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Proteasome inhibitor lactacystin augments natural killer cell cytotoxicity of myeloma via downregulation of HLA class I

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ABSTRACT

Modulation of inhibitory and activating natural killer (NK) receptor ligands on tumor cells represents a promising therapeutic approach against cancer, including multiple myeloma (MM). Human leukocyte antigen (HLA) class I molecules, the NK cell inhibitory killer cell immunoglobulin-like receptor (KIR) ligands, are critical determinants of NK cell activity. Proteasome inhibitors have demonstrated significant anti-myeloma activity in MM patients. In this study, we evaluated the effect of proteasome inhibitors on the surface expression of class I in human MM cells. We found that proteasome inhibitors downregulated surface expression of class I in a dose- and time-dependent manner in MM cell line and patient MM cells. No significant changes in the expression of the MHC class I chain-related molecules (MIC) A/B and the UL16-binding proteins (ULBPs) 1–3 were observed. Downregulation of class I by lactacystin (LAC) significantly enhances NK cell-mediated lysis of MM. Furthermore, the downregulation degree of class I was associated with increased susceptibility of myeloma cells to NK cell killing. HLA blocking antibody produced results that were similar to the findings from proteasome inhibitor. Taken together, our data suggest that proteasome inhibitors, possible targeting inhibitory KIR ligand class I on tumor cells, may contribute to the activation of cytolytic effector NK cells in vitro, enhancing their anti-myeloma activity. Our findings provide a rationale for clinical evaluation of proteasome inhibitor, alone or in combination, as a novel approach to immunotherapy of MM.

1. Introduction

Natural killer (NK) cells play an important role as a first-line defense in the control of malignancies [1,2]. NK cell activity is regulated by a dynamic balance between inhibitory and activating receptors that recognize ligands (L) on target cells, with the inhibitory signals being dominant. NK cells recognize and kill tumor cells that lack or have altered human leukocyte antigen (HLA) class I expression [2]. Emerging evidence suggests that NK cells have potent antitumor effects on the setting of allogeneic (allo) hematopoietic cell transplantation (HCT) [3].

Multiple myeloma (MM) is a malignant neoplasm. Despite promising results with novel agents, such as immunomodulatory drugs and proteasome inhibitor (bortezomib), the majority of patients are incurable due to the persistence of drug resistant tumor cells. Recent studies both in vitro and in vivo have shown that chemo-resistant myeloma cells can be killed by killer cell immunoglobulin-like receptor (KIR) ligand mismatched NK cells from an allogeneic, haploidentical donor [4]. Unfortunately, not all NK cells transfused are allo-reactive. In fact, the majority of the NK cells are inhibited by patient class I, which is the principle inhibitory KIR Ligands for NK cells. In contrast, NK cells avidly lyse tumor cells that do not display such inhibitory KIR ligands. The lack of class I expression is usually associated with an increased susceptibility of target cells to NK cell cytotoxicity [5].

HLA class I molecules consist of a major histocompatibility complex (MHC)-encoded heavy chain, light chain β_2 microglobulin (β_2M), and a peptide. The proteasome is responsible for the generation of the majority of peptides presented by class I molecules.

Abbreviations: NK, natural killer; MM, multiple myeloma; HLA, human leukocyte antigen; KIR, killer cell immunoglobulin-like receptor; MIC, MHC class I chain-related molecules; ULBP, UL16-binding proteins; LAC, lactacystin; L, ligand; Allo, allogeneic; HCT, hematopoietic cell transplantation; MHC, major histocompatibility complex; M, microglobulin; TAP, transporters associated with antigen processing; LLnL, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; Ada, AdaAhx3L3VS; Ab, antibody; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; min, minutes; h, hour; MFI, mean fluorescence intensity; RT, room temperature; DAPI, 2,4-diamidino-2-phenylindole; E, effector; T, target.

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These peptides are transported into the endoplasmic reticulum from the cytosol by the transporters associated with antigen processing (TAP). The peptides that bind to the class I molecules trigger their release from TAP, allowing their transport to the cell surface. The class I molecules that are without a peptide are unstable and fall off the cell surface [6,7].

We hypothesized that proteasome inhibition would block the generation of peptides and consequently decrease class I expression at the cell surface. Lactacystin (LAC, a natural product isolated from streptomyces) has been shown to be a proteasome inhibitor [8]. In this study, we investigated the effect of proteasome inhibitors on the surface expression of class I in human MM cells. We also studied whether exposure to proteasome inhibitor LAC could render myeloma cells to be more susceptible to NK cell-mediated lysis and the possible triggering mechanism.

2. Materials and methods

2.1. Cell culture

OPM2 was purchased from American Type Culture Collection (Manassas, VA, USA). OPM2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 2 mmol/L glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. Peripheral blood samples and bone marrow samples were obtained from myeloma patients or healthy donors after informed consent. $CD56^+/CD3^-$ NK cells and $CD138^+$ myeloma cells were isolated from peripheral blood or bone marrow using magnetic bead selection (Miltenyi Biotec Shanghai Office, Shanghai, China). The purity of selected cells was more than 95%.

2.2. Reagents and antibodies

Bortezomib (Velcade, PS-341) was purchased from Xian–Janssen Pharmaceutical Ltd. (Beijing, China). LAC, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL), and AdaAhx3L3VS (Ada) were purchased from China Sigma Co., Ltd. Protein expression on the cell surface was examined by flow cytometry using FITC, PE or APC conjugated antibodies (Ab) to: CD3, CD56, CD138, MICA/B, ULBP1–3, annexin V (BD PharMingen, San Diego, CA), HLA-ABC, tumor necrosis factor related apoptosis-inducing ligand (TRAIL)-R1/DR4 and TRAILR-2/DR5 (eBiosciences, San Diego, CA). Anti-human TRAIL Ab (eBiosciences) was used at a concentration of 10 μg/mL to block NK cell surface TRAIL molecular. Propidium iodide (PI) was commercially from Sigma (China Sigma Co., Ltd.).

2.3. Acid treatment and flow cytometry

Cells were centrifuged and the resulting pellet was resuspended gently in 2 mL of 300 mM glycine (pH 2.5)/1% (w/v) BSA and incubated for 4 min at 37 °C. The suspension was neutralized by dilution with 100 mL of cell medium containing 0.5 N NaOH and 0.2 M HEPES and centrifuged. Cells post acid stripping were resuspended into 15 mL of cell medium in the presence or absence of LAC and incubated for 16 h at 37 °C to allow class I re-expression. All antibody staining for flow cytometry were done according to the following protocol. The cells were washed once with PBS and incubated with appropriate amounts of antibody at 4 °C for 30 min. Following the incubation, cells were washed twice, resuspended in 400 μl of FACS medium, and analyzed on a flow cytometer (BD Biosciences). Live cells were gated on PI and annexin V double negative cell, and the mean fluorescence intensity (MFI) was recorded. Class I decrease % = 100 × (MFI of control − MFI of treated cells)/MFI of control.

2.4. Immunofluorescence analysis by confocal microscopy

OPM2 cells were incubated with anti-human class I Ab (W6/32; Serotec, Oxford, UK) or mouse IgG control (1 μg/mL) for 1 h at room temperature (RT). The cells were then incubated with alexa fluor 594-conjugated anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) for 1 h at RT, followed by 2,4-diamidino-2-phenylindole (DAPI) staining for 15 min at RT and three PBS–Tween (1× PBS, 0.5% Tween 20) washes. Cells were then washed twice with ice-cold PBS, immobilized on polylysine-treated coverslips at 4 °C for 20 min and fixed with 4% paraformaldehyde. The fluorescence signal was detected with an inverted fluorescence microscope (Olympus), and the images were captured by a digital imaging system (Nikon). The plasma membrane of class I protein in OPM2 was visualized using a confocal microscope (LSM 510, Zeiss, USA).

2.5. Cytotoxicity assay

The cytotoxic capacity of NK cells against LAC treated and untreated cells was evaluated in vitro using a standard 4-h ^{51}Cr release assay. Target cells were labeled with 100 μ Ci (3.7 MBq) ^{51}Cr for 1 h at 37 °C, washed twice with PBS, and resuspended in 1 mL RPMI medium. A total of 2×10^4 target cells in 100 μL medium was placed in triplicates into V-bottom 96-well plates and incubated for 4 h with 100 μL effector (E) cells at appropriate concentrations to obtain effector:target (T) ratios from 1:1 to 10:1. Aliquots of supernatants were then counted using a Packard Cobra Auto-Gamma 5000 Series Counting System (Meriden, CT). The percentage of specific lysis was calculated as: percentage specific release = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

2.6. Statistical analysis

Values presented are the mean ± SD of at least three experiments. Comparison between two groups was performed using Student's *t*-test. *P* values of .05 or less were considered to be significant.

3. Results

3.1. Proteasome inhibitors decrease HLA class I surface expression on human myeloma cells

Proteasome inhibitors have been clinically used in MM for their strong anti-myeloma activities. To investigate the effect of proteasome inhibitors on the surface expression of class I on human MM cells, we initially performed a flow cytometric analysis on OPM2 cell line after treatment with LAC, LLnL, Ada and bortezomib. As shown in Fig. 1A, fluorescence histograms showed that OPM2 cells treated with all these tested inhibitors were able to downregulate the surface expression of class I in a dose-dependent manner. FACS analysis also revealed that LAC decreased class I expression in a time-dependent manner on OPM2. Immunofluorescence analysis was used to examine class I distribution on the plasma membrane of OPM2 before and after LAC treatment. Reduced class I expression was observed in OPM2 after LAC treatment (Fig. 1B). We then tested the effect of LAC on class I expression of patient MM cells. Decreased class I expression was found on patient myeloma cells post LAC treatment. Four micromolar of LAC downregulated surface level of class I by a mean of 49% ($n = 4$), compared to untreated control (Fig. 1C). Finally, we examined the effect of LAC on surface expression of other NK cell ligands, such as MICA/B, ULBP1–3, DR4 and DR5 of OPM2. We found that LAC treatment upregulated DR4 and DR5, but did not have any significant effect on MICA/B, and ULBP1–3 molecules (data not shown).

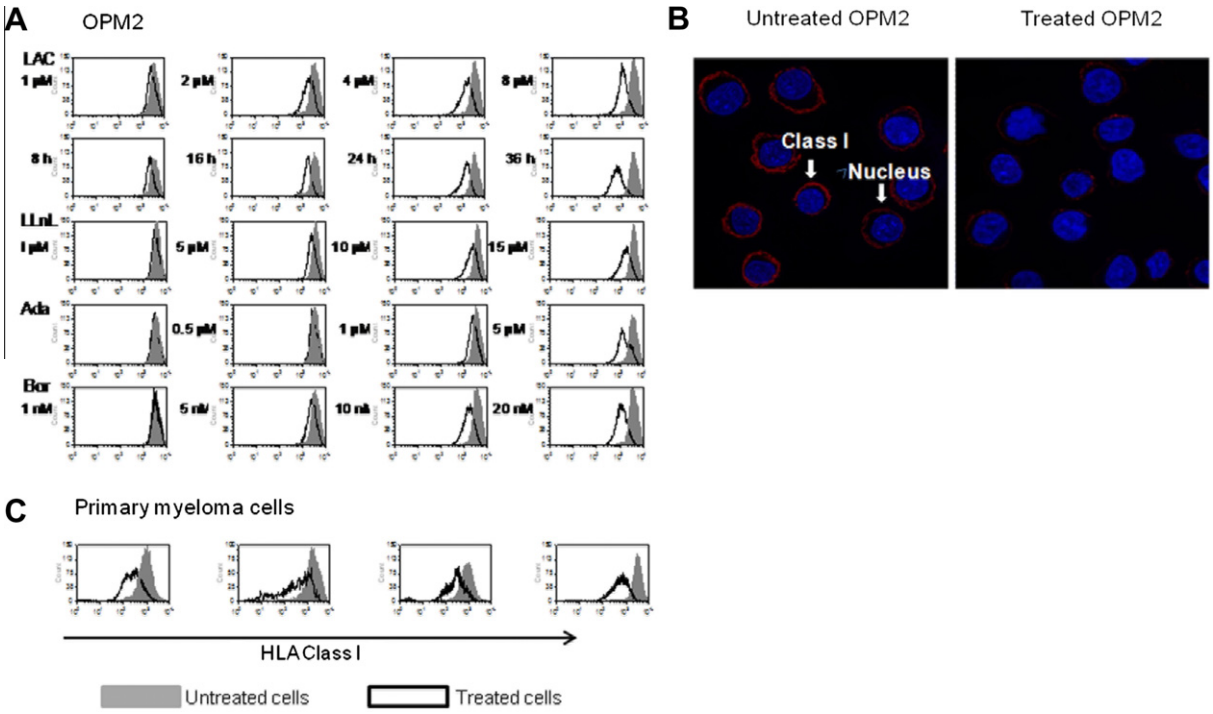

Fig. 1. Proteasome inhibitors downregulate surface expression of HLA class I on myeloma cells. (A) LAC, LLnL, Ada and bortezomib (Bor) decreased class I expression in a dose- and/or time-dependent manner on both OPM2 and patient MM cells. Cells were cultured in the medium with or without indicated concentrations of drugs for various times. Class I expression was measured by FACS analysis. (B) Using immunofluorescence analysis, reduced class I expression was observed in OPM2 after LAC treatment. The cells were treated with 4 μM LAC for 24 h. (C) LAC decreased class I expression in patient MM cells. Four patient MM cells were treated with 4 μM LAC for 24 h.

3.2. LAC inhibits surface re-expression of newly synthesized class I in OPM2 cells after acid treatment

Treatment of cells with acid makes it possible to selectively measure the surface expression of newly generated class I complexes. To examine whether LAC affects cell surface re-expression of class I, OPM2 cells were treated with glycine (pH 2.5) to remove HLA class I complexes from the cell surface. We then measured the surface expression of newly generated class I in the presence or absence of LAC for 16 h using flow cytometry. Immediately after glycine treatment, the expression of class I (MFI = 139) was nearly undetectable, compared to an untreated control (MFI = 3029). In the absence of LAC, the cells re-expressed class I (MFI = 931). Exposure to LAC for 16 h inhibited class I re-expression (MFI = 464) in OPM2 (Fig. 2). These data suggest that LAC blocks re-expression of newly synthesized class I in myeloma cells.

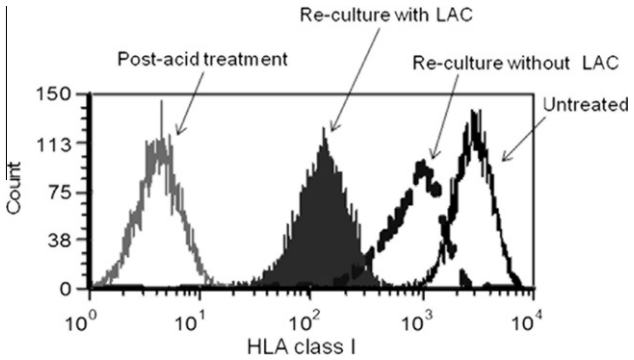

Fig. 2. LAC blocks surface re-expression of newly generated class I in OPM2 after acid treatment. Cells were treated with glycine (pH 2.5) as described in materials and methods, and then incubated for 16 h in the absence or presence of 4 μM LAC. Expression of class I on OPM2 was analyzed by flow cytometry.

3.3. LAC treatment enhances NK cell-mediated lysis of myeloma cells

Because changes in the expression of inhibitory KIR ligand class I in tumor cells can affect the activation of NK cells, we tested whether LAC treatment on myeloma cells could lead to an increased NK cell-mediated cytolysis of MM cells. The cells were cultured in medium with 4 μM LAC for 24 h. Treated and untreated cells were then used as targets at various E/T ratio in ^{51}Cr release assays. LAC treatment of OPM2 greatly increased its sensitivity to NK cell-mediated lysis, compared to untreated control [specific lysis (mean ± SD): 64.7 ± 3.8% vs. 33.3 ± 4.5%, $p < 0.05$] at E:T ratio of 10:1 (Fig. 3A). This suggests that exposure to LAC was able to significantly enhance the sensitivity of OPM2 to healthy donor NK cell killing. Similar results were obtained using patient MM cells as targets. Primary MM cells exposed to 4 μM LAC for 24 h significantly augmented the sensitivity of tumor cells to NK cell-mediated killing, compared to untreated patient MM cells (specific lysis: 42.5 ± 5.1% vs. 15.4 ± 3.5%, $n = 4$, $p < 0.05$) at E:T ratio of 10:1 (Fig. 3B). These results indicated that proteasome inhibitor has the ability to make the patient myeloma cells more susceptibility to NK cell lysis.

3.4. Augmented NK cell cytotoxicity against LAC treated myeloma cells correlates with decreased expression of HLA class I

To elucidate the underlying triggering mechanisms, we further investigated whether downregulation of class I on myeloma cells by proteasome inhibitor might be associated with increased susceptibility of tumor cells to NK cell-mediated lysis. OPM2 cells were treated with LAC at various concentrations for 24 h and then subjected to cytotoxicity assays. A remarkable decrease of class I expressions was observed in OPM2 cells that were exposed to the drug (Fig. 1A). The degree of class I downregulation in OPM2

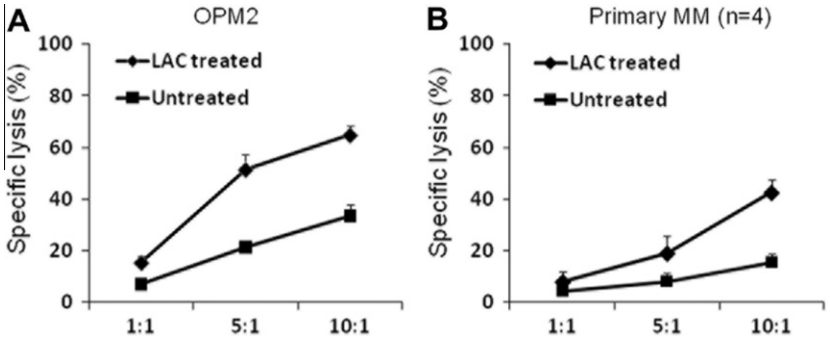

Fig. 3. LAC treatment renders MM cells to be more sensitive to NK cell cytolysis. (A) LAC treatment enhanced NK cell-mediated lysis of OPM2 cells. OPM2 cells were cultured in the medium with and without 4 μM LAC for 24 h. Treated and untreated cells were then used in cytotoxicity assays. (B) LAC treatment augmented NK cell killing of patient MM cells. Four patient MM cells were treated with and without LAC for 24 h. Target cells were placed in triplicates into V-bottom 96-well plates. NK cells from healthy donors were used as effectors. Data were presented as means (±SD).

was correlated with an increased sensitivity to NK cell-mediated cytolysis (Fig. 4A). Two microliters of LAC downregulated class I expression by 31% and therefore enhanced the susceptibility of OPM2 cells to lysis by NK cells, increasing their killing up to 24.3 ± 2.5%. LAC at 4 μM for 24 h downregulated class I by 45% and also induced a higher level of susceptibility to lysis when compared to 2 μM LAC treatment. Furthermore, treating OPM2 cells with class I blocking antibody exhibited similar results with this drug. Treatment with 4 μM LAC downregulated class I by approximately 55% in OPM2, therefore increasing NK lysis. Blocking 58% of class I with class I-specific antibody resulted in an increase in NK lysis that was almost identical to the increase obtained by 4 μM LAC treatment (Fig. 4B). We have shown that LAC treatment did not change the expression of MICA/B, and ULBP1–3 in OPM2.

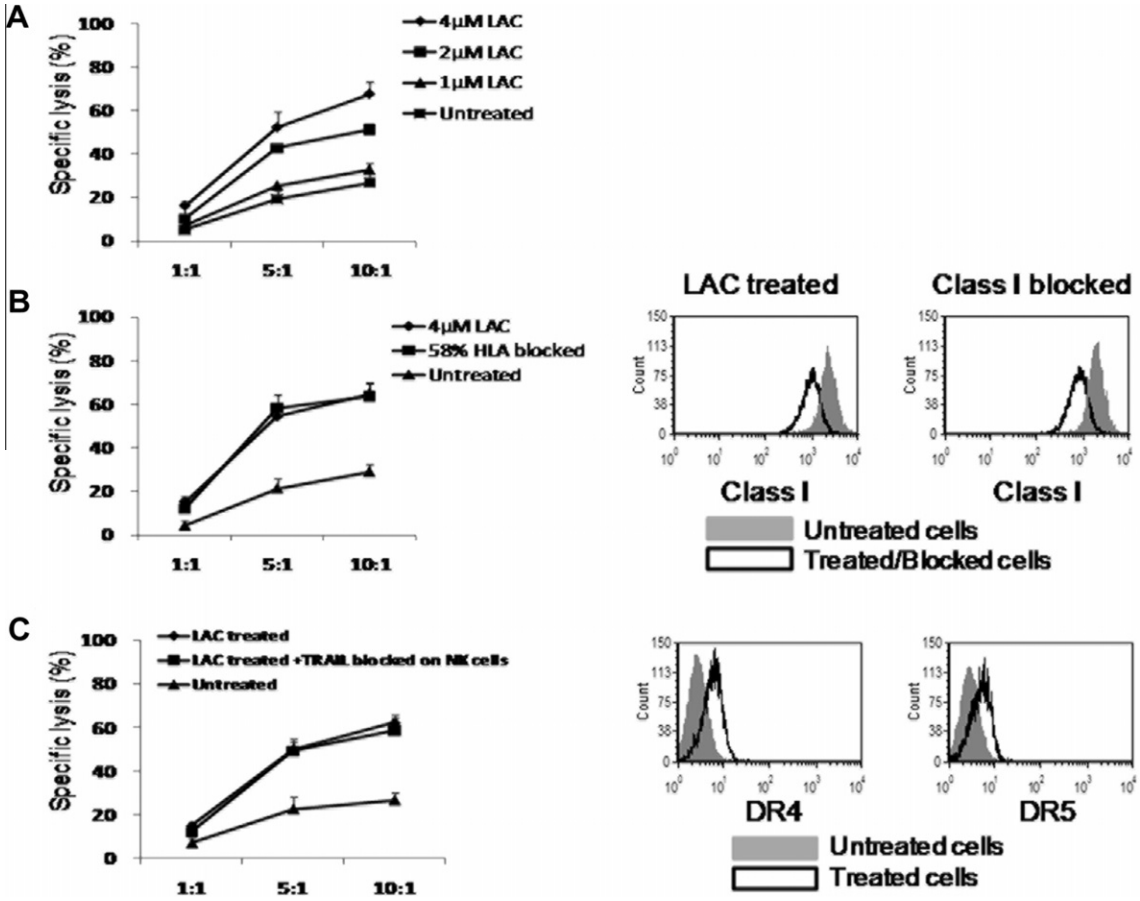

Fig. 4. Decreased level of class I by LAC is associated with enhanced NK cell cytotoxicity against LAC treated myeloma cells. (A) The degree of class I downregulation in OPM2 was correlated with their enhanced sensitivity to NK cell-mediated cytolysis. OPM2 cells were treated with indicated concentrations of LAC for 24 h. Untreated and treated cells were then used in cytotoxicity assay. (B) Blocking class I with the specific antibody in OPM2 produced the similar results with OPM2 after LAC treatment. 4 μM LAC treatment downregulated class I by 55% in OPM2. 0.5 μg/mL anti-class I Ab treatment of OPM2 reduced 58% class I expression. (C) Blocking TRAIL in NK cells did not change the sensitivity of treated OPM2 to NK cell killing. LAC treatment upregulated expression of DR4 and DR5 in OPM2. OPM2 cells were cultured in the medium with and without 4 μM LAC for 24 h, and then used in cytotoxicity assays. NK cells blocked by anti-TRAIL Ab were used as effectors. Targets were placed in triplicates into V-bottom 96-well plates. Data were presented as means (±SD).

Although we observed a notable upregulated expression of DR4 and DR5 in OPM2 post 4 μM LAC treatment, blocking TRAIL on NK cells did not diminish the sensitivity of treated OPM2 to NK cell killing (Fig. 4C). Taken together, these data indicate that the reduction of class I by proteasome inhibition may be as a possible mechanism for augmenting NK cell activity in human myeloma.

4. Discussion

Proteasomes play a critical role in cellular homeostasis through its function in ubiquitin-dependent protein turnover of target proteins that regulate signal transduction, cell cycle progression and apoptosis. More importantly, cancer cells appear to be particularly dependent on these proteasome-regulated homeostatic pathways [9]. Bortezomib, a drug that targets proteasomes, has shown strong anti-myeloma activity in the treatment of MM. The use of bortezomib has dramatically improved survival for MM patients. Preclinical and clinical studies with bortezomib and other proteasome inhibitors, including carfilzomib (PR-171) [10], salinosporamide (NPI-0052) [11] and CEP18770 [12], also have established proteasomes as a therapeutic target in patients with mantle cell lymphoma and other select subtypes of non-Hodgkin's lymphoma. New proteasome inhibitors with enhanced specificity and efficiency were designed and assessed in clinical trials, which might open the door to new drug candidates for treating a range of cancer. Although these drugs differ somewhat from each other chemically, proteasome specificity, the ultimate target of proteasome inhibition, is the same among all [13].

Proteasomes are also involved in producing peptides, which are used by the immune system and presented as antigenic peptides at the cell surface by class I molecules. The inhibition of proteasomes will block the generation of peptides from cytoplasmic proteins and prevent the subsequent conjunction of peptides and class I, resulting in reduced class I expression on the cell surface [14]. LAC has been shown to be an effective proteasome inhibitor [8]. In this study, we investigated the effect of proteasome inhibitors on the surface expression of class I in myeloma cells. We found that proteasome inhibition by LAC was able to decrease class I in a dose- and time-dependent manner in myeloma cell line as measured by FACS. The reduced expression of class I in MM cell line after treatment was confirmed by immunofluorescence analysis. LAC also downregulated class I expression on patient MM cells. Furthermore, class I expression reduction was observed when the cells were treated with other well-characterized proteasome inhibitors LLnL, Ada and bortezomib. Expression of class I decreased in myeloma cells exposed to proteasome inhibitors in vitro, implying that the proteasome is responsible for the stability of cell surface expression of class I by generating class I bounding peptide. In agreement with our findings, previous studies showed that the generation of stable heterodimers of class I molecules was markedly inhibited in cells treated with proteasome inhibitors [4,6,7,15,16].

It has been demonstrated that transplantation across HLA class I barriers can trigger allo-reactive NK cell responses and graft-versus-leukemia effects in allo-HCT. The exciting results obtained in haploidentical transplantation have prompted attempts to exploit NK cell alloreactivity as immunotherapies of human cancer [17]. However, the beneficial clinical effect of NK cell alloreactivity has not been uniformly demonstrated [18,19]. Human NK cells possess clonally-distributed inhibitory receptors that recognize allotypic determinants (KIR ligands) shared by certain groups of HLA class I alleles [20,21]. HLA class I molecules are critical determinants of NK cell activity. NK cells effectively lyse cells lacking expression of some or all HLA molecules [2]. Cells that lack expression of HLA molecules fail to deliver inhibitory signals to NK cells and therefore promote cytolytic activity of NK cells. Modulation of NK cell recognition of tumor cells by downregulating cell surface inhibitory ligand class I may augment NK cell activity. Here we reported that proteasome inhibition induced downregulation of class I, resulting in reduced NK inhibitory KIR ligand on tumor cells, and then enhanced NK cell-mediated killing. Furthermore, we described that decreased expression of class I on myeloma cells was associated with enhanced NK cell killing of tumor cells. HLA blocking antibody produced results that were similar to the findings from proteasome inhibitor. Although LAC treatment induced elevated expression of DR4 and DR5 on OPM2, NK cell function study showed that blocking TRAIL on NK cells did not decrease the sensitivity of treated OPM2 cells to NK cell killing. Furthermore, LAC treatment had no effect on expression of MICA/B and ULBP1–3 on OPM2 cells. These data suggest that proteasome inhibition may downregulate expression of inhibitory KIR ligand class I on tumor cells, thus facilitating NK cell activation and enhancing the eradication of drug treated tumor cells. It is postulated that proteasome inhibitors may play an important role in potentiating the antitumor activity of allogeneic KIR ligand mismatched NK cells. Proteasome inhibitor alone or in combination with other approaches may help maximize the potential of NK cell for the treatment of MM.

In summary, this study provides that proteasome inhibition downregulates HLA class I expression on myeloma cells in a dose- and time-dependent manner and significantly enhances the sensitivity of myeloma cells to NK cell-mediated lysis. We also demonstrate that the degree of reduction in HLA class I expression correlated with increasing susceptibility to lysis by NK cells. Taken together, we conclude that proteasome inhibitor may be used as an immunomodulating agent by modulating the NK-receptor ligand repertoire on targets and sensitizing chemo-resistant tumor cells to NK cell-mediated killing. Furthermore, the feasibility of using proteasome inhibitor to potentially optimize the NK cell therapy for the management of MM patients, particularly aiming at the eradication of drug resistant tumor cells, is worthy of further exploration in order to verify its clinical potential.

Acknowledgments

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