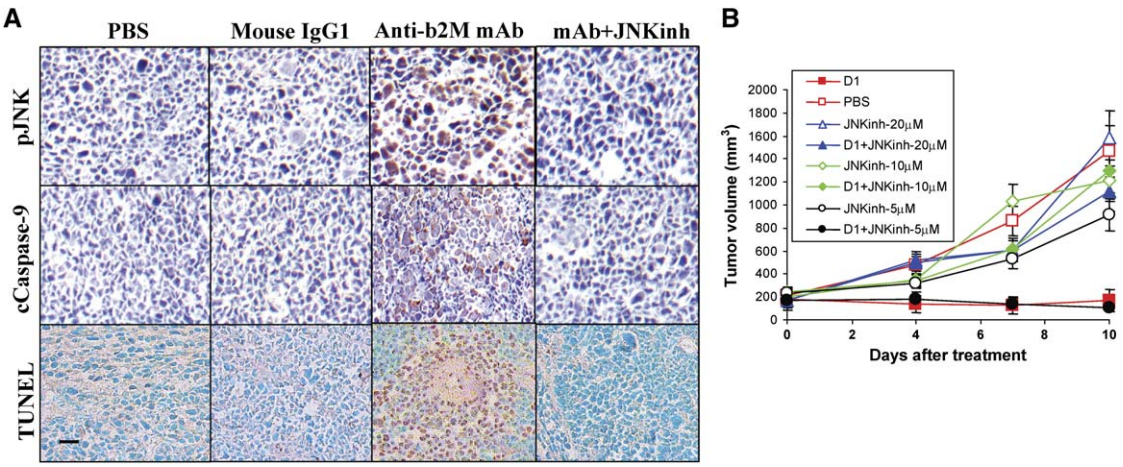

Figure 7. In vivo validation of signaling and apoptosis mechanisms

A: Myeloma (MM.1S)-bearing mice were treated as previously indicated, sacrificed, and had tumors removed for immunohistochemical staining for phosphorylated JNK (pJNK), cleaved caspase-9 (cCaspase-9), and apoptotic cells by in situ TUNEL assay. Shown are staining of tumors after one treatment with intraperitoneal injection of the mAbs. In some experiments, JNK inhibitor (JNK inhibitor II, 20 μM) was injected around the tumors before injecting the mAbs. Scale bar, 20 μM. **B**: Effect of the JNK inhibitor in abrogating the therapeutic efficacy of β_2M mAbs on established myeloma (MM.1S) in SCID mice. PBS contained DMSO. It is evident that the JNK inhibitor at ≥10 μM was effective. Representative results of three experiments are shown. The same results were also obtained with other tumor cells lines in SCID mice. Error bars = SEM.

et al., 2002; Vaickus et al., 1989; Vidovic and Toral, 1998). These mAbs were shown to be selective to activated or tumor-transformed cells, and caused no long-lasting hematological toxicity in primates (Nagy et al., 2002). Consequently, HLA-DR-specific mAbs are currently being evaluated for the treatment of lymphoid malignancies. On the other hand, ligation of MHC class I molecules on T and B lymphocytes triggered signal transduction, which induced responses ranging from anergy and apoptosis to cell proliferation and IL-2 production (Skov, 1998). These data suggested that MHC class I molecules alone or in association with other signal transducing molecules may be involved in regulation and fine-tuning of immune responses. By secondary cross-linking via plastic immobilization (Skov et al., 1997; Woodle et al., 1997) or use of secondary antibodies (Pedersen et al., 1999), these MHC class I-specific mAbs, including 5H7, induced apoptosis in B cell lymphoma and other cells in vitro. Soluble 5H7 also inhibited T cell proliferation (Smith et al., 1994). In contrast, mAbs specific for β_2M in this study induced tumor apoptosis without the need for secondary cross-linking, and were therefore effective in killing tumor cells in vivo.

Previous studies have indicated that β_2M/MHC class I can serve as important signal-transducing molecules (Skov, 1998). We show here that β_2M-specific mAbs targeted surface β_2M/MHC class I, and binding of the mAbs to tumor cells resulted in internalization and downmodulation of these molecules and induction of apoptosis of the cells. This finding is supported by siRNA experiments in which knockdown of surface β_2M and MHC class I molecules abrogated induction of apoptosis of tumor cells induced by the mAbs. We also showed that β_2M-specific mAb binding to surface β_2M/MHC class I molecules activated the JNK signaling pathway and inhibited PI3K/Akt and ERK pathways; cleaved capase-9, -3, -7, and PARP; upregulated Bad and Bax protein expression; induced phosphorylation of Bcl-2 and decreased phosphorylation of Bad; and released cytochrome c into the cytosol. Consistent with these results, it has been shown that JNK activation can lead to phosphorylation of Bcl-2 and upregulation of Bax protein expression (Maundrell et al., 1997). Phosphorylation of Bcl-2 blocked the antiapoptotic activity of this protein, partly due to a loss of ability to bind to Bax (Srivastava et al., 1998), which increased the permeability of mitochondria (Tsujimoto, 1998). On the other hand, inhibition of the PI3K/Akt and ERK pathways abrogated the phosphorylation of Bad and increased its binding or dimerization with Bcl-xL, which in turn blocked the antiapoptotic activity of Bcl-xL and again led to increased permeability of mitochondria (Franke et al., 1997, 2003). Consequently, cytochrome c was released into cytosol, caspase-9 cascade activated, and apoptosis induced. In vivo experiments using tumor-SCID mouse models validated these findings.

In this study, we elucidated the mechanisms underlying β_2M-specific mAb-induced apoptosis and selectivity toward tumor cells, and identified the signaling molecules between MHC class I and the downstream JNK, PI3K/Akt, and ERK kinases. We showed that following treatment of myeloma cells with the mAbs, MHC class I relocated to lipid rafts, where they recruited and activated kinases Lyn and PLCγ2. Indeed, both Lyn and PLCγ2 were shown to be physically associated with MHC class I and phosphorylated in myeloma cells (Figure 5). Furthermore, the importance of lipid rafts in myeloma cell apoptosis was confirmed by treatment with lipid raft-disrupting agent MCD (Anderson et al., 2000). Activation or phosphorylation of Lyn and PLCγ2 led to JNK activation and inhibition of PI3K/Akt and ERK pathways (Kurosaki, 1999; Shangary et al., 2003), which in turn induced apoptosis (Franke et al., 1997; 2003). Also, there was a differential expression of β_2M/MHC class I molecules by normal and cancer cells (Figure 3). Together with our findings in normal B cells that Lyn was not associated with lipid rafts and β_2M-specific mAbs did not trigger MHC class I relocation to the rafts, and that JNK, PI3K/Akt, and ERK activities remained unchanged after the mAb treatment, these data provide a plausible explanation for the selectivity and sensitivity of β_2M-specific mAb-mediated apoptosis of normal versus malignant cells.

Concerns may be raised with future clinical application of these mAbs, such as the possibility of NK cell killing of normal

cells due to potential blockade of cell surface MHC class I molecules by the mAbs. We investigated this possibility by examining human NK cell cytolysis of normal cells in the presence or absence of the mAbs in both in vitro and in vivo situations, and found no killing or damage to human cells or tissues, even though the NK cells were cytolytic to their target K562 cells. Another concern is the possible formation of damaging immune complexes formed by soluble β_2M binding the mAbs, and if they are present, whether our animal models could capture the complexes. We examined sera of myeloma-bearing mice and found that after myeloma was established in SCID or SCID-hu mice, circulating human β_2M levels could reach 3 μg/ml, which is equal to levels seen in about 50% of newly diagnosed myeloma patients. Yet, these mAbs were therapeutic and caused no damage to normal tissues in these mice. Thus, to a large extent, the animal models represent human disease and are well suited for the preclinical studies. Other barriers for future clinical application may include blocking effects of soluble and cell-expressing β_2M, safety and toxicity of the mAbs, and generation of anti-mouse IgG. These issues warrant further studies to compare SCID and HLA-A2-transgenic SCID mice (in which every tissue and all cells express human β_2M) to examine the efficacy of the mAbs to kill tumor cells in these hosts, use nonhuman primates for safety and toxicity studies, and generate and examine chimeric or humanized mAbs against β_2M for their capacity to induce apoptosis of tumor cells. Furthermore, we will also use in vitro assays and mouse models to examine the cytolytic effects of mAb-mediated ADCC and CDC on tumor and normal cells.

In summary, we show for the first time that β_2M-specific mAbs alone are tumoricidal to tumor cells of many hematological malignancies. These mAbs selectively induced apoptosis of tumor cells in vitro and eradicated established tumors in xenograft mouse models of myeloma, including primary myeloma cells and other hematological tumors. The mAbs induced cell death via recruiting MHC class I molecules to lipid rafts, recruiting and activating Lyn and PLCγ2, in turn activating JNK and inhibiting PI3K/Akt and ERK, which then leads to compromised mitochondrial integrity, cytochrome c release, and activation of the caspase-9 cascade. We anticipate that these mAbs may be even more effective in the presence of immunological effectors such as complement and NK cells.

Experimental procedures

Experimental animals

Balb/c mice and CB.17 SCID mice were purchased from Harlan (Indianapolis, IN), and human β_2M/HLA-A2-transgenic C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in American Association of Laboratory Animal Care-accredited facilities, and studies were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and The University of Texas M. D. Anderson Cancer Center.

Cell lines, primary tumor cells, and reagents

Human myeloma cell lines ARP-1 and ARK-RS were established at the Arkansas Cancer Research Center from bone marrow aspirates of patients with MM (Hardin et al., 1994), and MM.1S and MM.1R were kindly provided by Dr. Steven Rosen of Northwestern University (Chicago, IL). The other tumor cell lines were purchased from ATCC. Primary myeloma cells were isolated from bone marrow aspirates obtained from patients during a routine clinic visit. $CD138^{++}$ myeloma cells and $CD56^{+}$ NK cells were isolated by magnetic-bead sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Purified human β_2M and mouse IgG and IgG1 were purchased from Sigma (St. Louis, MO). Monoclonal antibodies against β_2M (clone B2) and HLA-ABC (clone W6/32) were purchased from Serotec Ltd., and mAb against HLA-ABC (clone LY5.1) was purchased from Acris Antibodies GmbH (Hiddenhausen, Germany).

Generation of D1 and E6 monoclonal antibodies

Female Balb/c mice were immunized with purified human β_2M protein. Lymphocytes from spleens were fused with SP2/0 myeloma cells, and positive hybridomas were screened against β_2M by ELISA. Large-scale antibody production of selected clones, such as D1 and E6 (both are IgG1), was achieved by purifying mAbs from culture media using a protein G column (Amersham Biosciences, Piscataway, NJ) or by intraperitoneal injection of 2×10^6 hybridoma cells into Balb/c mice to produce ascites. Isotyping of mAbs was performed using a kit purchased from Serotec Ltd.

Generation of osteoclasts and coculture with myeloma cells

The method has been described previously (Yaccoby et al., 2004). PBMCs were cultured in 24-well plates in α-MEM medium supplemented with 10% fetal calf serum, antibiotics, RANK ligand (50 ng/ml), human M-CSF (25 ng/ml), and 10 nM dexamethasone for 10 to 14 days to generate tartrate-resistant acid phosphatase $(TRAP)^{+}$ and vitronectin $receptor^{+}$ osteoclasts. Before coculture, osteoclasts were washed, and freshly isolated myeloma plasma cells from patients (0.5×10^6 cells/ml) were added to the wells and cocultured with osteoclasts, with or without the addition of β_2M-specific mAbs. At the end of the studies, myeloma cells were detached from osteoclasts, and both myeloma and osteoclasts were subjected to analysis.

Apoptosis assays, caspase inhibitor, RNA interference, flow cytometry analysis of cell surface antigens, histology, immunohistochemistry, immunofluorescence analyses, immunoprecipitation, western blotting, and cytotoxicity assay

See Supplemental Experimental Procedures.

Isolation of lipid rafts

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 (Rietveld et al., 1999). Briefly, 5×10^7 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 four layers of 30% to 0% OptiPrep, and centrifuged at 200,000 × g using a TFT 65.13 rotor (Kontron Instruments) for 4 hr at 4°C. Nine fractions (1 ml each) were collected from the top to the bottom of the gradients. The lipid rafts determined with a caveolin-1-specific antibody were found in fractions 2 to 5.

In vivo antitumor activity of β_2M-specific monoclonal antibodies

Six- to eight-week-old female CB.17 SCID mice were subcutaneously inoculated in the right flank with 1×10^6 ARP-1, 2×10^6 MM.1S myeloma, 5×10^6 CA46 Burkitt's lymphoma, Granta 519 mantle cell lymphoma, or KG1 acute myelogenous leukemia cells in 50 μl RPMI-1640 medium. Three to four weeks later, when palpable tumors (≥5 mm in diameter) developed, mice (n = 5 for all groups) were treated with either subcutaneous (around tumors) injections of 200 μg purified D1 β_2M-specific mAb (or 100 μl ascites containing about 200 μg D1) or intraperitoneal injections of 250 μl ascites containing the mAb every 3 days for a total of four injections. Control mice received equal amounts of mouse IgG1 or PBS injected the same way as the mAb. To deplete murine NK cells, mice were injected intraperitoneally with 25 μl of anti-asialo GM1 antiserum (Wako Chemicals, Richmond, VA) 2 days before mAb treatment. This was repeated each week throughout the experiment in all mice. Tumor size was measured every 3 days in two dimensions using a caliper, and tumor volume (mm^3) was calculated as $4\pi/3 \times$ (tumor width/2)$^2 \times$ (tumor length/2) (LeBlanc et al., 2002). Serum was also collected from mice twice a week and used for detection by ELISA of myeloma-secreted M-proteins (human immunoglobulins) or their light chains. Mice were humanely sacrificed when moribund or when subcutaneous tumors reached 15 mm in diameter. Survival was evaluated from the day of tumor inoculation until death.

SCID-hu mouse model was established in CB.17 SCID mice as described before (Yaccoby et al., 1998). Freshly isolated primary myeloma cells

(1×10^6/mouse) from patients were directly injected to implanted human bones, and once myeloma was established (circulating human M-proteins or their light chains reaching >50 μg/ml), treatment began, which was the same as for SCID mice. In some experiments, 100 μg of D1 mAb or IgG1 was injected directly into implanted human bones that contained no tumor cells, followed by injection of purified human NK cells (2×10^6 cells), and the injections were repeated once again 3 days later. Mice were sacrificed 3 days after the final injection and human bones were removed for immunohistochemical examination.

Statistical analysis

All data are shown as means ± SEM. The Student's t test was used to compare various experimental groups. In mouse experiments, overall survival was measured using the Kaplan-Meier method, and the log-rank test was used for group comparison. Significance was set at $p < 0.05$.

Supplemental data

The Supplemental Data for this article can be found online at http://www.cancercell.org/cgi/content/full/10/4/295/DC1/.

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