Direct and Natural Killer Cell-Mediated Antitumor Effects of Low-Dose Bortezomib in Hepatocellular Carcinoma

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Abstract

Purpose: Hepatocellular carcinoma (HCC) displays particular resistance to conventional cytostatic agents. Alternative treatment strategies focus on novel substances exhibiting antineoplastic and/or immunomodulatory activity enhancing for example natural killer (NK) cell antitumor reactivity. However, tumor-associated ligands engaging activating NK cell receptors are largely unknown. Exceptions are NKG2D ligands (NKG2DL) of the MHC class I-related chain and UL16-binding protein families, which potently stimulate NK cell responses. We studied the consequences of proteasome inhibition with regard to direct and NK cell – mediated effects against HCC.

Experimental Design: Primary human hepatocytes (PHH) from different donors, hepatoma cell lines, and NK cells were exposed to Bortezomib. Growth and viability of the different cells, and immunomodulatory effects including alterations of NKG2DL expression on hepatoma cells, specific induction of NK cell cytotoxicity and IFN-γ production were investigated.

Results: Bortezomib treatment inhibited hepatoma cell growth with IC_{50} values between 2.4 and 7.7 nmol/L. These low doses increased MICA/B mRNA levels, resulting in an increase of total and cell surface protein expression in hepatoma cells, thus stimulating cytotoxicity and IFN-γ production of cocultured NK cells. Importantly, although NK cell IFN-γ production was concentration-dependently reduced, low-dose Bortezomib neither induced NKG2DL expression or cell death in PHH nor altered NK cell cytotoxicity.

Conclusions: Low-dose Bortezomib mediates a specific dual antitumor effect in HCC by inhibiting tumor cell proliferation and priming hepatoma cells for NK cell antitumor reactivity. Our data suggest that patients with HCC may benefit from Bortezomib treatment combined with immunotherapeutic approaches such as adoptive NK cell transfer taking advantage of enhanced NKG2D-mediated antitumor immunity.

Hepatocellular carcinoma (HCC), being one of the most common cancers worldwide (1), typically displays a particular resistance to conventional cytostatic agents (2, 3). Therefore, advanced disease stages urgently require alternative treatment strategies that focus on immunotherapeutic approaches and/or targeting cellular pathways playing a major role in tumor development and maintenance (4 – 7). One potential target is the cellular turnover of proteins, which is a tightly regulated process. Interestingly, some types of cancers are exquisitely prone to undergo apoptosis in response to inhibition of the ubiquitin-proteasome pathway, a phenomenon that, at present, still lacks a precise explanation (8, 9).

Proteasome inhibition, e.g. with Bortezomib, has successfully been evaluated in a number of published and ongoing trials for solid and hematologic malignancies. Besides its prominent role in multiple myeloma, Bortezomib is presently approved by the Food and Drug Administration for use in relapsed and refractory mantle cell lymphoma. The activity of Bortezomib in other forms of cancer remains still to be determined with the exception of non – small cell lung cancer, where it displayed substantial activity (9). In HCC, short-term exposure with intermediate doses of Bortezomib was shown to sensitize the tumor cells to apoptotic stimuli, whereas healthy primary human hepatocytes (PHH) are not affected (10). In addition, prolonged exposure of hepatoma cells to higher doses of Bortezomib has been reported to induce apoptosis on its own (11).

Although these promising reports suggested a potentially selective effect of Bortezomib on hepatoma cells, a recent study

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described that high concentrations of Bortezomib inhibited natural killer (NK) cell cytotoxicity (12). These observations raise the concern that a potential antitumor effect of Bortezomib in the context of HCC might be annulled by a negative influence on immune effector cells such as NK cells. NK cells as a central component of the cytotoxic lymphocyte compartment substantially contribute to antitumor immune responses (13), and the adoptive transfer of *ex vivo* stimulated NK cells has been proposed as a strategy to prevent HCC recurrence after liver transplantation (14).

NK cell activity is governed by the complex interplay of multiple activating, inhibitory, and costimulatory receptors. The function of inhibitory NK cell receptors, e.g. recognizing MHC class I, is meanwhile well characterized. However, the tumor-associated ligands engaging activating NK cell receptors are still largely unknown. Exceptions are several highly diversified MHC class I–related molecules that have been shown to bind to the activating, homodimeric C-type lectin-like NKG2D receptor (13). NKG2D ligand (NKG2DL) expression potently stimulates antitumor responses that are critically dependent on NKG2DL expression levels (15, 16). In this study, we addressed the dual effect of a prolonged exposure of low Bortezomib doses on HCC cells and the NK cell–mediated immunosurveillance. Besides a direct antiproliferative effect of Bortezomib on HCC cells under these conditions, we surprisingly found that proteasome inhibition specifically induces expression of NKG2DL in malignant but not in healthy liver cells, leading to a specific stimulation of NK cell antitumor activity. Due to this dual response pattern, we suggest the treatment with low-dose Bortezomib or substances with similar activities in combination with immunotherapeutic approaches such as adoptive NK cell transfer as a promising novel option for the treatment of HCC patients.

Materials and Methods

Reagents and antibodies. Bortezomib/Velcade was a gift of Peter Brossart, Eberhard-Karls-University, Tuebingen, Germany and was diluted in PBS. The proteasome inihibitors MG132 and Lactacystine were from Sigma. Antimouse IgG2a–horseradish peroxidase was from Southern Biotechnology. The goat anti-mouse phycoerythrin conjugate, mouse-IgG1, and mouse-IgG2a were from Jackson Immunoresearch. The monoclonal antibodies (mAb) AMO1 (anti-MICA), BMO1 (anti-MICB), BAMO1 (anti-MICA/B), BMO2 (anti-MICB) and BAMO3 (anti-MICA/B), and W6/32 (anti-MHC class I) and recombinant MICA*04 and MICB*02 were produced as previously described (17). $F(ab')_2$ fragments were generated by pepsin digestion.

Cells. Human hepatoma cells Hep3B, HepG2, HuH7, and PLC/PRF/5 were maintained in 10% FCS-DMEM. The NK cell line NK92 was maintained in α-MEM with 12.5% horse serum, 12.5% FCS and Interleukin-2 (10 ng/mL). Primary NK cells were generated by incubation of non–plastic-adherent peripheral blood mononuclear cells with irradiated RPMI8866 feeder cells (ratio 4:1) in 10% FCS-RPMI containing 25 U/mL Interleukin-2 for 10 d. Experiments were done when purity of NK cells was above 90% as determined by flow cytometry. Tissue samples from human liver resection for PHH preparations were obtained from three male patients undergoing partial hepatectomy. Experimental procedures were done according to the guidelines of the Ethics Committee, University Hospital of Regensburg, with informed consent. The tissues were screened by the Human Tissue and Cell Research foundation, PHH were isolated using a modified two-step EGTA/collagenase perfusion procedure, and cultured on type I collagen with DMEM and 125 mU/mL insulin, 60 ng/mL hydrocortisone, 10 ng/mL glucagon, 100 μg/mL streptomycin, and 100 mU/mL penicillin as described (18). For treatment, 100,000 cells per well were incubated in 6-well plates for the indicated times.

Real-time PCR. Real-time PCR analysis was done as described previously (17). Samples were normalized to 18S rRNA to account for the variability in the initial concentration of the total RNA and conversion efficiency of the reverse transcription reaction. Primers for 18S rRNA were 5-CGGCTACCACATCCAAGGAA-3 and 5-GCTGGAATTACCGCGGCT-3; for MICA, 5-CCTTGGCCATGAACGTCAGG-3 and 5-CCTCTGAGGCCTCGCTGCG-3; and for MICB, 5-ACCTTGGCTATGAACGTCACA-3 and 5-CCCTCTGAGACCTCGCTGCA-3.

Immunofluorescence staining. Fluorescence microscopy was done using the anti-MICA/B mAb BAMO1 followed by the secondary phycoerythrin (PE) conjugate and 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Dianova) as previously described (19).

Flow cytometry. Hepatoma cells were incubated with the indicated NKG2DL- or MHC class I–specific mAb or the respective isotype controls followed by the secondary phycoerythrin conjugate and analyzed on a FACScalibur (Becton Dickinson).

Determination of hepatoma cell proliferation. Hepatoma cells or PHH were cultured for 24 h before addition of Bortezomib at the indicated concentrations and culture for additional 5 d. Growth inhibition was evaluated by sulforhodamin B assay being the standard assay of the US National Cancer Institute to screen for all kinds of cytotoxic substances as described (20, 21). Data represent means of absorbance measurements related to untreated cells. Inhibition concentration IC_{50} was calculated from four independent experiments using an equation for a sigmoidal dose-response curve with variable slope with GraphPad Prism 4.00 (GraphPad Software).

Determination of MIC levels by ELISA. Detection of MICA and MICB in cell lysates was done as previously described (19). In brief, for detection of MICA the mAb AMO1 and BAMO3 with recombinant MICA*04 as a standard were used. MICB levels were determined using the mAb BAMO1 and BMO2 with recombinant MICB*02. MICA/B protein levels were related to total protein contents as determined by BSA assay (Pierce). Cells were treated in triplicates and ELISA were done in duplicates for each cell sample. Two-way ANOVA with Bonferroni posttest was done for statistical analysis using GraphPad Prism 4.00.

Cellular cytotoxicity assay. Cytotoxicity was analyzed by a standard chromium release assay as previously described (17). In blocking experiments, anti-MICA/B $F(ab')_2$ fragments and IgG1 $F(ab')_2$ fragments as isotype control were added at 5 μg/mL 30 min before the coculture. Analysis of results was done by two-way ANOVA.

Determination of IFN-γ and lactate dehydrogenase. IFN-γ production of NK cells was analyzed using OptEIA sets from PharMingen, and release of lactate dehydrogenase (LDH) was measured using LDH-P mono (Biocon Diagnostic) according to manufacturer's instructions. Concentrations in supernatants are expressed as means and SDs of triplicates. Analysis of results was done with Student's *t* test using GraphPad Prism 4.00.

Results

Low-dose Bortezomib treatment inhibits proliferation of hepatoma cells and induces expression of the NKG2DL MICA/B. First, the human-derived hepatoma cell lines HuH7, HepG2, Hep3B, PLC/PRF/5, and PHH were incubated with increasing concentrations of Bortezomib (1.25-100 nmol/L) for 5 d. Analysis with all hepatoma cell lines revealed a clear dose-dependent inhibition of viability (Fig. 1A), with low IC_{50} values between 2.4 nanomol/liter (nmol/L) for HuH7 and 7.7 nmol/L for Hep3B cells (Table 1). To assess whether Bortezomib also had a direct toxic effect on the hepatoma cells, we further did LDH release assays, which showed a substantial increase of cellular lysis at 25 nmol/L but not at low concentrations such as

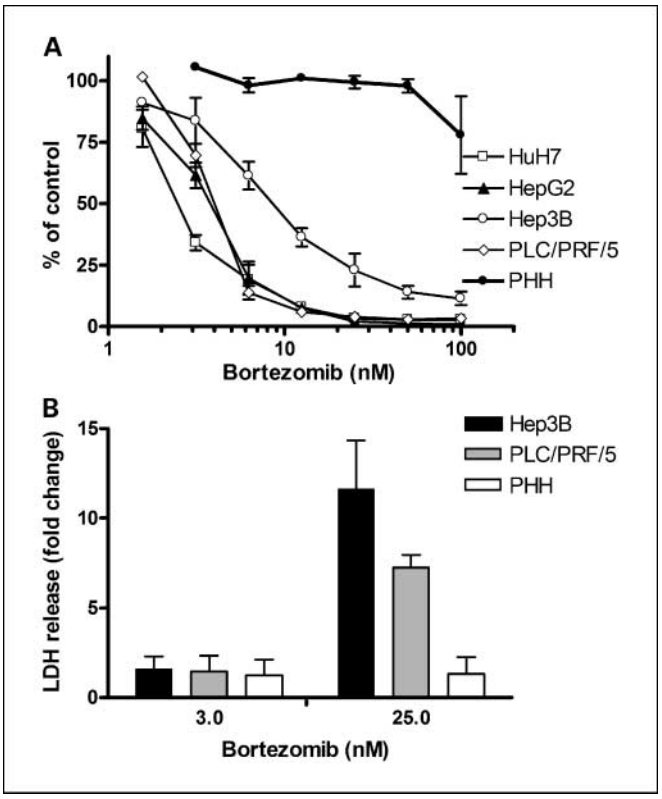

Fig. 1. Effect of Bortezomib on growth and cellular integrity of human hepatoma cell lines and primary hepatocytes. *A,* hepatoma cell lines and PHH were cultured for 5 d in the presence of the indicated concentrations of Bortezomib before analysis of cell viability compared with untreated cells by sulforhodamin B assay. Points, means of four independent experiments; bars, SD. *B,* the indicated hepatoma cell lines and PHH were cultured for 2 d in the presence of either 3 or 25 nmol/L Bortezomib. Then LDH release was determined and compared with untreated control cells. Columns, mean PHH of three different donors and from three independent experiments with the hepatoma cell lines; bars, SD.

3 nmol/L (Fig. 1B). It is of importance that in contrast to the effects on hepatoma cells, PHH from three different donors were not affected by Bortezomib, neither in the sulforhodamin B nor in the LDH release assay (Fig. 1A and B). This shows that even low doses of Bortezomib, in the range of 2.4 to 7.7 nmol/L according to the IC_{50} values determined with the hepatoma cells, are capable to mediate substantial effects during a prolonged exposure time period by a direct inhibition of tumor cell growth. Therefore, we chose 3 and 6 nmol/L as a potential low-dose Bortezomib treatment for subsequent studies addressing additional effects on NK cell antitumor reactivity.

Accordingly, we determined the effect of low-dose Bortezomib treatment on the expression of ligands for the activating immunoreceptor NKG2D. Using antibody BAMO1 that recognizes both NKG2DL MICA and MICB, weak MICA/B expression was detected on untreated ("0 nmol/L") Hep3B and PLC/PRF/5 cells by immunofluorescence analysis (Fig. 2). Treatment with Bortezomib for 48 h increased MICA/B expression by Hep3B and PLC/PRF/5 cells; notably, nuclei were not stained. MIC expression levels detectable by immunofluorescence analysis seemed to be higher in PLC/PRF/5 compared with Hep3B both prior ("0 nmol/L") and after ("3 and 6 nmol/L") Bortezomib treatment (Fig. 2).

To confirm these results and to investigate whether changes of NKG2DL expression could also be observed at the cell surface where it is relevant for NKG2D-mediated recognition by NK cells, we used flow cytometry. We found that incubation for 48 h with 3 and 6 nmol/L Bortezomib induced a substantial up-regulation of MICA/B surface expression on both Hep3B and PLC/PRF/5 cells (Fig. 3A, *top* and *middle* histograms). Importantly, MHC class I surface expression was not altered by Bortezomib treatment (Fig. 3A, *bottom* histograms) demonstrating that the observed changes were due to alterations in NKG2DL surface expression and not due to an unspecific effect of the compound. In contrast to MICA/B, the NKG2DL ULBP1-3 were not detectable on untreated or Bortezomib-treated hepatoma cells by specific antibodies (data not shown).

MIC molecules can be released in soluble form from the cell surface of various tumor cells including hepatoma cells, and the release is altered during the induction of MIC surface expression (19, 22, 23). To extend these studies to the effect of Bortezomib on MIC molecule expression, we treated Hep3B and PLC/PRF/5 cells with Bortezomib for 48 h and analyzed the culture supernatants by ELISA. Treatment concentration dependently increased the levels of both sMICA and sMICB released into the culture supernatants (Supplementary Fig. S1). These results confirm that human-derived hepatoma cells are able to release soluble MIC proteins, and this release is altered by therapeutic intervention.

To determine whether the increased MICA/B protein expression in hepatoma cells after proteasome inhibition was associated with alterations of MICA/B mRNA levels, we cultured PLC/PRF/5 cells for 12 hours in the presence or absence of 3 nmol/L Bortezomib and subsequently did MICA and MICB real-time PCR analysis. MICA and MICB mRNA levels in treated and untreated cells were quantified by standardization with 18S rRNA levels (Fig. 3B). Compared with untreated control cells, we found significantly higher levels of MICA and MICB mRNA in treated hepatoma cells, which suggests that the enhanced MICA/B protein expression is, at least in part, due to a Bortezomib-induced increase in mRNA levels. To further investigate whether a MIC up-regulation still occurred despite an inhibition of translation, cycloheximide was added in a further experiment and MIC protein contents were determined by ELISA and by immunofluorescence analysis. The Bortezomib-induced increase of MIC proteins could be inhibited by cycloheximide (Supplementary Fig. S2; Fig. 3C). This further supports our hypothesis that the well-known direct inhibitory effect of Bortezomib on the proteasome itself, which can lead to an accumulation of intracellular proteins, is not solely responsible for the observed MIC regulation patterns.

Table 1. Growth inhibition of human HCC-derived cell lines with Bortezomib

HCC cell line	IC_{50} (nmol/L)	95% CI
HuH7	2.4	2.2-2.7
Hep3B	7.7	6.4-8.8
HepG2	3.9	3.4-4.0
PLC/PRF/5	3.6	3.5-3.8

NOTE: IC_{50}, 50% growth inhibition determined by sulforhodamin B-assay.
Abbreviation: 95% CI, 95% confidence intervals.

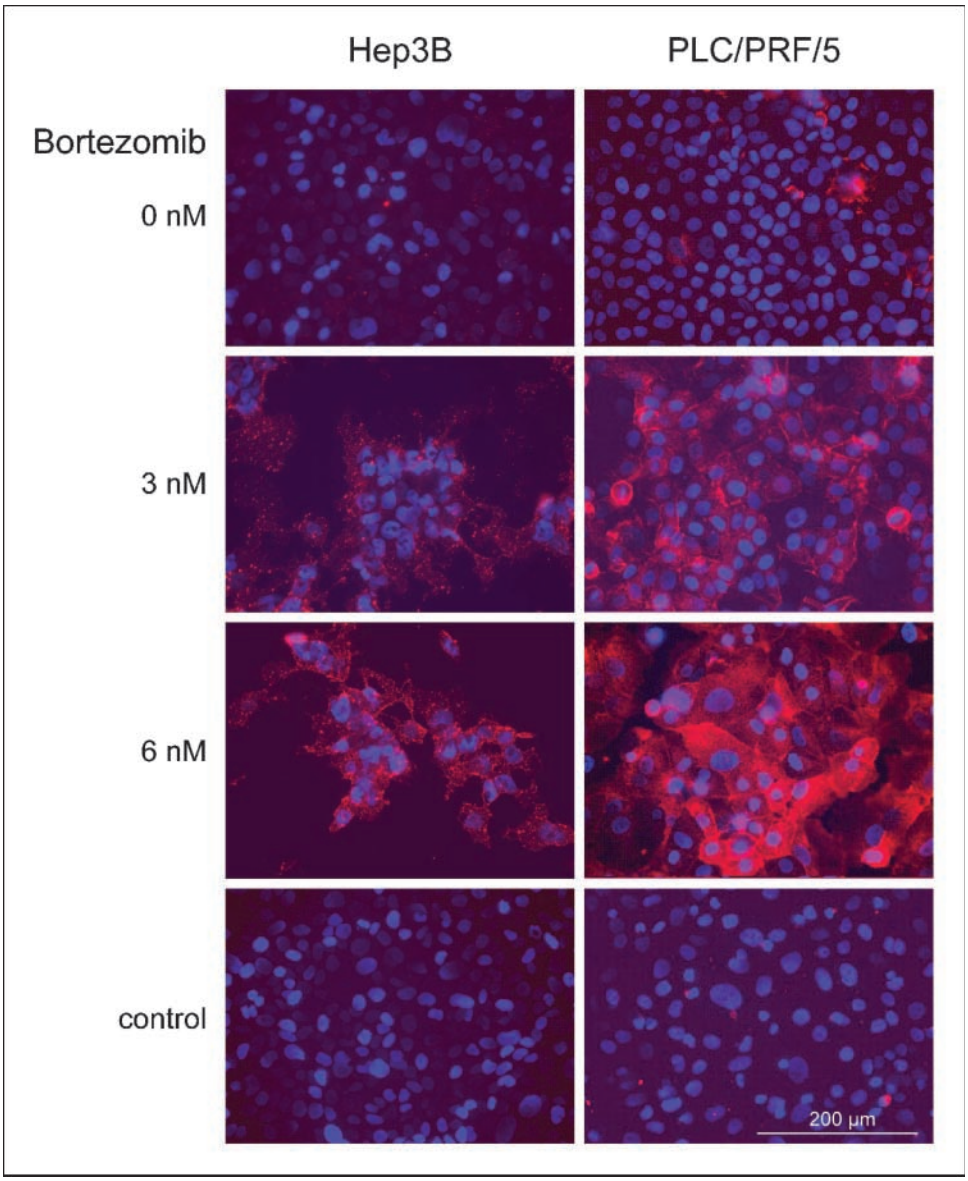

Fig. 2. Increase of MICA/B protein in hepatoma cells after Bortezomib treatment. Hep3B and PLC/PRF/5 cells were cultured for 48 h in the presence or absence of the indicated concentrations of Bortezomib. Subsequently, immunofluorescence analysis using the MICA/B specific mAb BAMO1 followed by the secondary PE conjugate and DAPI for nuclear staining was done. As a control, hepatoma cells incubated without primary mAb are shown. One representative experiment of a total of three is shown.

Differential expression of MICA and MICB in hepatoma cells and PHH after Bortezomib treatment. To quantify the up-regulation of MICA/B proteins and to discriminate between MICA and MICB protein induction after Bortezomib treatment, we used our previously described ELISA (19). Equal numbers of Hep3B and PLC/PRF/5 cells and PHH were cultured in the presence or absence of 3 or 6 nmol/L Bortezomib. After 48 hours, MICA and MICB protein levels were determined and related to total protein contents to account for differences in cell densities in the cultures (Fig. 4). In each hepatoma cell line, we observed a substantial and significant increase of MIC protein expression compared with untreated control cells (all $P < 0.01$; two-way ANOVA). In detail, in Hep3B cell lysates MICA protein levels were increased after Bortezomib treatment from 1.9 to 2.8 and 2.4 ng/mg protein (3 and 6 nmol/L, respectively). With PLC/PRF/5 cells, we found a constitutive expression of 1.5 ng/mg protein MICA, which was increased to 3.6 and 5.4 ng/mg protein (3 and 6 nmol/L, respectively; Fig. 4A). Regarding MICB, a higher constitutive amount of protein (4.2 ng/mg protein) was observed in Hep3B cells, and after Bortezomib treatment, cells contained 6.4 and 10.7 ng/mg protein (3 and 6 nmol/L, respectively; Fig. 4B). In PLC/PRF/5 cells, we measured 0.7 ng/mg protein MICB in untreated cells, which was increased to 1.6 and 2.3 ng/mg protein (3 and 6 nmol/L, respectively) by Bortezomib treatment.

In contrast, when PHH from two different donors were analyzed, we did not detect any substantial levels of either MICA or MICB, and importantly, we did also not detect an increase in MICA or MICB protein content after incubation with Bortezomib (Fig. 4; bars in grey). This shows that Bortezomib may specifically prime hepatoma cells but not nonmalignant PHH for NK cell recognition via NKG2D. To determine whether the up-regulation of MIC molecules also occurred with other agents than Bortezomib, we incubated Hep3B and PLC/PRF/5 cells with MG132 and Lactacystine, two further widely used proteasome inhibitors. In line with the results obtained with Bortezomib, similar concentration-dependent regulation patterns were observed by investigation

of whole cellular protein content using the ELISA (Fig. 4C and D), and this effect was also confirmed by immunofluorescence analysis (Supplementary Fig. S3). Thus, the observed MIC regulation in hepatoma cells seems to be the result of a complex cellular reaction pattern to proteasome inhibition in general and not only a substance-specific effect of Bortezomib.

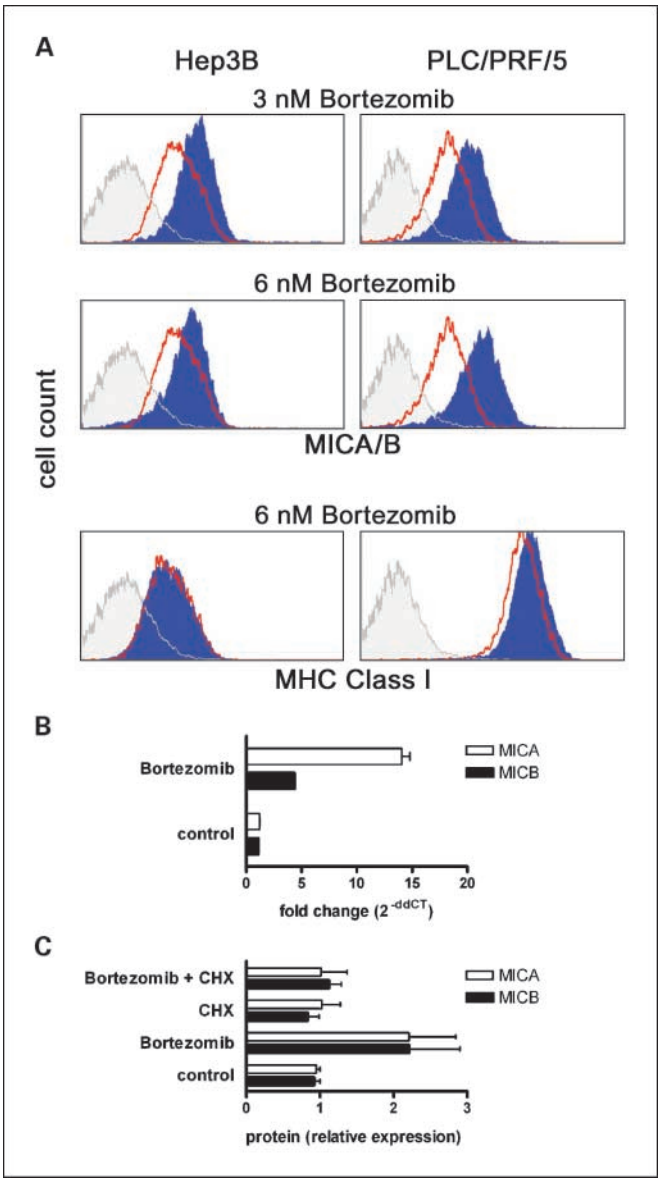

Fig. 3. Bortezomib stimulates MICA and MICB transcription and cell surface expression. *A,* Hep3B and PLC/PRF/5 cells were cultured for 48 h with 3 or 6 nmol/L Bortezomib or control medium before flow cytometric analysis of MICA/B and MHC class I surface expression. *Red histograms,* staining of untreated cells; *shaded/blue histograms,* expression on Bortezomib-treated cells; *gray,* isotype control stainings. *B,* PLC/PRF/5 cells were incubated for 12 h with 3 nmol/L Bortezomib or control medium, then total RNA was isolated and reverse transcribed. Relative copy numbers of MICA and MICB were determined by real-time PCR and normalized with 18S rRNA expression. Data are shown as fold change with SD from triplicates. *C,* PLC/PRF/5 cells were cultured for 48 h in the presence or absence of 6 nmol/L Bortezomib and/or 10 μg/mL cycloheximide before the determination of MICA and MICB levels in whole cellular lysates by ELISA. Columns, means of triplicates as relative cellular MIC protein content compared with untreated controls; bars, SD. One representative experiment each a total of at least three is shown.

Low-dose Bortezomib treatment stimulates NKG2D-dependent effector functions of NK cells against hepatoma cells. In initial experiments, we observed that hepatoma cell killing by NK cells was in part mediated via NKG2D (data not shown). Therefore, we addressed the functional relevance of altered NKG2DL expression on hepatoma cells after treatment with low-dose Bortezomib using the human NK cell line NK92, which is exquisitely suitable to study NKG2D-mediated NK cell functions (19). Pretreatment with 3 nmol/L Bortezomib for 48 hours caused a statistically significant ($P < 0.01$; two-way ANOVA) increase in the NK-mediated killing of hepatoma cells of >50% (Fig. 5A). The Bortezomib-mediated increase in cytotoxicity was critically dependent on NKG2D/NKG2DL interaction because it could be abrogated by the addition of blocking anti-MICA/B $F(ab')_2$ fragments, whereas the addition of isotype control $F(ab')_2$ fragments did not influence the lysis of Bortezomib-treated hepatoma cells.

To investigate whether increased MIC expression after Bortezomib treatment of hepatoma cell lines also influenced production of IFN-γ by NK cells, PLC/PRF/5 cells were again treated with 3 nmol/L Bortezomib. Subsequently, we cultured NK92 cells for 24 hours alone or in the presence of untreated and Bortezomib-treated hepatoma cells and analyzed the culture supernatants by ELISA. Presence of PLC/PRF/5 cells significantly increased the levels of IFN-γ produced by NK cells, and IFN-γ production was significantly higher when the HCC cells had been pretreated with Bortezomib ($P < 0.01$; Student's t test). This increase of IFN-γ production by NK cells in response to Bortezomib-treated hepatoma cells was found to be critically dependent on MIC-NKG2D interaction: IFN-γ production was significantly ($P < 0.01$) decreased by addition of anti-MICA/B $F(ab')_2$ fragments, whereas isotype control had no significant effect (Fig. 5B). The fact that not only tumor cell lysis but also IFN-γ production by NK cells is increased in response to Bortezomib pretreated hepatoma cells strongly suggests that induction of MICA/B on hepatoma cells and the resulting enhanced NKG2D stimulation is, at least in part, responsible for the augmented NK cell reactivity toward hepatoma cells.

Bortezomib differentially affects NK cell cytotoxicity and IFN-γ production. As mentioned above, high doses of Bortezomib have been reported to inhibit NK cell cytotoxicity (12), which could potentially counteract the observed beneficial effects of MIC induction on the hepatoma cells. Therefore, we treated primary NK cells of healthy donors with different concentrations of Bortezomib for 24 hours and examined NK cell cytotoxicity and IFN-γ production in response to K562 target cells, which are the classic target cells for studying NK cell functions. We found that low doses of Bortezomib as applied in our experiments did not affect NK cell cytotoxicity (Fig. 5C), whereas a significant ($P < 0.01$; two-way ANOVA test) inhibition was observed at higher Bortezomib levels starting at 25 nmol/L.

Next, we analyzed IFN-γ production of NK cells alone and in cocultures with K562 target cells at an effector to target ratio of 5:1. As expected, presence of K562 cells substantially increased the IFN-γ release of untreated NK cells. However, even low levels of Bortezomib concentration-dependently inhibited the IFN-γ production of NK cells. At the concentrations used for hepatoma cell treatment, NK cell IFN-γ release was reduced ~50% (Fig. 5D). Thus, our data indicate that Bortezomib

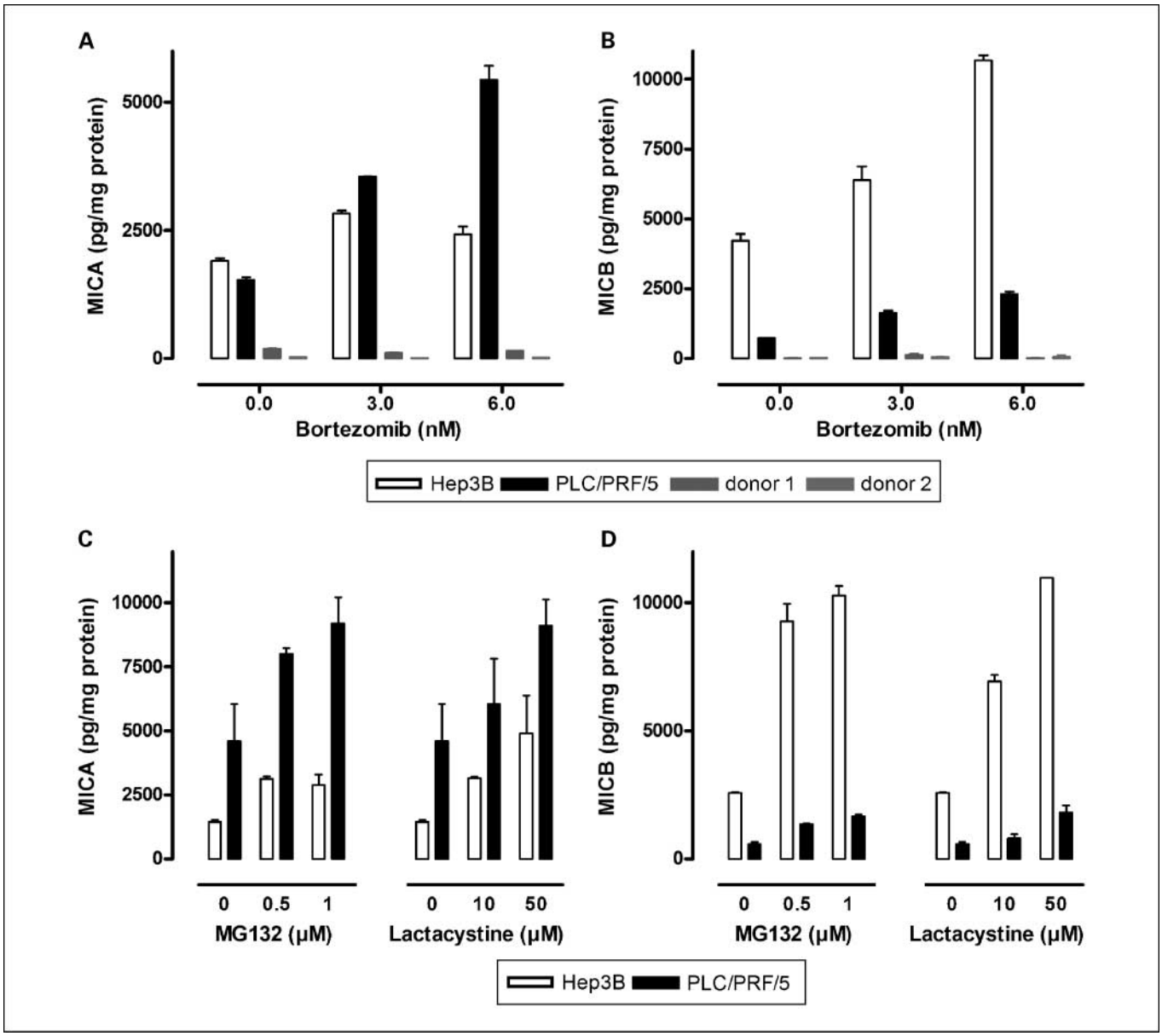

Fig. 4. Bortezomib induces MIC expression in human-derived hepatoma cell lines but not in PHH. PHH from two different donors as well as Hep3B and PLC/PRF/5 cells were incubated in the presence or absence of 3 or 6 nmol/L Bortezomib for 48 h. Subsequently, MICA (*A*) and MICB (*B*) protein levels in whole cell lysates were analyzed by ELISA and related to total protein contents to account for differences in cell densities in the cultures. In addition, the effect of different concentrations of the proteasome inhibitors MG132 and Lactacystine on MICA (*C*) and MICB (*D*) levels in hepatoma cells was determined. Columns, means of triplicates of one representative experiment each from a total of three; bars, SD.

differentially affects the two main effector functions of NK cells and show that low concentrations of Bortezomib prime hepatoma cells for NK cell lysis without affecting NK cell cytotoxicity but may partially impair IFN-γ production.

Discussion

Currently the value of proteasome inhibition for the treatment of HCC is under early clinical investigation. This is due to the fact that hepatoma cells display a marked resistance to conventional cytostatic agents resulting in disappointing clinical outcomes when currently available chemotherapeutic treatment strategies are used. It has been shown in hepatoma cells that proteasome inhibition can lead to a shift in the balance of proapoptotic and antiapoptotic factors resulting in profound apoptosis or sensitization of hepatoma cells to the proapoptotic cytokine TNF-related apoptosis-inducing ligand (10, 11, 24, 25).

In contrast to available reports regarding Bortezomib effects in HCC, we used a prolonged exposure of hepatoma cells to low concentrations of Bortezomib and observed a profound inhibition of tumor cell growth with IC_{50} values between 2.4 and 7.7 nmol/L. Short-term exposure of tumor cells to high concentrations revealed a direct toxic effect, which was not observed with the low-dose Bortezomib concentration used further in our study. Pharmacokinetic studies in humans with

single injections between 1.0 and 2.0 mg/m^2 Bortezomib according to the doses that are currently clinically applied led to plasma peak concentrations between 29 and 547 nmol/L (26). Our *in vitro* analysis of PHH cultures did not reveal an increase of LDH release as a surrogate marker for cellular damage with low Bortezomib concentrations up to 25 nmol/L. In line, other investigators even did not observe any cytotoxicity for PHH applying >100-fold higher concentrations (10). Considering this differential reaction pattern of PHH and hepatoma cells and the substantial effect on tumor cell growth, it is tempting to speculate that low doses of Bortezomib might be suitable for usage as a new HCC treatment option. It is noteworthy that liver toxicity seems not to limit the clinical use of this drug in general.

Besides the direct effect on tumor cells, several studies revealed that proteasome inhibitors may also affect immune and inflammatory responses including NK cell reactivity (9). This is of great importance because NK cells as a central component of the cytotoxic lymphocyte compartment substantially contribute to antitumor immunity, and adoptive transfer of NK cells has been implicated as a strategy in the treatment of HCC (14). NK cell activity results of an integrative response emerging from multiple activating and inhibitory NK cell receptors (13). In recent years, the role of the activating NKG2D receptor in the antitumor reactivity of NK cells especially received considerable interest. NKG2DL are inducibly expressed in cells subjected to genotoxic stress and are associated with malignant transformation but usually are not expressed in normal healthy tissues and can render cells susceptible to NK cell cytotoxicity depending on the NKG2DL expression levels despite the expression of substantial levels of MHC class I (13, 15, 16). In fact, it has been shown that NKG2D provides protection from spontaneous tumors *in vivo* (27, 28), and we showed recently that NKG2DL are expressed on hepatoma cells *in vivo* and *in vitro* (19). These earlier results and the fact that the ubiquitin-proteasome pathway plays an essential role in regulating

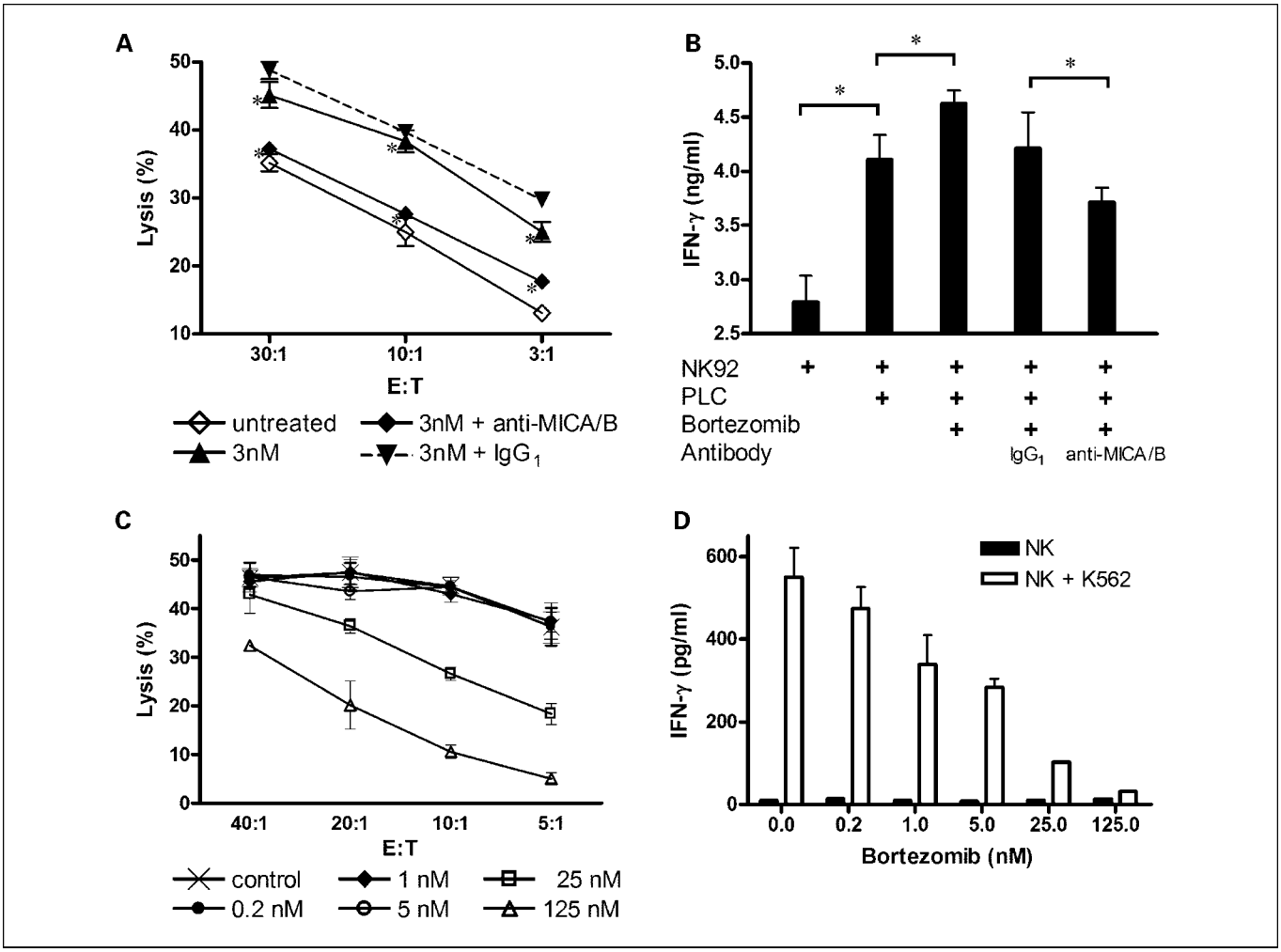

Fig. 5. Low-dose Bortezomib induces an NKG2D-dependent stimulation of NK cell effector functions against hepatoma cells. *A,* PLC/PRF/5 cells were incubated for 48 h with 3 nmol/L Bortezomib (3 nmol/L) or control medium (untreated) prior to 4-h chromium release assays with the NK92 cell line at the indicated effector to target ratios (E:T). Where indicated, anti-MICA/B BAMO1 (3 nmol/L + anti-MICA/B) or isotype control F(ab')$_2$ fragments (3 nmol/L + IgG1) were added before addition of NK cells. *B,* PLC/PRF/5 cells were incubated for 24 h with control medium or 3 nmol/L Bortezomib. Then NK92 cells were cocultured for additional 24 h in the presence or absence of anti-MICA/B or isotype control F(ab')$_2$ fragments. Afterwards, supernatants were harvested and analyzed for IFN-γ by ELISA. *C* and *D,* primary human-derived NK cells were cultured for 24 h in the presence or absence of the indicated concentrations of Bortezomib, then NK cells were cocultured with K562 cells as target cells. Subsequently, cytotoxicity of NK cells (*C*) was analyzed by chromium release assays and IFN-γ production of NK cells (*D*) was determined after 24 h by ELISA of culture supernatants. Columns, means of triplicates of one representative experiment each of a total of three; bars, SD. *, significant differences ($P < 0.01$).

protein levels during cell cycle, apoptosis, intracellular signal transduction, and response to cellular stress (i.e., DNA damage, hypoxia; refs. 8, 9), prompted us to study whether the proteasome inhibitor Bortezomib was capable to stimulate NK cell reactivity in the context of HCC.

We show that MICA and MICB protein expression in hepatoma cells is markedly increased upon Bortezomib treatment. Similar results were obtained using two further prototype proteasome inhibitors. Flow cytometry revealed an up-regulation of MICA/B surface expression, which correlated with the increase in total cellular MIC protein. In contrast, the NKG2DL ULBP1-3 were absent on hepatoma cells, and expression was not induced by Bortezomib.

Recently, we reported that hepatoma cells, such as tumor cells of many other histologic origins, release MIC molecules in soluble form. Treatment with Bortezomib increased the levels of sMIC in the culture supernatants, which paralleled our findings regarding the effect of the histonedeacetylase-inhibitor Valproate on hepatoma cells. Release of NKG2DL by shedding from the tumor cell surface has been proposed by various investigators to mediate evasion from immunesurveillance by e.g., systemically reducing NKG2D expression on cytotoxic lymphocytes (ref. 22). However, in other studies, NKG2D down-regulation required direct contact between cancer cells and cytotoxic lymphocytes, whereas soluble factors such as recombinant NKG2DL or tumor cell supernatants did not affect NKG2D expression, indicating that this process may be more complex than suspected (e.g., refs. 29, 30). Further studies will be required to clarify the specific role and function of sNKG2DL in tumor immunesurveillance.

Because it has been reported that NKG2DL expression may also be regulated on a posttranscriptional level (31), we wanted to determine whether the increase of MIC protein was associated with a Bortezomib-induced alteration of MIC mRNA levels. In fact, quantitative PCR analysis revealed an increase of MIC mRNA levels in treated compared with untreated hepatoma cells, which points to a yet unknown effect of proteasome inhibition. Furthermore, inhibition of translation by addition of cycloheximide blocked the Bortezomib-induced increase of MIC proteins. This suggests an interrelation between the Bortezomib induced mRNA up-regulation and the observed increase of MIC proteins. Two previous studies showed that after Bortezomib treatment of hepatoma cells, a relevant number of apoptotic cells was observed above concentrations of 25 to 50 nmol/L (10, 11). We observed a substantial MIC up-regulation already at 3 nmol/L Bortezomib and after 12 h, suggesting that MIC induction seems not to be linked to apoptosis.

Subsequently, we determined the functional significance of enhanced NKG2DL surface expression for priming HCC cells for NK cell recognition. To eradicate tumors, NK cells perform two critical tasks, namely cytotoxicity and production of IFN-γ, the latter participating in cancer elimination by e.g., inhibiting cellular proliferation and angiogenesis, promoting apoptosis, and stimulating the adaptive immune system (32). We found that NK cell lysis of HCC cells was significantly enhanced after treatment with 3 nmol/L Bortezomib, and the increase in NK cell cytotoxicity was critically dependent on NKG2D/NKG2DL interaction. Our data are seemingly in contrast to the results of Lundqvist and coworkers (33), who did not observe an up-regulation of MIC cell surface expression on a renal cell carcinoma line by Bortezomib. However, these authors observed a stimulation of NK cell cytotoxicity against Bortezomib-treated tumor cell lines only in about half of the various tumor lines investigated in their study, which did not include HCC-derived cells. In addition, they determined MIC surface expression early after 18 hours of treatment, which might explain the discrepancy to our data obtained at later time points. Our findings regarding the effect of Bortezomib-induced NKG2DL expression for NK cell cytotoxicity are mirrored by our observation that increased MIC protein expression also stimulated the production of IFN-γ,the second major antitumor effector function of NK cells.

Theoretically, an induction of MIC proteins in healthy, nonmalignant liver cells could interfere with a potential NK cell–based antitumor effect of Bortezomib treatment. However, in contrast to hepatoma cells, we did not detect a relevant expression or up-regulation of MICA or MICB in PHH, suggesting that Bortezomib is able to specifically up-regulate "danger signals" for NK cells in malignant but not in healthy liver cells.

It needs to be pointed out that partially conflicting results regarding the effect of proteasome inhibitors on NK cell antitumor reactivity have been reported thus far: high doses of Bortezomib in the range of 650 to 3,250 nmol/L have been shown to inhibit NK cell killing of tumor cells (12), and in rats, several previous studies revealed that proteasome inhibitors can reduce the cytotoxicity of NK cells (34). On the other hand, treatment with Bortezomib and dendritic cells displayed substantial antitumor efficacy in a mouse model, which was dependent on the activity of cytotxic lymphocytes, suggesting that NK cells may be involved in the antitumor effects of Bortezomib (35). In addition, pretreatment with Bortezomib has been shown to sensitize tumor cells to TRAIL–mediated NK cell lysis (33), which constitutes one component of NK cell antitumor effector mechanisms. However, the initiation of NK cell reactivity requires recognition of "missing self" and/or "induced self," the latter being mediated, for example, by up-regulation of NKG2DL on tumor cells, which determines whether NK cell effector functions are initiated or not (13, 31). Our findings implicate that Bortezomib-induced MICA/B expression on hepatoma cells shifts the balance of activating and inhibitory signals for NK cells, favoring their antitumor reactivity.

We further show that low concentrations of Bortezomib do not impair NK cell cytotoxicity, which is in line with the data of Lundqvist et al. (33), whereas concentrations of 25 nmol/L and above displayed an inhibitory effect in agreement with the data of Markasz and coworkers (12). We further investigated IFN-γ production of NK cells and observed a strongly dose-dependent reduction of this cytokine. Although it seems surprising that the two effector functions of NK cells are differentially affected by proteasome inhibition, it needs to be considered that cytotoxicity and cytokine production of NK cells are mediated by tightly regulated and at least partially independent signaling pathways involving various kinases, phosphatases, and transcription factors. Although an activation of the extracellular signal-regulated kinase signal cascade is crucial for NK cell–mediated lytic functions (36), the production of IFN-γ is influenced by diverse pathways leading to activation of transcription factors such as nuclear factor-κB or members of the signal transducers and activators of transcription (STAT) family (37). In addition, very little is yet known regarding the role of the proteasome,

especially its therapeutic modulation, in the regulation of NK cell effector functions. Therefore, it might well be that low doses of Bortezomib differentially affect cytotoxicity and IFN-γ production of NK cells. The underlying molecular mechanisms are presently subject of an ongoing study.

Our results indicate that the mechanisms of the antitumor activity of Bortezomib, which up to now have focused on the immediate effects on tumor cells, have to be extended to Bortezomib-induced stimulation of NK cell antitumor reactivity. Remarkably, both effects, the direct inhibition of tumor cell growth and immunostimulation, could be achieved by Bortezomib doses as low as 3 nmol/L. This is of importance because the available data suggest that Bortezomib treatment doses should be carefully considered with regard to therapeutic approaches. Although Bortezomib doses required for MIC molecule induction partially reduced NK cell IFN-γ production, NK cell cytotoxicity was not altered. Our results point to a differential effect of proteasome inhibition on NK cell cytotoxicity and cytokine production and suggest that Bortezomib, in addition to its direct effect on HCC cells, might be able to prime tumor cells *in vivo* for NK cell recognition and lysis. This opens up the possibility of an immunotherapy of HCC using adoptive NK cell transfer in combination with low-dose Bortezomib treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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