Selective Induction of Expression of a Ligand for the NKG2D Receptor by Proteasome Inhibitors

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Abstract

The interaction of the activating receptor NKG2D with its ligands plays an important role in immunosurveillance of tumors and infectious pathogens, but dysregulation of this system may lead to autoimmunity. The expression of NKG2D ligands is induced by cellular "stress." However, the regulation of expression of these molecules is not well understood. Here, we show that cells treated with proteasome inhibitors can become more susceptible to cytotoxicity mediated by natural killer cells because of the induction of expression of ligands for NKG2D, specifically ULBP2, but not down-regulation of MHC class I. Treatment with proteasome inhibitors led to up-regulation of ULBP2 expression in multiple, but not all, cell lines tested. This increase in expression of ULBP2 at the cell surface correlated with induction of transcription of the *ULBP2* gene and synthesis of ULBP2 protein. In contrast, treatment with inhibitors of histone deacetylases led to increased levels of mRNA and protein, for both ULBP2 and MHC class I–related chain A/B molecules. Thus, different types of stress can trigger up-regulated expression of different sets of NKG2D ligands. Proteasome inhibitors are proving to be of significant value in the treatment of hematologic malignancies and these observations may help to better understand the biology of therapy with these compounds. [Cancer Res 2008; 68(5):1546–54]

Introduction

The activation of the immune system mediated by the interaction of NKG2D with its ligands is crucial in the regulation of both innate and specific immune responses, and this interaction is thought to play an important role in immunosurveillance of both pathogens and tumors (1). NKG2D is an activating receptor expressed on all natural killer (NK) cells, but it is also expressed on TCR$\gamma\delta^+$ and CD8$^+$ TCR$\alpha\beta^+$ T cells (2) where it can costimulate the activation of naïve T cells (3) and can even trigger cytotoxicity in the absence of NK ligation (4). In humans, NKG2D binds to MHC class I–related chain (MIC) A, MICB, and UL16-binding proteins (ULBP), all of them distantly related to MHC molecules and encoded within the MHC region (1, 5). The expression of NKG2D ligands (NKG2D-L) is restricted or absent on normal tissues; instead, they are up-regulated in situations of stress and disease. The molecular basis of this altered expression is not well understood, although it has been described that stress situations such as heat shock, oxidative stress, and DNA damage can up-regulate NKG2D-L expression (6–8). Further, although the expression of NKG2D-L has been described in multiple types of tumors (1, 9), there is considerable variation between donors in cell surface expression of these molecules (10). In summary, although the regulation of the cellular expression of NKG2D-L is not well understood, increased surface expression of NKG2D-L can be triggered by a number of signals, including tumorigenesis and infection (11).

Another poorly understood feature of the biology of NKG2D is the existence of multiple ligands for one receptor. NKG2D binding to these various molecules depends on the conservation of an overall shape complementarity and binding energy, but the different ligands do not share a common set of chemical interactions (12). It has been suggested that the appearance of all these different ligands could be a response to pressure exerted by infectious organisms (5). It is also possible that different stimuli provoke a selective up-regulation of different NKG2D-L (13), although only limited evidence supports this idea. We have noted that treatment of cells with proteasome inhibitors leads to the selective up-regulation of ULBP2 and here characterize this phenomenon.

The 26S proteasome is a multisubunit protease complex responsible for the recognition and subsequent degradation of proteins targeted for proteolysis (14) and its proper function is essential for cell viability. Aberrations in the ubiquitin-proteasome system have been related with a number of diseases, including several types of cancer, and neurodegenerative diseases such as Alzheimer's (15, 16). For this reason, proteasome inhibitors have recently become targets for drug development and one compound, bortezomib (PS-341, Velcade), a reversible inhibitor affecting the chymotryptic activity of the proteasome, has been licensed for use in therapy of multiple myeloma and is currently in trials to assess efficacy as therapy for other malignancies (17). Although growth inhibition and induction of apoptosis of myeloma cells have been proposed to be responsible for the response of patients treated with proteasome inhibitors, the molecular mechanisms important for the antitumor activity of these drugs are not completely understood (17–19). Here, we identify a new mechanism of action of proteasome inhibitors that involves enhancement of the immune response mediated by the activating receptor NKG2D. Specifically, we show that treatment with doses of proteasome inhibitors too low to induce cell death provokes the selective up-regulation of ULBP2, whereas treatment with inhibitors of histone deacetylases (HDAC) led to increased levels of mRNA, and protein, for both ULBP2 and MICA/MICB molecules. Thus, these data support the hypothesis that different cellular stresses can selectively trigger up-regulation of expression of different sets of NKG2D ligands.

Note: M. Valés-Gómez and S.E. Chisholm contributed equally to this work.
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Materials and Methods

Cells, reagents, and antibodies. The human foreskin fibroblast cell lines Hs27 and HFFF2 were obtained from the European Collection of Animal Cell Cultures and maintained in DMEM supplemented with 10% FCS and antibiotics (DMEM/10% FCS). ATR-Seckel fibroblasts, characterized by a markedly reduced expression of ataxia telangiectasia and Rad3-related protein (ATR; ref. 20; a gift of Dr. P. Jeggo University of Sussex, Brighton, United Kingdom), were cultured in DMEM supplemented with 15% FCS. Ataxia-telangiectasia mutated (ATM)–deficient cells transfected with either empty vector or ATM (ref. 21; gift of Dr. S.P. Jackson, University of Cambridge, Cambridge, United Kingdom) were cultured in DMEM supplemented with 20% FCS and 100 μg/mL hygromycin B. HeLa cells were cultured in DMEM/10% FCS. HCT116, 721.221, Raji, Daudi, Jurkat, U937, THP-1, and MonoMac 6 cells were cultured in RPMI 1640 with 10% FCS and antibiotics.

NK cell lines were prepared from buffy coats isolated from anonymous healthy adult donors as described (22). NK cells used in the experiments presented were more than 95% $CD3^-CD56^+$. All the NK cells expressed the activating receptors NKG2D, NKp46, NKp44, and NKp30.

The anti–HLA class I antibody HP-1F7 (23) was a gift from Prof. M. López-Botet. Other monoclonal antibodies (mAb) were purchased from BD PharMingen (CD3 and CD56), Immatics (MICA and MICB), R&D Systems (NKG2D; MICA/MICB; and ULBP1, ULBP2, and ULBP3), and Beckman Coulter (NKp30, NKp44, and NKp46). mAbs specific for ULBP1, ULBP2, ULBP3, and ULBP4 were a kind gift of Amgen, Inc. Isotype control mouse mAbs were purchased from Sigma.

The production of recombinant NKG2D-Ig fusion protein has been described (24). MG132, LiCl, and kenpaullone were purchased from Sigma; epoxomicin, ALLN, Ro106, Ro106C, IKK2, nuclear factor-κB (NF-κB) activation inhibitor, sodium butyrate, sodium valproate, tunicamycin, and thapsigargin were purchased from Calbiochem. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2, an inhibitor of multiple steps in the NF-κB signaling pathway (25), was purchased from Cayman Chemical. Bortezomib (Millenium Pharmaceuticals) was obtained from the Department of Pharmacy (Addenbrookes Hospital, Cambridge, United Kingdom).

PCR. Total RNA was isolated from equal numbers of cells using Trizol (Invitrogen) and cDNA synthesized using oligo dT and Superscript II reverse transcriptase (Invitrogen). After ethanol precipitation, semiquantitative PCR reactions were set up using serial (5-fold) dilutions of this cDNA. Amplifications were performed using *Taq* DNA polymerase (Qiagen) and the following oligonucleotides:

GAPDH, forward: 5′-ACCACAGTCCATGCCATCAC-3′;
reverse: 5′-TCCACCACCCTGTTGCTGTA-3′
MICA/MICB, forward: 5′-GCGAATTCGCCATGGGGCTGGGCC-3′;
reverse: 5′-CGGAATTCTAGGCGCCCTCAGTGGA-3′
ULBP2, forward: 5′-CCTAGCGCTCTGGGTCC-3′;
reverse: 5′-GTCAAAGAGGAGGAAGAACTGC-3′

Western blot. Equal numbers of viable cells were washed in PBS and lysed [in 20 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP40, and 0.1% SDS with Pepstatin A (1 μg/mL), leupeptin (1 μg/mL), and 5 mmol/L iodoacetamide]. Equal quantities of lysate were run on 10% SDS-PAGE gels and transferred to Immobilon-P membrane (Millipore). Western blots were performed using the goat anti-MICA/MICB or goat anti-ULBP2 antibodies (both from R&D Systems) and a β-actin–specific mAb (Sigma), followed by horseradish peroxidase–conjugated secondary antibody (Dako). Blots were visualized using the enhanced chemiluminescence system (Amersham Pharmacia).

Flow cytometry. For flow cytometry, 10^5 cells were preincubated in PBS containing 1% bovine serum albumin, 0.1% sodium azide, and 10% human serum. Cells were then incubated with mouse mAbs and bound antibody was visualized using either phycoerythrin- or FITC-labeled $F(ab')_2$ fragments of goat anti-mouse immunoglobulin (Dako). For experiments with NKG2D-Ig, cells were blocked with sodium azide/10% goat serum and bound immunoglobulin-fusion protein was visualized with $F(ab')_2$ fragments of goat anti-human IgG1 coupled to FITC (binding site). Samples were analyzed using a FACScan II flow cytometer (Becton Dickinson). Dead cells were excluded from all analyses by staining with propidium iodide.

Cytotoxicity assays. The cytolytic activity of NK lines against treated and untreated target cell lines was assessed in 4 h ^{51}Cr release assays as previously described (22). In experiments of antibody blocking, the NK cells or target cells were preincubated with either a mixture of natural cytotoxicity receptor (NCR)–specific mAbs (each at 10 μg/mL), an NKG2D-specific mAb, or an isotype control CD56-specific mAb (also at 10 μg/mL) for 30 min, before mixing NK and target cells.

Assays were performed in triplicate, and data differed by <10% (on average ~5%) of the mean. In all presented cytotoxicity assays, the spontaneous release of ^{51}Cr is indicated in the figure. All cytotoxicity assays were performed 6 to 8 days after restimulation of the NK cells and the data are representative of multiple experiments done with polyclonal NK lines generated from multiple donors.

Results

Treatment of human fibroblasts with proteasome inhibitors increases their susceptibility to NK-mediated cytotoxicity. MG132 and ALLN are reversible proteasome inhibitors that affect primarily the chymotrypsin-like activity of the proteasome, but also inhibit, at higher concentrations, other cellular proteases such as cathepsins and calpains. Epoxomicin is an irreversible inhibitor that affects all three activities of the proteasome and does not inhibit other nonproteasomal proteases. In the course of a set of experiments to investigate an unrelated phenomenon, we noticed that treatment of primary human fibroblasts with MG132 led to an increase in the susceptibility of these cells to NK-mediated lysis (Fig. 1*A*). This effect was dependent on the concentration of the inhibitor used and the time of incubation. At least 6 h of treatment were needed to observe an increase in susceptibility to NK cytotoxicity of fibroblasts treated with 2.5 μmol/L MG132. A concentration-dependent increase in sensitivity to lysis by NK cells was also observed in fibroblasts treated with epoxomicin and ALLN (Fig. 1*B* and *C*). Because three compounds targeting different enzymatic activities of the proteasome provoked susceptibility to NK cytotoxicity, it seems reasonable to conclude that the altered sensitivity to NK lysis depends on inhibition of the proteasome and not some other activity of these inhibitors.

Enhanced NK lysis of proteasome inhibitor–treated fibroblasts depends on recognition via the activating receptor NKG2D. NK cells become activated either through lack of inhibition, e.g., loss of recognition of self-MHC class I molecules, or through ligation of activating receptors. At the concentrations of proteasome inhibitor used, treatment of fibroblasts did not decrease the amount of MHC class I detectable by flow cytometry (Fig. 2*A*), suggesting that proteasome inhibition did not make cells better targets for NK cell cytotoxicity by reducing surface expression of MHC class I molecules. Thus, we next explored, in antibody blocking experiments, whether known NK activating receptors such as NKG2D or the NCRs NKp30, NKp44, and NKp46 were involved in the increased cytotoxicity of NK cells against proteasome inhibitor–treated cells. Preincubation of the NK cells with NKG2D-specific antibody (Fig. 2*B*), but not a mixture of NCR-specific mAbs or an isotype control, CD56-specific mAb, completely blocked the increased NK cell cytotoxicity. The involvement of NKG2D in NK cell recognition of the fibroblasts treated with MG132 was confirmed in flow cytometry experiments showing an increase in binding of NKG2D-Ig to viable, as judged by exclusion of propidium iodide, inhibitor-treated cells (Fig. 2*C*). Thus, fibroblasts treated with MG132 show a higher expression of

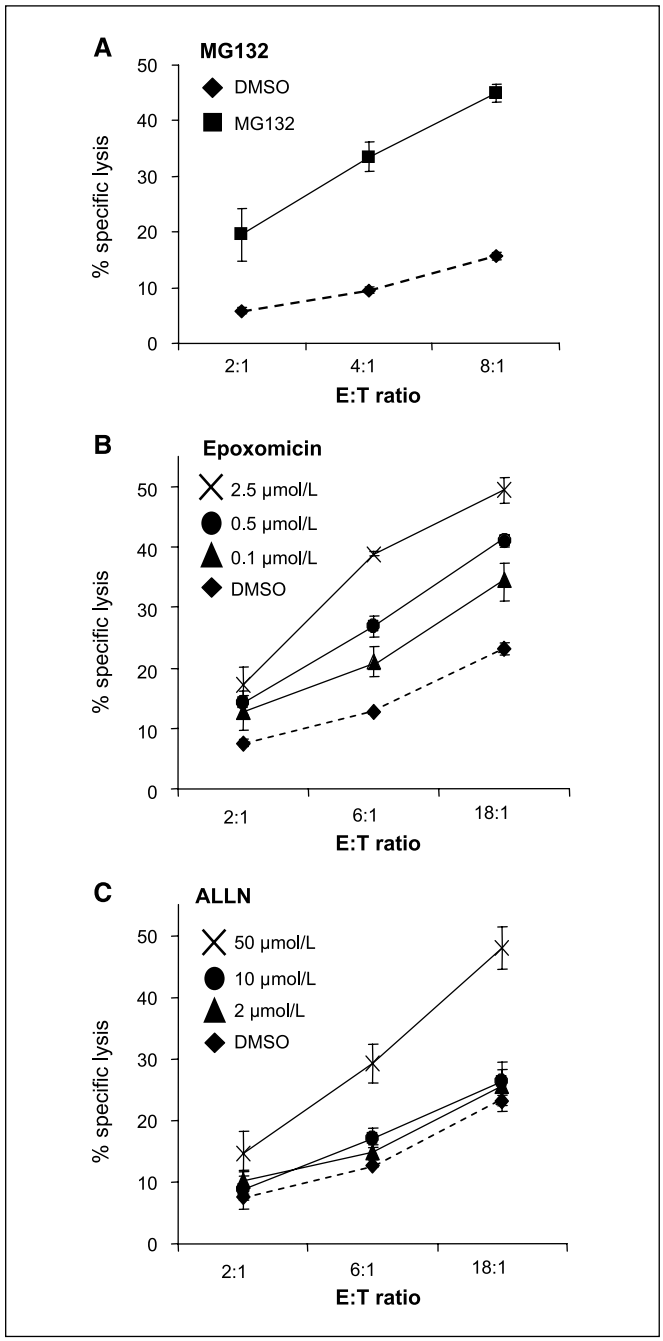

Figure 1. Treatment of fibroblasts with proteasome inhibitors leads to increased susceptibility to lysis by NK cells. Hs27 fibroblasts were treated for 16 h with proteasome inhibitors or DMSO (vehicle control) and used as targets in 4 h ^{51}Cr-release assays at the indicated effector-to-target ratios (*E:T*). *A,* 2.5 μmol/L MG132 or DMSO control. Spontaneous lysis: DMSO, 6.3%; MG132, 9.5%. *B* and *C,* increasing concentrations of epoxomicin and ALLN, as indicated. Spontaneous lysis: DMSO, 6.6%; epoxomicin (0.1 μmol/L, 11.7%; 0.5 μmol/L, 17.4%; 2.5 μmol/L, 19.3%); ALLN (2 μmol/L, 7.8%; 10 μmol/L, 8.0%; 50 μmol/L, 13.9%).

NKG2D-L than non-treated cells, leading to an increased susceptibility to cytotoxicity by NK cells.

ULBP2 expression is increased in various cell types in response to proteasome inhibitor treatment. To test whether the phenomenon described in fibroblasts occurred in other cell types, the Jurkat cell line was treated with increasing amounts of MG132 and the expression of NKG2D-L was assayed. To control for differences between cell lines in sensitivity to apoptosis induction after proteasome inhibition, cell death provoked by inhibitor treatment was measured by costaining with propidium iodide and only viable cells were analyzed for NKG2D-L expression. Optimal induction of NKG2D-L on human fibroblasts or HCT116 cells required 2.5 μmol/L of MG132, whereas at these concentrations of MG132 the majority of Jurkat cells were dead or dying. Instead, only 0.25 μmol/L MG132 was required to trigger an increase in surface

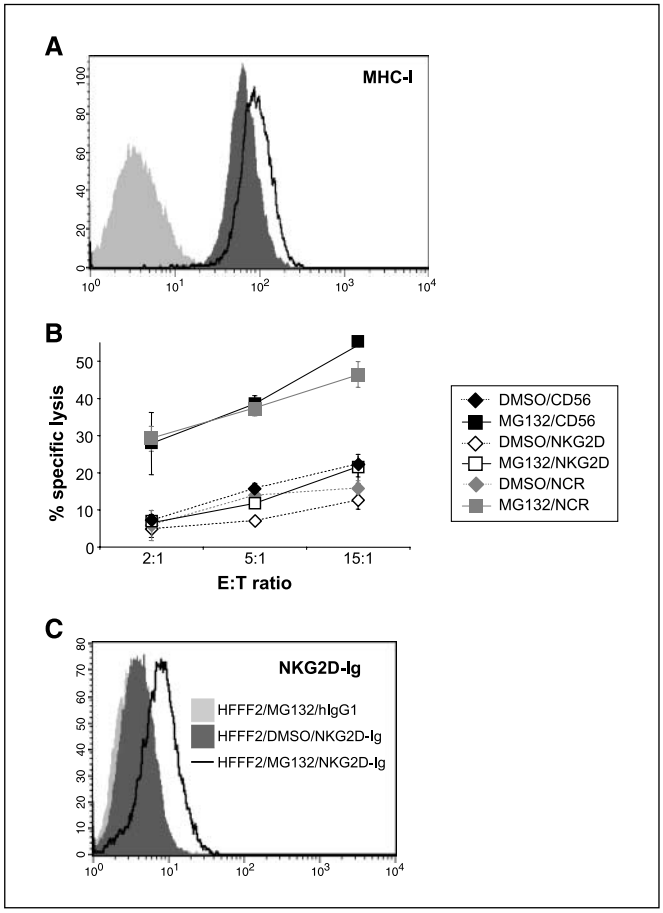

Figure 2. Increased NK susceptibility of proteasome inhibitor–treated cells depends on ligation of NKG2D. *A,* treatment of fibroblasts with proteasome inhibitors does not down-regulate MHC class I. Flow cytometry was performed on Hs27 fibroblasts treated with either 2.5 μmol/L MG132 or DMSO control for 16 h. MHC class I molecules were stained using mAb HP-1F7 and normal mouse serum was used as a negative control. *Light gray histogram,* cells treated with MG132 and stained with normal mouse serum; *dark gray histogram,* cells treated with DMSO and stained with HP-1F7; *bold line,* cells treated with MG132 and stained with HP-1F7. *B,* antibody blocking experiment. Target cells were cultured with either MG132 or DMSO before use in a ^{51}Cr release assay. Preincubation of NK cells with NKG2D-specific mAb, but not a mix of mAbs specific for the NCR or an isotype-matched control mAb (CD56-specific) blocked the increased lysis of cells treated with proteasome inhibitors. Target cells treated with vehicle control (DMSO); target cells treated with 2.5 μmol/L MG132. *Black fill,* NK cells pretreated with control CD56 mAb; *gray fill,* NK cells pretreated with NCR-specific mAb; *white fill,* NK cells pretreated with NKG2D-specific mAb. Spontaneous lysis: DMSO/CD56, 7%; MG132/CD56, 8.3%; DMSO/NKG2D, 7.7%; MG132/NKG2D, 9.4%; DMSO/NCR, 7.4%; MG132/NCR, 9.5%. *C,* cells treated with proteasome inhibitors express NKG2D ligands. Flow cytometry experiments show increased binding of NKG2D-Ig to viable fibroblasts after culture overnight with 2.5 μmol/L MG132. Data show one representative experiment of three. *Light gray histogram,* cells treated with MG132 and stained with control human IgG1; *dark gray histogram,* cells treated with DMSO and stained with NKG2D-Ig; *bold line,* cells treated with MG132 and stained with NKG2D-Ig.

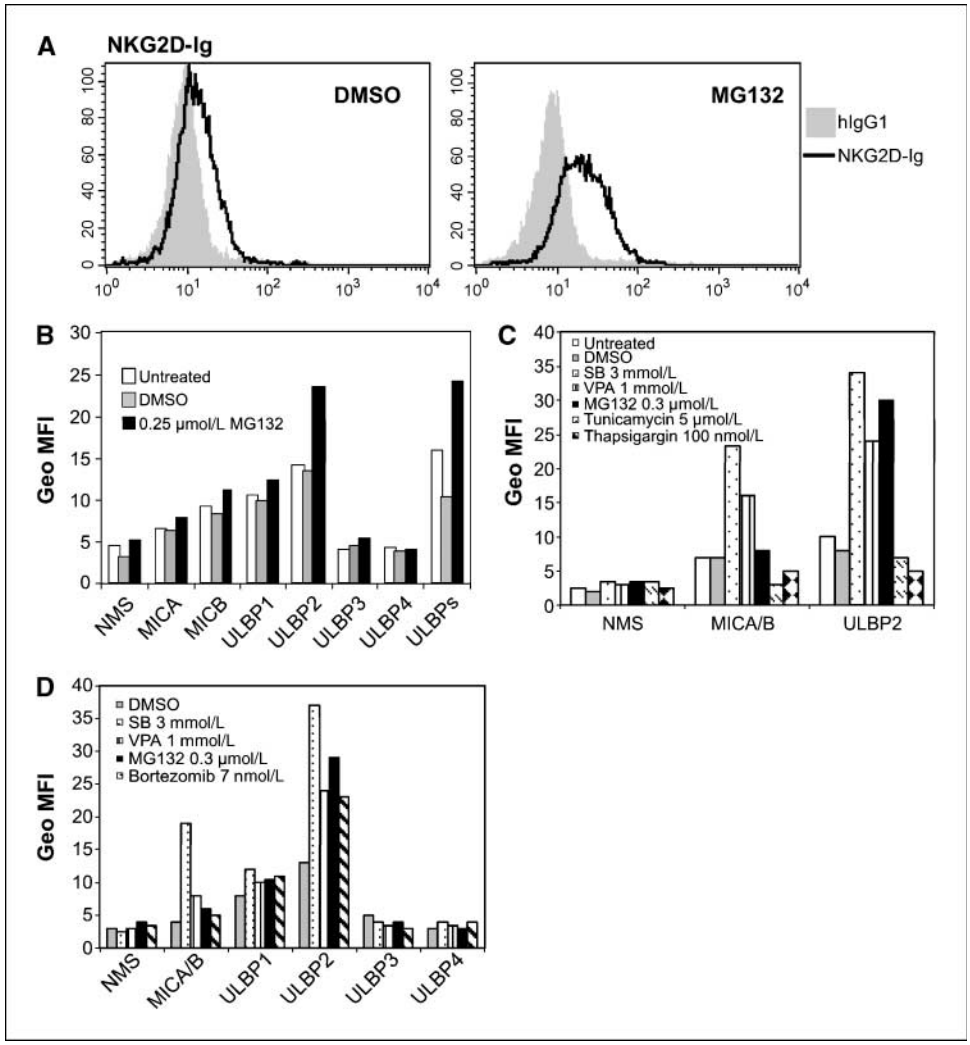

Figure 3. Jurkat cells up-regulate NKG2D ligands, specifically ULBP2, after treatment with MG132. Jurkat cells were treated for 16 h with either DMSO or 0.25 μmol/L MG132 and then stained with either (*A*) NKG2D-Ig or (*B*) mAbs specific for MICA; MICB (Immatics); or ULBP1, ULBP2, ULBP3, and ULBP4 (mAbs were gifts of Amgen). *C,* comparison of proteasome inhibitors with other stress-related reagents. HDAC inhibitors: sodium butyrate, sodium valproate; proteasome inhibitor (MG132); ER stress: tunicamycin, thapsigargin. *Geo MFI,* geometric mean fluorescence intensity. Cell viability after the different treatments was around 80% and dead cells were excluded from the analysis by propidium iodide staining. Data show one representative experiment of five using mAbs specific for MICA/MICB (R&D Systems) or ULBP2 (mAb was a gift of Amgen). *D,* induction of the expression of ULBP2 by bortezomib. Jurkat cells were either untreated or treated for 16 h with the various inhibitors at the indicated concentrations and then stained for flow cytometry with mAbs specific for MICA/MICB (R&D Systems) or ULBP1, ULBP2, ULBP3, and ULBP4 (mAbs were gifts of Amgen).

NKG2D-L on Jurkat cells (Fig. 3*A*) and at this concentration the majority of cells were viable. Flow cytometry experiments using mAb specific for individual NKG2D-L revealed that only ULBP2 was clearly up-regulated upon MG132 treatment of Jurkat cells (Fig. 3*B*). To check that selective ULBP2 up-regulation was not due to an inability of this cell line to up-regulate other NKG2D-L, the cells were also treated with sodium valproate, a HDAC inhibitor known to up-regulate expression of MICA/MICB molecules on some cell types (26) and another inhibitor of HDAC, sodium butyrate. Because proteasome inhibitors are known to produce endoplasmic reticulum (ER) stress (27, 28), and eventually apoptosis, two other compounds known to cause ER stress, tunicamycin and thapsigargin (29), were included in the study to test whether treatment with these compounds could also trigger ULBP2 up-regulation. The results of these experiments are summarized in Fig. 3*C*: The surface expression of both MICA and MICB could be induced on Jurkat after treatment with HDAC inhibitors but not in cells treated with proteasome inhibitors, tunicamycin or thapsigargin, suggesting that up-regulation of ULBP2 depends on inhibition of the proteasome. It was thus of interest to test whether bortezomib, a proteasome inhibitor approved for use in the clinic, could induce expression of NKG2D-L because MG132 and bortezomib have been reported to display differential specificities toward the subunits of the constitutive and immunoproteasome (19). Treatment of Jurkat cells (Fig. 3*D*) and THP-1 cells (data not shown) with low doses of bortezomib led to increased expression of ULBP2 molecules on viable cells.

We next used the panel of mAbs specific for individual NKG2D-L to explore whether this increase in ULBP2 occurred in other tumor cells treated with MG132. Interestingly, the effects of the different compounds used varied significantly in different cells (Table 1). The viability of the different cell lines varied significantly after treatment with the same concentration of MG132 (0.25 μmol/L); however, analysis of viable cells showed that although overnight culture of Monomac 6 (MM6), THP1, and U937 (all monocyte/macrophage tumor cell lines) with MG132 led to increased staining for ULBPs, this treatment did not change the levels of NKG2D-L expressed on 721.221, Raji, or Daudi cells (all B cell lines). In separate experiments, HeLa (cervical carcinoma) and HCT116 (colon carcinoma) cells also up-regulated ULBP2 after treatment with 2.5 μmol/L MG132. It is interesting to note that this effect occurs independently of the presence of a background expression of NKG2D-L. For example, untreated Jurkat, HCT116, and HeLa express low background levels of ULBPs, whereas MM6 does not express any NKG2D-L (9).

Another interesting observation of this set of data is the fact that the response to HDAC inhibitors also depends on cell type: For example, only ULBPs, but not MICA/MICB, were induced on Raji

Table 1. Induction of NKG2D-L expression by HDAC and proteasome inhibitors in a panel of different cell lines

Treatment	Monomac 6		THP-1		U937		721.221		Raji		Daudi		HeLa		HCT 116	
	MICA/B	ULBP2	MICA/B	ULBP2	MICA/B	ULBP2	MICA/B	ULBP2	MICA/B	ULBP2	MICA/B	ULBP2	MICA/B	ULBP2	MICA/B	ULBP2
Control*	3	3	3	4	2	2	3	3	4	2	2	2	65	3	27	2
SB	12	17	3	10	6	16	15	5	4	10	2	4	98	18	25	16
VPA	6	12	2	10	4	8	15	3	3	4	2	4	ND	ND	ND	ND
MG132	6	12	4	15	4	12	4	5	4	3	2	3	72	15	25	15

Abbreviations: SB, sodium butyrate; VPA, sodium valproate; ND, not done.
*Geometric mean fluorescence intensities.

cells after treatment with sodium butyrate and no NKG2D-L was up-regulated after treatment of these cells with sodium valproate. In conclusion, NKG2D-L can be selectively induced at the surface of tumor cells after treatment with various types of inhibitors at doses below those that induce cell death.

Treatment of cells with proteasome and HDAC inhibitors induces an increase in transcription of specific NKG2D ligands. The increased surface expression of NKG2D-L could represent mobilization of intracellular protein or enhanced transcription of the genes encoding these molecules. To distinguish between these possibilities, reverse transcription-PCR experiments were performed to assay the amount of mRNA encoding MICA/MICB and ULBP2 present in Jurkat cells treated with MG132, tunicamycin, thapsigargin, sodium butyrate, sodium valproate, and DMSO (control). RNA was extracted from equal numbers of viable cells and serial dilutions of the cDNA prepared were used as template in specific PCR reactions (Fig. 4*A*). Analysis of the housekeeping gene *G3PDH* confirmed that similar amounts of mRNA were present in each sample. The levels of mRNA for MICA/MICB increased significantly in cells treated with sodium butyrate and sodium valproate, and this correlated with the increase in surface expression observed after these treatments. Some ULBP2 mRNA could be detected in all the samples, but the levels expressed were clearly increased in cells treated with MG132, sodium butyrate, and sodium valproate. Interestingly, there was also an increase in mRNA for ULBP2 in cells treated with tunicamycin but this did not lead to an increase in the cell surface expression of this molecule. Similar data were obtained when cDNA prepared from Monomac 6 cells treated with the same panel of compounds was analyzed (data not shown). This discrepancy between the levels of mRNA of a given NKG2D-L and its expression at the cell surface prompted the question of whether the increase in transcription of NKG2D-L necessarily correlated with translation into protein. To address this issue, the quantity of NKG2D-L present in equal amounts of lysates of treated and untreated cells was analyzed by Western blot. Figure 4*B* shows that MICA/MICB is increased in sodium butyrate– and sodium valproate–treated cells and ULBP2 is increased in sodium butyrate–, sodium valproate–, and MG132-treated cells, but not those cells treated with tunicamycin, arguing against the possibility that tunicamycin treatment disrupts trafficking of ULBP2

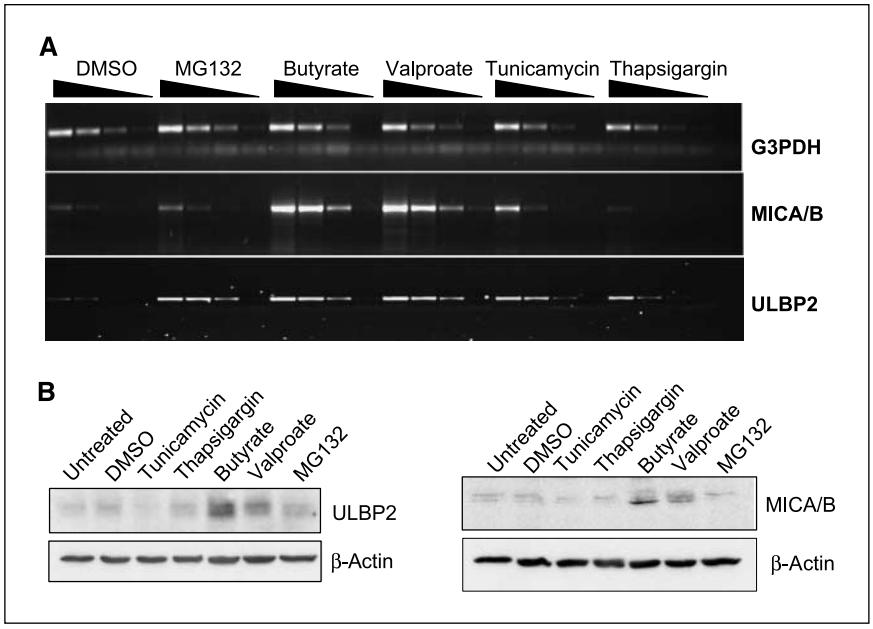

Figure 4. Induction of the expression of NKG2D-L with various stress-causing reagents. Jurkat cells were treated for 16 h with the various inhibitors at the same concentrations as in Fig. 3. *A,* RNA extraction and semiquantitative PCR. cDNA was extracted from equal amounts of cells and used as template in PCR reactions using oligonucleotides specific for the housekeeping gene *G3PDH*, MICA/MICB and ULBP2. The amount of RNA for *G3PDH* was equal in all the cells, but not the RNA for NKG2D-L. *B,* SDS-PAGE separation and Western blot. Lysates corresponding to equal number of cells treated with the indicated inhibitors were loaded in an SDS-PAGE gel and analyzed by Western blot using polyclonal antibodies specific for ULBP2 or MICA/MICB. β-Actin was used as control for equal loading.

to the cell surface. Thus, not all the mRNA encoding NKG2D-L induced after cellular stress translates into protein, suggesting post-transcriptional control of the expression of NKG2D-L. The involvement of post-transcriptional, and post-translational, modifications in the regulation of MICA has been suggested previously (30, 31).

Induction of ULBP2 by proteasome inhibitor treatment depends on expression of ATM and ATR, but is independent of inhibition of NF-κB or glycogen synthase kinase-3. The transcription and expression of NKG2D-L has been shown to be induced by stimuli that activate the systems of DNA damage repair, specifically the kinases ATM and ATR, which are key triggers of the DNA damage transduction cascade (32). We therefore tested the effect of treatment with proteasome inhibitors on the susceptibility to NK lysis of cells deficient in either ATM or ATR. As a control, ATM-deficient cells transfected with ATM, to restore function, were also included in these experiments. Proteasome inhibition did not trigger NKG2D-dependent lysis of cells deficient in ATM and cells deficient in ATR were lysed only poorly (Fig. 5*A*), whereas efficient induction of susceptibility to lysis via NKG2D after treatment with proteasome inhibitors was observed in cells expressing both ATM and ATR.

Because treatment with MG132 blocks NF-κB activation (33) as well as proteasome activity, fibroblasts were treated with either MG132 or other specific inhibitors of NF-κB signaling and then tested as targets in killing assays with NK cells (Fig. 5*B*). Only MG132, but not inhibitors of NF-κB, triggered increased target cell sensitivity to NK lysis, suggesting that treatment with proteasome inhibitors provokes susceptibility to NK cytotoxicity via a NF-κB–independent mechanism.

It has been reported that the induction of MICA/MICB expression by HDAC inhibitors depends on glycogen synthase kinase-3 (GSK-3) activity (34). Indeed, treatment with LiCl and kenpaullone, two structurally different inhibitors of GSK-3 kinase activity (35), reduced MICA/MICB expression induced by butyrate (Fig. 5*C*) and valproate (not shown). However, kenpaullone treatment had no effect on the induction of ULBP2 by either butyrate or MG132 in these experiments, whereas treatment with very high doses (75 mmol/L) of LiCl was required to block induction of ULBP2 expression. These experiments suggest that the induction of MICA/

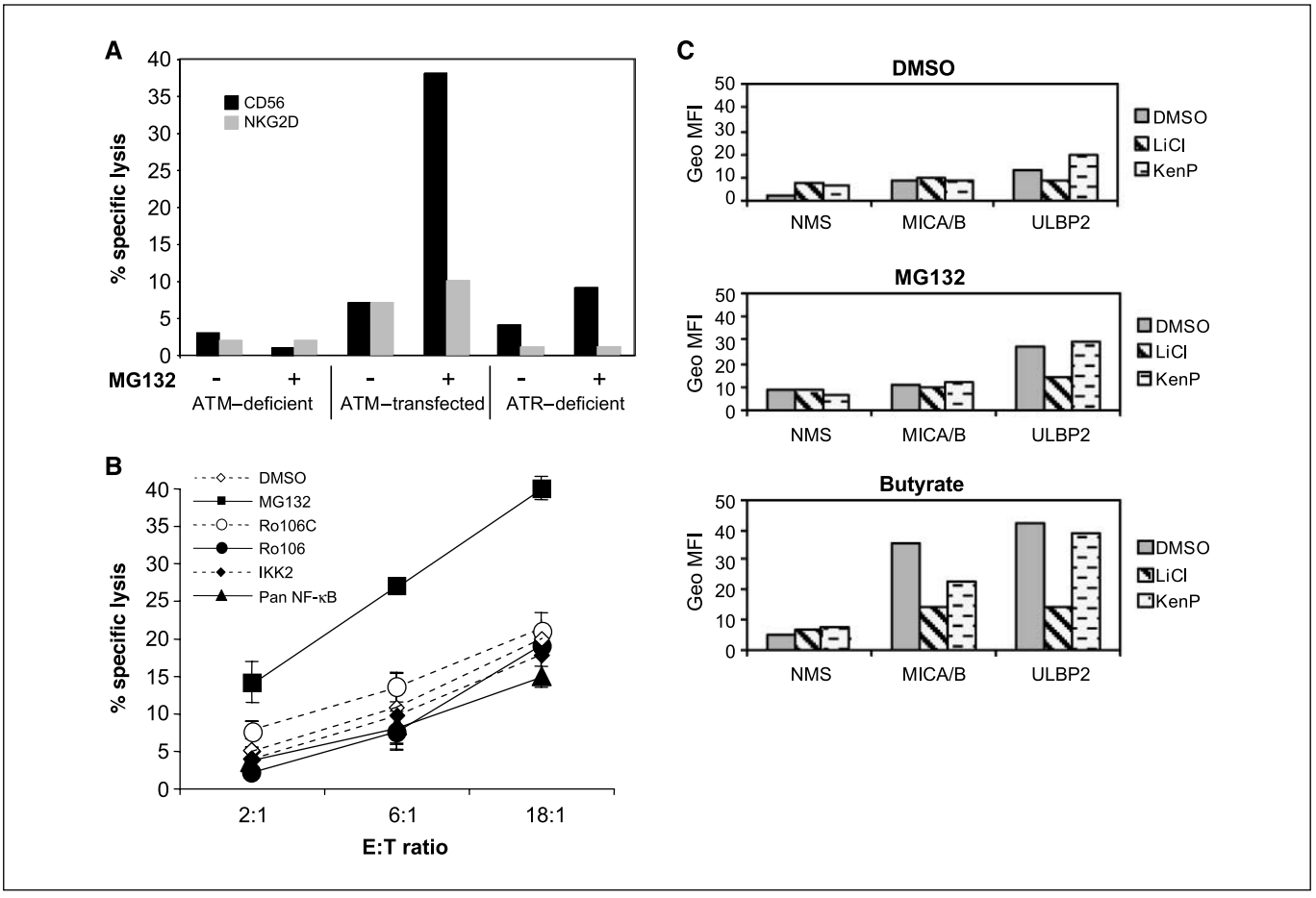

Figure 5. Induction of ULBP2 by proteasome inhibitor treatment depends on ATM and ATR expression, but is independent of inhibition of NF-κB or GSK-3. *A,* ATM-deficient fibroblasts, transfected with either empty vector (ATM-deficient) or ATM (ATM-transfected), and ATR-Seckel fibroblasts (ATR-deficient) were treated for 16 h with 2.5 μmol/L MG132 and used as targets in 4 h ^{51}Cr-release assays at an effector-to-target ratio of 5:1 with NK cells that had been preincubated with either isotype control CD56 or NKG2D-specific mAb as indicated. Spontaneous lysis: ATM/empty vector, DMSO, 15%; MG132, 16%; ATM/ATM, DMSO, 9.5%; MG132, 11.3%; ATR-Seckel, DMSO, 10.2%; MG132, 19.6%. *B,* human foreskin fibroblasts were treated for 16 h with 2.5 μm MG132 (—■—), the indicated NF-κB inhibitors [Ro106 (—●—) IKK2 (- - -◆- - -), pan NF-κB (—▲—)] or controls [DMSO (- - -◇- - -), Ro106C (- - -○- - -)] and used as targets in 4 h ^{51}Cr-release assays at the indicated E:T ratios. Spontaneous lysis: DMSO, 9.7%; MG132, 12.7%; Ro106C, 10.3%; Ro106, 15.5%; Ikk2, 11.1%; pan NF-κB, 10.8%. *C,* cell lines were treated with DMSO (*top*), 3 mmol/L sodium butyrate (*bottom*), or 0.25 μmol/L MG132 (*middle*) alone, or in combination with either LiCl (75 mmol/L) or kenpaullone (*KenP*, 10 μmol/L) as indicated for 16 h and then stained for flow cytometry with mAbs specific for MICA/MICB (R&D Systems) or ULBP2 (gift of Amgen).

MICB and ULBP2 depend on separate pathways and that GSK-3 is unlikely to be involved in ULBP2 expression induced by either inhibitors of HDAC or the proteasome.

Discussion

Proper function of the ubiquitin-proteasome system is critical to the maintenance of appropriate levels of short-lived and regulatory proteins as important and diverse as those involved in cellular metabolism, heat shock and stress response, antigen presentation, modulation of cell surface receptors and ion channels, cell cycle regulation, transcription, and signaling factors. Thus, it is not surprising that proteasome inhibition is proving to be an effective therapy for some human cancers (17), although the factors important for this therapeutic effect are only beginning to be understood. Proteasome inhibitors may exert an anticancer effect by inducing apoptosis via inhibition of the transcription factor NF-κB, a key survival factor, or by stimulating proapoptotic ER stress (36). Here, we describe a third mechanism of action for proteasome inhibitors that promote activation of the immune system when these drugs are used at doses too low to induce apoptosis. Moreover, the effect triggered by this treatment, selective up-regulation of certain NKG2D-L, also occurs after treatment with HDAC inhibitors and, in both cases, the quality and quantity of the response is very sensitive to the dosage and cell type. Thus, our data provide evidence for a relationship between the nature of the cellular stress and the selectivity of the cellular response.

Treatment with proteasome inhibitors increases susceptibility to NK cytotoxicity. NK cell recognition is controlled by a balance of signals from inhibitory and activating receptors (37), but here the increased susceptibility to lysis of cells treated with proteasome inhibitors is not due to the loss of key inhibitory ligands such as MHC class I molecules. Instead, inhibitor-treated cells show increased reactivity with NKG2D-Ig fusion protein, and preincubation of the effector NK cells with mAbs specific for NKG2D largely blocks NK recognition of proteasome inhibitor–treated target cells. These data lead to the conclusion that inhibitor-treated cells become sensitive to NK cell lysis because of up-regulation of ligands for the activating receptor NKG2D. These observations have profound implications for our understanding of cellular immunity to cancer, because forced expression of NKG2D-L by tumor cells not only leads to enhanced immune-mediated elimination of these tumor cells (38–40) but can also induce adaptive immunity to parental tumor cells that do not express these molecules (38). In addition, the activation of the immune system provoked by treatment with low doses of proteasome inhibitors might provide an alternative explanation for some previously described effects of these drugs. For example, the treatment with the proteasome inhibitor bortezomib has been shown to sensitize tumor cells to the lytic effects of effector NK and $CD8^+$ T-cells (41), and it was hypothesized that the cancer cells were more sensitive to immune effector cells because of proapoptotic changes induced by the treatment with proteasome inhibitor. However, our data suggest that increased expression of NKG2D-L could have contributed to enhanced immune recognition. In another report, tumor cells treated with the proteasome inhibitor bortezomib up-regulated the death receptor DR5 and this enhanced NK cell tumor cytotoxicity via the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) molecule expressed on NK cells (42). However, most freshly isolated leukocytes, including NK cells, do not express a detectable level of TRAIL on their surface until after activation by cytokines, principally IFN-γ (43). Therefore, this mechanism is more likely to be important in potentiating cytotoxicity mediated by already activated NK cells rather than initiating NK cell recognition of proteasome inhibitor–treated cells. Lundqvist et al. (42) excluded NKG2D-dependent activation as a mechanism for enhanced NK recognition because the expression of MICA/MICB did not change. However, they did not study the expression of other NKG2D-L.

In our experiments, proteasome inhibitor treatment specifically triggers up-regulation of ULBP2, and not other ULBPs or MICA/MICB. The selective induction of ULBP2 expression by proteasome inhibitors is striking because although differential induction of NKG2D-L genes in response to infection by different pathogens has been suggested to explain the existence of multiple ligands that bind the NKG2D receptor with high affinity, only limited evidence is available to support this idea (13, 44). Indeed, in general, infection with bacteria (both Gram positive and Gram negative) or viruses, such as cytomegalovirus, has been shown to induce transcription of all NKG2D-L examined (13, 45). However, our data suggest that a further level of regulation exists. Treatment with HDAC inhibitors leads to increased expression of MICA and MICB as well as ULBP2. These data exclude the possibility that the cell lines tested were unable to induce NKG2D-L other than ULBP2 and show that different cellular stresses can selectively trigger up-regulation of expression of different NKG2D-L. In fact, the induction of expression of MIC and ULBP molecules in response to the various proteasome and HDAC inhibitors varied in different cells. This might reflect that only a single dose of inhibitor was used in these experiments and it is possible that different cells are differentially sensitive to treatment with the various inhibitors, for example the concentration of proteasome inhibitor required to induce significant apoptosis in different multiple myeloma cell lines varied >10-fold between the different cell lines tested (28). Consistent with this idea, optimal induction of NKG2D-L on human fibroblasts or HCT116 cells required 2.5 μmol/L of MG132, whereas only 0.25 μmol/L MG132 was optimal for Jurkat cells. The hypothesis that different cell types are differentially sensitive to treatment with the various inhibitors might also explain why valproate treatment triggered expression of MICA/MICB, but not ULBPs, in hepatoma cells (26), whereas in our experiments exposure of Jurkat cells to the same concentration of valproate induced expression of MICA/MICB and ULBP2. Similarly, it has been shown that the degree of induction of MICA/MICB by HDAC inhibitors can differ markedly between different cell lines (34).

The regulation of expression of NKG2D-L is not well understood. The transcription and expression of multiple NKG2D-L is induced by stimuli that damage the DNA and activate the systems of DNA damage repair (32), including the ATM and ATR kinases. Interestingly, a growing body of evidence shows that the proteasome is involved in various aspects of gene transcription (46), including the repair of DNA double-strand breaks (47). Our data show that ATM and ATR are required for ULBP2 expression after proteasome inhibition, but it is not clear how this pathway triggers selective induction of ULBP2 expression. This will be an important topic for further study.

Skov et al. (34) reported that treatment with the HDAC inhibitor induced GSK-3 activity essential for the induction of expression of MICA/MICB proteins, although it is not clear where GSK-3 might act in the pathway regulating MICA/MICB expression. In our experiments, we could confirm that treatment with LiCl and kenpaullone, two structurally different inhibitors of GSK-3 kinase

activity (35), could inhibit butyrate-induced MICA/MICB expression. However, kenpaullone treatment did not block the induction of ULBP2 by either butyrate or MG132 in these experiments, whereas very high doses of LiCl were required to abrogate ULBP2 expression. These data indicate that GSK-3 is unlikely to be involved in the induction of ULBP2 expression by either inhibitors of HDAC or the proteasome and therefore the induction of MICA/MICB and ULBP2 by these compounds depends on distinct pathways. HDAC inhibitors such as butyrate and trichostatin A can suppress proteasome activity by reducing expression of the catalytic subunits of the proteasome at both the protein and mRNA levels (48). Thus, it seems reasonable to propose that HDAC inhibitors induce ULBP2 expression indirectly, via a pathway similar to that activated by proteasome inhibitors.

However, the mechanism by which proteasome inhibition triggers expression of ULBP2 is not clear. Proteasome inhibitors generally also block activation of the transcription factor NF-κB, but a range of specific inhibitors of NF-κB activation did not induce susceptibility to NK lysis. Inhibition of proteasome activity is also known to induce ER stress and apoptosis (49), but treatment with compounds that provoke ER stress, such as tunicamycin or thapsigargin, did not lead to enhanced expression of NKG2D-L at the cell surface. Thus, activation of the multiple signaling pathways associated with the unfolded protein response is not sufficient to induce NKG2D-L expression. Interestingly, although tunicamycin treatment did not induce synthesis of ULBP2 protein, increased levels of transcription of the *ULBP2* gene could be detected in treated Jurkat cells. This observation is consistent with other data suggesting that the regulation of NKG2D-L expression at the cell surface is a complex process depending on a variety of mechanisms that can act post-transcriptionally and post-translationally (30, 31), as well as at the level of transcription (8). Further study of induced NKG2D-L expression by proteasome inhibitors may provide new insight into the complex web of factors that regulate cell surface expression of ligands for the activating receptor NKG2D.

Taken together, our results indicate that the mechanisms of action of two different classes of antineoplastic drugs, which up to now have focused on their immediate effects on tumor cell apoptosis, can be extended to include enhancement of immune recognition of tumor cells by NKG2D-expressing cytotoxic effector cells. These observations raise the possibility of priming tumor cells *in vivo* for lysis by NK cells or activated $CD8^+$ T cells, perhaps in combination with adoptive immunotherapy, although more investigation will be required to test to what extent an induction of NKG2D-L proteins in healthy, nonmalignant cells could complicate this enhanced immune surveillance of cancer. It will also be important to test if enhanced expression of NKG2D-L leads to enhanced shedding of soluble ligands because chronic exposure to NKG2D-L–expressing cells, or soluble NKG2D-L, can lead to a loss of NKG2D expression and NK cell cytotoxicity (22, 50).

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References

1. Gonzalez S, Groh V, Spies T. Immunobiology of human NKG2D and its ligands. Curr Top Microbiol Immunol 2006;298:121–38.
2. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285:727–9.
3. Maasho K, Opoku-Anane J, Marusina AI, Coligan JE, Borrego F. NKG2D is a costimulatory receptor for human naive $CD8^+$ T cells. J Immunol 2005;174:4480–4.
4. Verneris MR, Karami M, Baker J, Jayaswal A, Negrin RS. Role of NKG2D signaling in the cytotoxicity of activated and expanded $CD8^+$ T cells. Blood 2004;103:3065–72.
5. Bahram S, Inoko H, Shiina T, Radosavljevic M. MIC and other NKG2D ligands: from none to too many. Curr Opin Immunol 2005;17:505–9.
6. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A 1996;93:12445–50.
7. Yamamoto K, Fujiyama Y, Andoh A, Bamba T, Okabe H. Oxidative stress increases MICA and MICB gene expression in the human colon carcinoma cell line (CaCo-2). Biochim Biophys Acta 2001;1526:10–2.
8. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature 2005;436:1186–90.
9. Pende D, Rivera P, Marcenaro S, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res 2002;62:6178–86.
10. Salih HR, Antropius H, Gieseke F, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 2003;102:1389–96.
11. Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. Nat Immunol 2004;5:996–1002.
12. Radaev S, Kattah M, Zou Z, Colonna M, Sun PD. Making sense of the diverse ligand recognition by NKG2D. J Immunol 2002;169:6279–85.
13. Hamerman JA, Ogasawara K, Lanier LL. Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. J Immunol 2004;172:2001–5.
14. Nandi D, Tahiliani P, Kumar A, Chandu D. The ubiquitin-proteasome system. J Biosci 2006;31:137–55.
15. Ciechanover A, Schwartz AL. Ubiquitin-mediated degradation of cellular proteins in health and disease. Hepatology 2002;35:3–6.
16. Pagano M, Benmaamar R. When protein destruction runs amok, malignancy is on the loose. Cancer Cell 2003;4:251–6.
17. Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. Annu Rev Med 2006;57:33–47.
18. Hideshima T, Mitsiades C, Akiyama M, et al. Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. Blood 2003;101:1530–4.
19. Crawford LJ, Walker B, Ovaa H, et al. Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132. Cancer Res 2006;66:6379–86.
20. O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet 2003;33:497–501.
21. Jazayeri A, Falck J, Lukas C, et al. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol 2006;8:37–45.
22. Roda-Navarro P, Vales-Gomez M, Chisholm SE, Reyburn HT. Transfer of NKG2D and MICB at the cytotoxic NK cell immune synapse correlates with a reduction in NK cell cytotoxic function. Proc Natl Acad Sci U S A 2006;103:11258–63.
23. Perez-Villar JJ, Melero I, Rodriguez A, et al. Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. J Immunol 1995;154:5779–88.
24. Vales-Gomez M, Browne H, Reyburn HT. Expression of the UL16 glycoprotein of human cytomegalovirus protects the virus-infected cell from attack by natural killer cells. BMC Immunol 2003;4:4.
25. Straus DS, Pascual G, Li M, et al. 15-deoxy-δ 12,14-prostaglandin J2 inhibits multiple steps in the NF-κB signaling pathway. Proc Natl Acad Sci U S A 2000;97:4844–9.
26. Armeanu S, Bitzer M, Lauer UM, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. Cancer Res 2005;65:6321–9.
27. Bush KT, Goldberg AL, Nigam SK. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J Biol Chem 1997;272:9086–92.
28. Obeng EA, Carlson LM, Gutman DM, Harrington WJ, Jr., Lee KP, Boise LH. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood 2006;107:4907–16.
29. Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. Nat Cell Biol 2001;3:E255–63.

30. Boissel N, Rea D, Tieng V, et al. BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. J Immunol 2006;176:5108–16.
31. Borchers MT, Harris NL, Wesselkamper SC, Vitucci M, Cosman D. NKG2D ligands are expressed on stressed human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 2006;291:L222–31.
32. Gasser S, Raulet DH. The DNA damage response arouses the immune system. Cancer Res 2006;66:3959–62.
33. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 1994;78:773–85.
34. Skov S, Pedersen MT, Andresen L, Straten PT, Woetmann A, Odum N. Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. Cancer Res 2005;65:11136–45.
35. Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. Biochem J 2003;371:199–204.
36. Nawrocki ST, Carew JS, Dunner K, Jr., et al. Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. Cancer Res 2005;65:11510–9.
37. Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J 2004;23:255–9.
38. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature 2001;413:165–71.
39. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor *in vivo*. Proc Natl Acad Sci U S A 2001;98:11521–6.
40. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by γδ T cells. Science 2001;294:605–9.
41. Schumacher LY, Vo DD, Garban HJ, et al. Immunosensitization of tumor cells to dendritic cell-activated immune responses with the proteasome inhibitor bortezomib (PS-341, Velcade). J Immunol 2006;176:4757–65.
42. Lundqvist A, Abrams SI, Schrump DS, et al. Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. Cancer Res 2006;66:7317–25.
43. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H. Nature's TRAIL-on a path to cancer immunotherapy. Immunity 2003;18:1–6.
44. Vankayalapati R, Garg A, Porgador A, et al. Role of NK cell-activating receptors and their ligands in the lysis of mononuclear phagocytes infected with an intracellular bacterium. J Immunol 2005;175:4611–7.
45. Welte SA, Sinzger C, Lutz SZ, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol 2003;33:194–203.
46. Collins GA, Tansey WP. The proteasome: a utility tool for transcription? Curr Opin Genet Dev 2006;16:197–202.
47. Krogan NJ, Lam MH, Fillingham J, et al. Proteasome involvement in the repair of DNA double-strand breaks. Mol Cell 2004;16:1027–34.
48. Place RF, Noonan EJ, Giardina C. HDAC inhibition prevents NF-κB activation by suppressing proteasome activity: down-regulation of proteasome subunit expression stabilizes IκBα. Biochem Pharmacol 2005;70:394–406.
49. Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annu Rev Biochem 2005;74:739–89.
50. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2002;419:734–8.