Abstract. Natural killer (NK) cells recognize stress-activated NK group 2, member D (NKG2D) ligands in tumors. In the present study, the expression levels of NKG2D ligands were examined in four lung cancer cell lines (A549, PLA801D, NCI-H157 and NCI-H520). In the A549 cells, the expression of MHC class I polypeptide-related sequence (MIC)A/B and UL16 binding protein (ULBP)1 was weak, the expression of ULBP2 was typical, and neither ULBP3 nor ULBP4 were expressed. The mechanism underlying the regulatory effect of a cancer treatment agent on the expression of NKG2D ligands was investigated using the proteasome inhibitor MG132. Following treatment for 8 h with MG132, the transcription levels of MICB and ULBP1 were upregulated 10.62- and 11.09-fold, respectively, and the expression levels of MICB and ULBP1 were increased by 68.18 and 23.65%, respectively. Notably, MICB exhibited significant time-dependent change. MG132 increased the transcription of MICB by acting at a site in the 480-bp MICB upstream promoter. The activity of the MICB promoter was upregulated 1.77-fold following treatment with MG132. MG132 treatment improved the cytotoxicity of NK cells, which was partially blocked by an antibody targeting NKG2D, and more specifically the MICB molecule. The expression of MICB induced by MG132 was inhibited by KU-55933 [ataxia telangiectasia mutated (ATM) kinase inhibitor], wortmannin (phosphoinositide 3 kinase inhibitor) and caffeine (ATM/ATM-Rad3-related inhibitor). The phosphorylation of checkpoint kinase 2 (Chk2), an event associated with DNA damage, was observed following treatment with MG132. These results indicated that MG132 selectively upregulates the expression of MICB in A549 cells, and increases the NKG2D-mediated cytotoxicity of NK cells. The regulatory effect of MG132 may be associated with the activation of Chk2, an event associated with DNA damage. The combination of MG132 with NK cell immunotherapy may have a synergistic effect that improves the therapeutic effect of lung cancer treatment.

Introduction

Human natural killer (NK) cells are a subpopulation of lymphocytes that are important in innate immunity, adaptive immunity and reproduction (1). NK cells are major effector cells of the innate immune system, being important in tumor immunity, antiviral infection and the removal of ‘non-self’ cells (2-4). The T cell receptor is a specific marker on the surface of all T cells which recognizes specific antigens and exerts an immunological function. The difference between T cells and NK cells is that the latter are not dependent on the MHC-I molecules of tumor cells to initiate a toxic effect and their activity is the result of the interaction of various receptor molecules with corresponding ligands (5,6). The interaction between the NK cell lectin-like receptor, NK group 2, member D; NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ULBP, UL16 binding protein

Key words: MG132, MHC class I polypeptide-related sequence B, natural killer cells, immunotherapy, chemotherapy
(ULBP)1-5 (10). NKG2D ligands are expressed on the surface of tumor cells and MICs are induced cell surface antigens (11,12). Previous studies have revealed that numerous factors, including cell stress, heat shock, infection, DNA damage and transformation, can upregulate the expression of MICs (13-18).

Previous studies have demonstrated that specific MHC-restricted cytotoxic T cells cannot function when MHC-I molecules on tumor cells are lost or vary from the norm. In this case, the NKG2D-NKG2D ligand signaling pathway is important in antitumor immunity, with NKG2D as the main activating receptor of NK cells that induces anti-tumor effects (19,20). Following the interaction between NKG2D and corresponding induced ligands on target cells, NKG2D binds to an adaptor protein and transmits an activated signal to NK cells. In this way, NK cells obtain the ability to lyse the target cells (21). In addition, CD8+ T cells provide the necessary synergistic stimulating signals, and the activation of the NKG2D pathway largely determines the intensity of the cell's immune response against cancer.

At present, NK cell-based immunotherapy combined with chemotherapy is widely used clinically; however, the mechanism by which chemotherapy regulates the antitumor activity of NK cells, particularly NKG2D-mediated cell death, remains to be fully elucidated to the best of our knowledge. Although the coding regions of NKG2D ligands are conservative, the homology of the 5' non-translated region is low, which indicates that the regulation of their expression may be specific to different stimuli or lesions.

Therefore, the A549 cell line was used as the cell model in the present study in order to investigate the regulatory effect of a cancer treatment agent (the proteasome inhibitor MG132) on NKG2D ligands and the susceptibility of A549 cells to NK lysis. The possible regulatory mechanisms underlying the DNA damage response pathway were also examined.

Materials and methods

Cell culture and reagents. The A549 (catalog no. 3111CC0001CCC00002), NCI-H520 (catalog no. 3111CC0001CCC000197) and NCI-H157 (catalog no. 3111CC0001CCC000113) lung cancer cell lines were purchased from Peking Union Culture Collection (Beijing, China). The PLA801D lung cancer cell line (catalog no. CBP61005) was purchased from Nanjing Cobeioer Biotechnology Corporation (Nanjing, China). The cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Logan, UT, USA) cultured at 37°C for 24 h, following which they were treated with 10% FBS and 10% horse serum (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells (5x10⁴) were cultured at 37°C for 24 h, following which they were treated with 10 µM MG132 at 37°C for 8 h and then lysed (Promega Corporation, Madison, WI, USA). Luciferase and Renilla activity were measured as previously described (22).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (23). RT of 2 µg (20 µl) RNA into cDNA was performed using PrimeScript™ Reverse Transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). Luciferase and Renilla (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo version 7.6 software (FlowJo LLC, Ashland, OR, USA).

Promoter analysis. The genomic DNA of the A549 cells was isolated and the full-length MICB promoter was amplified using polymerase chain reaction (PCR). The promoter fragments were cloned into the pGL3 luciferase reporter plasmid. The A549 cells were transfected with 0.79 µg MICB promoter plasmid and 0.01 µg SV40-Revilla plasmid using Lipofectamine® 2000 and Plus reagents (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells (5x10⁴) were cultured at 37°C for 24 h, following which they were treated with 10 µM MG132 at 37°C for 8 h and then lysed (Promega Corporation, Madison, WI, USA). Luciferase and Renilla activity were measured as previously described (22).

Treatment of the cells with the inhibitors. A549 cells were treated with KU-55933 (10 µM) for 9 h or wortmannin (6 µM) or caffeine (10 mM) for 10 h, or with KU-55933 for 9 h or wortmannin or caffeine for 10 h, with the addition of MG132 during the final 8 h at 37°C. Then cells were harvested at 300 x g at room temperature for 5 min.

Flow cytometry. The cells were harvested, washed three times with PBS, and adjusted to a concentration of 5x10⁵ cells/ml with PBS. The cell suspensions (200 µl) were added to labeled tubes and stained with mouse anti-human MICA, MICB, ULBP1, ULBP2, ULBP3 and ULBP4 antibodies (1:200) or an isotype antibody for 30 min at 4°C, followed by incubation with goat anti-mouse IgG in the dark. Following incubation at 4°C for 30 min, the cells were washed three times in PBS. The samples were examined using FACScalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo version 7.6 software (FlowJo LLC, Ashland, OR, USA).

Luciferase and Renilla activity were measured as previously described (22).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (23). RT of 2 µg (20 µl) RNA into cDNA was performed using PrimeScript™ Reverse Transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). Luciferase and Renilla (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo version 7.6 software (FlowJo LLC, Ashland, OR, USA).
CTCCTCTTTTG-3' and downstream 5'-CGGAATTCTG
AGGGAAATGCTGCC-3'; and β-actin, upstream 5'-ATC
ATGTTTTAGACCTTCAACA-3' and downstream 5'-CAT
CTCTTGCTCGAAGTC-3'. The percentage change was
calculated using the following formula: 2^△△Cq (24).

**Cytotoxicity assays.** The cytotoxicity of the NK cells was
measured using a standard 51Cr-release assay (25). Briefly,
the target tumor cells were incubated for 1 h with 150 µCi
51Cr (PerkinElmer, Inc., Waltham, MA, USA) at 37°C in
5% CO2. The cells were then washed three times with media
and incubated for an additional 30 min. In order to detect the
differential lysis effect of different effector to target cell ratios,
labeled target cells (1x10^6 cells/well) were incubated with
effector cells in 96-well plates in 1% FCS-RPMI-1640 at a
total volume of 200 µl. The plates were centrifuged at 300 x g
at 37°C for 5 min following incubation for 4 h. Aliquots
(100 µl) of the supernatants from each well were transferred to
a new plate containing 100 µl/well of Optiphase Supermix
scintillation fluid. The NK cells were pre-incubated at 37°C
for 1 h with NKG2D antibodies (dilution 1:500) for antibody
blocking experiments. Radioactivity was measured using a
gamma counter. The percentage of cytotoxicity was calcu-
lated according to the following formula: 100x(experimental
release-spontaneous release)/(maximum release-spontaneous
release). Maximum release was determined by the addition of
100 µl 10% Triton X-100 and spontaneous release was deter-
imined by incubating the targets with 100 µl complete media.

**Comet assay.** The alkaline comet method of Singh et al (26)
was followed with minor differences, and the application steps
described. The cells were harvested following treatment with
10 µM MG132 for 8 h. The slides were pre-coated with 1%
regular agarose. A low-melting-point agarose (0.65%) suspen-
sion was added to the cell suspension at a ratio of 4:1 and the suspension was immediately transferred onto the slides. The
cells on the slides were lysed with ice-cold high-salt lysis
buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1%
Triton X-100 and 10% DMSO) in the dark at 4°C for 1 h for
disintegration of the cell and nuclear membranes. Following
the lysis phase, the slides were placed in an electrophoresis
tank with electrophoresis buffer (pH 10.0) and incubated in the
dark and 4°C for 30 min. Electrophoresis was then performed
at 25 V for 20 min. Subsequently, the slides were washed twice
for 5 min with neutralization buffer and allowed to air-dry
until analysis. All samples were evaluated within 24 h and
were not subjected to fixation. The samples were stained with
25 µg/ml propidium iodide and then visualized under a fluo-
rescence microscope (Leica Microsystems GmbH, Wetzlar,
Germany) and analyzed with CASP Lab version 1.2.2 software
(University of Wroclaw, Wroclaw, Poland) (27).

**Western blotting.** Tumor cells were collected, washed three
times with PBS and then lysed with RIPA buffer (Beyotime
Institute of Biotechnology, Shanghai, China) for 30 min at 4°C.
Subsequently, the suspension was centrifuged at 16,000 x g
for 15 min at 4°C. The concentration of proteins was detected
using a BCA assay kit (Sigma-Aldrich; Merck KGaA). Equal
quantities (30 µg) of protein were separated using
SDS-PAGE (10% gels) and transferred onto polyvinylidene
fluoride membranes under 100 V for 1 h. The membranes
were blocked using 5% non-fat dry milk for 1 h at room
temperature. Subsequently, the membranes were blotted with
an appropriate primary antibody (Anti-Chk2, dilution 1:1,000,
catalog no. 2662 and p-Chk2, dilution 1:1,000, catalog no. 2661,
both Cell Signaling Technology, Inc.) overnight at 4°C. The
membranes were washed with TBST (TBS, pH 7.5, containing
0.05% Tween-20) and then were incubated with horseradish
peroxidase-conjugated secondary antibodies (1:1,500) at room
temperature for 1 h. The blots were visualized using ECL
reagents (Beyotime Institute of Biotechnology).

**Statistical analysis.** All results presented are representative
of a minimum of three experiments. Statistical comparisons
between groups were made using Student's t-test or one-way
analysis of variance followed by NewmanKeul's post hoc test.
P<0.05 was considered to indicate a statistically significant
difference. Statistical analysis was calculated using GraphPad,
version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

A restricted set of NKG2D ligands are expressed in the A549
cell line. The expression levels of cell surface NKG2D ligands
were measured in the lung cancer cell lines using flow cytometry
(Fig. 1). The results demonstrated that the expression levels of
NKG2D ligands in PLA801D, NCI-H520, NCI-H157 and A549
cells were different (Table I). In the A549 cells, MICA/B and
ULBP1 were weakly expressed, ULBP2 showed typical expres-
sion, and neither ULBP3 nor ULBP4 were expressed (Table I).

NKG2D ligand expression is selectively induced by MG132.
To investigate whether the cancer treatment agent MG132
affects NKG2D ligands, the expression levels of NKG2D
ligands were measured following treatment with MG132 (22).
Following treatment with MG132 for 8 h, the transcription
levels of MICB and ULBP1 were upregulated by 10.62- and
11.09-fold, respectively (Fig. 2A), and the surface expression
levels of MICB and ULBP1 were increased by 68.18 and
23.65%, respectively (Fig. 2B and C). Among the ligands,
the expression of MICB exhibited the most marked change.
Following treatment with MG132, the mRNA levels of MICB
increased linearly between 0 and 8 h and slowly increased after
8 h (Fig. 3A). In addition, following stimulation with MG132,
MICB was not detectable between 2 and 8 h; however, the
expression of MICB rapidly increased between 4 and 8 h and
then slowly increased after 8 h (Fig. 3B), which indicated that
the MG132-induced expression of MICB is time-dependent.

MICB promoter is activated by a proteasome inhibitor.
The proteasome inhibitor induced the transcription of MICB,
whether this change is initiated at the MICB promoter was
subsequently investigated. Promoter fragments of MICB,
from the MICB translation start site ATG to 480-bp upstream,
were cloned into the luciferase reporter vector pGL3 (28).
The A549 cells transfected with the pGL3-luciferase vector
and incubated with MG132 for 8 h prior to harvesting exhib-
ted increased luciferase activity and a 1.77-fold increase in
promoter activity, as corrected for transfection efficiency using
the co-transfected pRL-SV40 (Renilla luciferase) plasmid.
These results indicate that MG132 increases the transcription of MICB and increases the activity of the MICB promoter.

NKG2D-mediated tumor cell lysis is enhanced by the upregulation of MICB. To determine the effect of increased levels of NKG2D ligands in tumor cells, the cytotoxicity of NK cells against A549 cells pretreated with MG132 was measured. As shown in Fig. 4, MG132 significantly increased the susceptibility of the A549 cell line to cytolysis by NK cells. When the NKG2D-NKG2D ligand interactions were blocked with anti-NKG2D antibody, the lysis of A549 cells treated with MG132 was markedly reduced (Fig. 4A). The increased lysis of the MG132-treated cells was partially blocked by the MICB antibody (Fig. 4B). These results indicate that the interaction between NKG2D and its ligands is important in the NK-mediated lysis of the A549 cell line, and that the increased susceptibility of MG132-treated cancer cells to the cytotoxicity of NK cells may be mediated by upregulation of the NKG2D ligand MICB.

MG132 induces DNA damage in A549 cells. Previous studies have demonstrated that genotoxic agents that activate the DNA damage response pathway are responsible for the upregulation of NKG2D ligand expression in numerous tumor cell lines (13,22,23). Several of the chemotherapeutic drugs used clinically have the ability to induce the activation of ATM. Therefore, it was hypothesized that the MG132-induced upregulation of MICB in A549 cells may be dependent on activation of the DNA damage response pathway. Following MG132 treatment, the results produced a ‘comet tail’ in the comet assay, which indicates DNA strand breakage (Fig. 5A-C).

Chk2 is activated by MG132 in A549 cells. Numerous types of cancer cell, including A549 cells, exhibit defective DNA repair mechanisms. Chk2 autophosphorylation at Thr68 is a key early signaling event in the DNA damage response cascade (22,29). Therefore, whether Chk2 was functionally activated in MG132-treated A549 cells was investigated in the present study. The A549 cells were treated with 10 µM MG132 for 8 h and lysed, following which the phosphorylation of Chk2 at Thr68 was determined.

Table I. Mean fluorescence intensity of NK group 2, member D ligands on non-small cell lung cancer cells.

| Cells       | MICA | MICB | ULBP1 | ULBP2 | ULBP3 | ULBP4 |
|-------------|------|------|-------|-------|-------|-------|
| A549        | 1.34 | 1.27 | 1.22  | 1.80  | 0.95  | 1.03  |
| PLA801D     | 1.77 | 1.49 | 2.11  | 1.14  | 1.47  | 1.04  |
| NCI-H157    | 1.03 | 3.83 | 1.10  | 1.92  | 1.10  | 1.04  |
| NCI-H520    | 1.76 | 1.14 | 2.04  | 2.79  | 2.99  | 1.06  |

MIC, MHC class I polypeptide-related sequence A; ULBP, UL16 binding protein.
was measured using western blots. The results demonstrated that the phosphorylation of Chk2 at Thr68 was induced by 10 μM MG132 (Fig. 6). Although other aspects of the DNA damage response pathway have not been excluded, these results indicate that the autophosphorylation of Chk2 is involved in the increased expression of MICB induced by MG132.

MG132-induced expression of MICB is eliminated following treatment with KU-55933 (ATM kinase inhibitor), wortmannin [phosphoinositide 3 (PI3) kinase inhibitor] and caffeine (ATM/R inhibitor). Gasser et al (30) demonstrated that the expression of NKG2D ligands is induced by ATM/ATM-Rad3-related (ATR) signaling in the DNA damage response pathway and that induction is prevented by ATM/ATR inhibitors, including caffeine. Therefore, whether the ATM/ATR inhibitors KU-55933, wortmannin and caffeine can prevent drug-induced MICB transcription was investigated in the present study. Treatment with KU-55933, wortmannin

Figure 2. MG132 selectively induces the expression of NKG2D ligands. A549 cells were incubated with 10 μM MG132 for 8 h, and then (A) the mRNA expression of NKG2D ligands was detected using reverse transcription-quantitative polymerase chain reaction analysis and the (B) cell surface expression of NKG2D ligands was assessed via (C) flow cytometry. Data are representative of three independent experiments. Comparisons of two groups was performed with Student's t-test. *P<0.05. NKG2D, NK group 2, member D; Con, control.

Figure 3. MG132 induces the expression of MICB and increases MICB promoter activity in A549 cells. (A) A549 cells were treated with DMSO solvent or MG132 for the durations indicated, followed by cell lysis and analysis of the mRNA expression of MICB. (B) Anti-MICB monoclonal antibody staining of A549 cells treated with MG132 for the durations indicated on a logarithmic scale. Expression of MICB at 8 h is at the top. (C) A549 cells were transfected with the indicated pGL3-luciferase plasmids. The co-transfected pRL-TK plasmid was used as a control for transfection efficiency. MICB promoter pGL3-luciferase activity was assessed. At 32 h post-transfection, the cells were cultured with DMSO or MG132 for an additional 8 h followed by lysis. The histogram shows the relative increase in activity. Comparison of two groups was performed using Student's t-test. Multiple comparisons were performed with one-way analysis of variance. *P<0.05, **P<0.01 and ***P<0.001. MIC, MHC class I polypeptide-related sequence; Con, control.
and caffeine inhibited the MG132-induced upregulation of MICB (Fig. 7A). Consistent with the RT-qPCR results, the flow cytometry revealed a similar trend (Fig. 7B). These results indicate that the ATM/ATR signaling pathway is a possible mechanism by which MG132 induces the expression of MICB.

Discussion

In experimental animals and patients with cancer, the expression of tumor NKG2D ligands is associated with tumor eradication and survival rate (22). The expression levels of NKG2D ligands are increased in tumor cells compared with those in the surrounding normal tissue (21), which can be induced further by cancer treatment agents (30,31). Therefore, effective cancer treatments may directly damage tumor cells and induce the expression of NKG2D ligands, causing NK cell attack. In the present study, the expression levels of NKG2D ligands in A549 cells and other lung cancer cell lines, including PLA801D, NCI-H520 and NCI-H157, were detected. The results demonstrated that different lung cancer cell lines express different NKG2D ligands and have different levels of sensitivity to NKG2D-mediated cell death by NK cells. The regulation of NKG2D ligands in human A549 cells, a tumor system that remains to be fully elucidated, was investigated. The regulation of NKG2D ligands by the proteasome inhibitor MG132 and the susceptibility of A549 cells to NK lysis were also investigated. The possible regulatory mechanisms involved were examined, focusing on the role of the DNA damage response pathway.
Proteasome inhibitors have attracted interest as a novel type of antitumor drug. MG132 is an aldosterone inhibitor that reversibly inhibits proteasome chymotrypsin-like activity, inhibiting its degradation of protein substrates, inhibiting tumor cell growth and inducing apoptosis. MG132 is a potential therapeutic and preventive agent for cancer cachexia (32,33). Butler et al (22) reported that proteasome inhibitors that regulate NKG2D ligands are multifaceted, multilevel and can selectively upregulate expression of the ULBP1 promoter, and mRNA and cell surface proteins in head and neck squamous cell carcinoma. As the role of MG132 is complicated, the regulation of MICB may also be multilinked and multilevel. Therefore, the effects of MG132 on the promoter activity, mRNA expression and cell surface expression of MICB were investigated in the present study. Whether MG132 can cause DNA damage and activate key molecules in the DNA damage response pathway were also examined. The results demonstrated that MG132 upregulated the activity of the MICB promoter, mRNA expression and cell surface protein expression. Additionally, MG132 induced DNA single-strand breaks and activated Chk2 molecules. KU-55933 (ATM kinase inhibitor), wortmannin (PI3 kinase inhibitor) and caffeine (ATM/R inhibitor) inhibited the expression of MICB that was induced by MG132. These results indicate that DNA damage induced by MG132 activates protein kinase molecules, including ATM and Chk2, and those of other DNA damage pathways, which may affect the expression of MICB on the cell surface.

Based on analysis with Gene Runner software and experimental data (28), it was hypothesized that the core region of the MICB promoter includes elements for transcription regulation, including TATA-like elements and HSE. These elements may regulate the transcription of MICB, and this requires further investigation. Winter et al (34) reported that, following DNA damage, ATM molecules can phosphorylate nuclear E3 ubiquitin ligase Siah-1 and inhibit Siah-1-mediated homeodomain-interacting protein 2 (HIPK2) ubiquitination and degradation. HIPK2 activates phosphorylated p53 and promotes apoptosis. Therefore, the role of MG132 is associated not only with the DNA damage response but also with the ubiquitination and degradation of signaling molecules. However, the detailed mechanism requires further investigation.

In conclusion, the present study demonstrates that MG132 selectively upregulates the surface expression of MICB in A549 cells, and increases the NKG2D-mediated cytotoxicity of NK cells. The regulatory effect of MG132 is associated with the activation of Chk2, an event associated with DNA damage. The combination of MG132 with NK cell immunotherapy may have a synergistic effect that improves the therapeutic effect of lung cancer treatment.

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Availability of data and materials
All data generated or analyzed during the present study are included in this published article.
Authors' contributions

ZNC, FL, and ZLW conceived and designed the experiments. DL, XWD and BY performed the experiments and drafted the manuscript. ML and JHY were involved in the data analysis. HL and THX assisted with the experiments. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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