Effect of platinum-based chemotherapy on the expression of natural killer group 2 member D ligands, programmed cell death-1 ligand 1 and HLA class I in non-small cell lung cancer

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Abstract. Platinum-based chemotherapy improves the clinical outcome of patients with non-small cell lung cancer (NSCLC), although tumors often become refractory after treatment. Immunohistochemical staining was performed to investigate the expression levels of natural killer group 2 member D (NKG2D) ligands, programmed cell death-1 ligand 1 (PD-L1), and human leucocyte antigen (HLA)-class I in tissue samples collected from 10 NSCLC patients who received platinum-based chemotherapy followed by surgery. Additionally, the effects of repeated exposure to cisplatin on the expression of NKG2D ligands, PD-L1 and HLA-class I in NSCLC cell lines were assessed by flow cytometry. We found upregulation of PD-L1 or downregulation of NKG2D ligands in 5 of the 10 NSCLC cases, leading to the attenuation of NK cell-mediated tumor cell death. Moreover, upregulation of PD-L1 or downregulation of HLA-class I were observed in 6 cases, supporting tumor escape from T cell immunity. An *in vitro* assay showed that repeated exposure to cisplatin enhanced the expression of PD-L1 and NKG2D ligands in NSCLC cell lines. Notably, interferon gamma (IFNγ) stimuli enhanced PD-L1 expression while attenuated that of NKG2D ligands in NSCLC cell lines, which mimicked the results of the clinical study. Both IFNγ-induced upregulation of PD-L1 and downregulation of NKG2D ligands were blocked by the JAK-STAT inhibitor tofacitinib. These findings suggested that the expression levels of NKG2D ligands, PD-L1 and HLA-class I in residual tumors after chemotherapy were affected by host immunity, resulting in an immunoescape phenotype. Blocking IFNγ-induced tumor immunoescape by a JAK-STAT inhibitor might be a promising treatment strategy for NSCLC.

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Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Clinical studies have established cisplatin-based chemotherapy as a standard chemotherapeutic regimen for patients with non-small cell lung cancer (NSCLC) (2). However, cisplatin can give rise to refractory tumors. Recent research has shown that tumor progression might be caused by tumor cell escape from immunosurveillance (3). Natural killer (NK) cells play an important role in immunosurveillance against cancer cells (4), and are recognized as a promising tool in cancer immunotherapy (5). NK cell-mediated tumor cytotoxicity is mainly promoted by NK group 2 member D (NKG2D) receptors on NK cells (6-8). The major histocompatibility complex (MHC) class I-related chains A and B (MICA and MICB, respectively) (6), as well as UL16-binding proteins (ULBPs) (9), are ligands of NKG2D and they are expressed at low levels in non-malignant cells and at high levels in transformed cells (10). NKG2D ligands render transformed cells susceptible to NK cell-mediated death (6,11); however, tumor cells can evade immune recognition by downregulating the expression of NKG2D ligands. NKG2D ligands have been described as stress-related proteins and their expression can be increased through activation of the DNA damage pathway (12). Although cytotoxic anticancer drugs can induce apoptosis of NSCLC cells by inhibiting DNA replication and mitosis (13), tumors develop resistance to chemotherapy in many cases. There is no study reporting the expression of NKG2D ligands in NSCLC tissues before and after chemotherapy. However, we recently reported that a single exposure to cisplatin upregulated the expression of MICA and MICB (MICA/B), resulting in enhanced NK cell-mediated cytotoxicity via NKG2D-NKG2D ligand interaction in NSCLC cell lines (14). Therefore, it is of particular interest to understand whether cisplatin-based chemotherapy affects the expression of ligands for NK cell-activated or inhibitory receptors in tumor tissues collected from patients with NSCLC.

Furthermore, immune checkpoint inhibitors targeting the programmed cell death-1 (PD-1)/PD-1 ligand 1 (PD-L1) axis have shown promising results in recent clinical studies of patients with NSCLC. It is also of particular interest to investigate the effect of chemotherapy on the expression of PD-L1 since PD-L1 is one major mechanism of the immunoevasion of tumor cells (15-17). It has been reported that chemotherapy increases PD-L1 expression in thymic epithelial tumors and ovarian cancer (18,19), but another study showed that platinum-based chemotherapy tends to decrease the expression of PD-L1 in lung cancer (20). Thus, the effect of chemotherapy on the expression of PD-L1 in tumor cells has not been fully elucidated. However, the addition of the anti-PD-1 antibody pembrolizumab to standard chemotherapy was found to improve overall survival and progression-free survival to a greater extent than chemotherapy alone (21), suggesting that this combination may be a promising strategy for patients with NSCLC.

In the present study, we showed that repeated exposure to cisplatin *in vitro* enhanced the expression of both NKG2D ligands and PD-L1 in NSCLC cell lines, whereas platinum-based chemotherapy attenuated the expression of NKG2D ligands and enhanced the expression of PD-L1 in patients with NSCLC, which mimicked the effects of interferon γ (IFNγ) stimuli on NSCLC cells *in vitro*. Our findings suggested that residual tumors are the result of immunoselection by host immunity, and that possible mechanisms of tumor escape from host immunity during platinum-based chemotherapy include both the induction of PD-L1 expression and reduction of NKG2D ligand expression, which inhibit both T cell- and NK cell-mediated cytotoxicity. Notably, IFNγ stimuli enhanced PD-L1 expression and attenuated NKG2D ligand expression in NSCLC cell lines, which could explain the benefit of chemotherapy combined with a PD-1/PD-L1 inhibitor. Moreover, in NSCLC cell lines, the JAK-STAT inhibitor tofacitinib blocked IFNγ-induced increase and decrease in the expression of PD-L1 and NKG2D ligands, respectively, suggesting that triplet therapy with chemotherapy, a PD-1/PD-L1 targeting immunocheckpoint inhibitor, and a JAK-STAT inhibitor is a promising strategy to enhance the host antitumor immunity for patients with NSCLC.

Materials and methods

Patients and specimens. The present study was approved by the Kawasaki Medical School Ethics Committee (no. 1673-3), and written informed consent was obtained from all patients for the use of specimens. This study included patients with NSCLC who received platinum-based chemotherapy followed by surgery at the Kawasaki Medical School Hospital between January 2006 and October 2013. Histologic diagnosis was confirmed using hematoxylin and eosin (H&E) staining according to the WHO 2004 criteria (22) and the IASLC/ATS/ERS classification of lung adenocarcinoma (23). Pathological stages were defined according to the 7th edition of the TNM classification (24).

Immunohistochemical staining. NSCLC tissue sections were stained according to a previously described protocol (14). The expression levels of MICA/B, ULBP-2/5/6, PD-L1, and HLA class I were determined by immunohistochemical staining (IHC) using a mouse anti-MICA/B antibody (1:50 dilution; clone no. D-8; Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-ULBP-2/5/6 polyclonal antibody (polyclonal, 1:20 dilution, cat. no. AF1298; R&D Systems, Minneapolis, MN, USA), mouse monoclonal anti-PD-L1 antibody (1:100 dilution; clone no. SP142; Spring Bioscience Corp., Pleasanton, CA, USA), or mouse monoclonal anti-HLA class I (HLA-A, B and C) antibody (1:100 dilution; clone no. EMR8-5; Medical Biological Laboratories Co., Ltd., Nagoya, Japan) followed by poly-HRP-conjugated goat anti-mouse/rabbit secondary antibody (cat. no. K5007; Dako, Santa Clara, CA, USA) or anti-goat HRP-DAB Cell & Tissue Staining kit (cat. no. CTS008; R&D Systems). To assess the expression level of each marker, the slides were evaluated by two investigators (RO and AM) who were blinded to the corresponding clinicopathological data. The intensity scoring for staining was defined as follows: 0, no staining; 1+, weak staining that was visible only with high magnification; 2+, moderate staining (between 1+ and 3+), and 3+, strong staining that was visible with low magnification.

NSCLC cell lines. The human NSCLC cell lines A549 and PC-9 were obtained from Riken BRC through the National Bio-Resource Project of MEXT (Tsukuba, Japan) and the IBL Cell Bank (Gunma, Japan), respectively. The genotypes of the cell lines were identified with the PowerPlex 16 STR System (Promega, Fitchburg, WI, USA). Both cell lines were maintained as previously described (25). For cell culture experiments, cisplatin (Wako Pure Chemicals Industries Ltd., Osaka, Japan) and tofacitinib (Selleck Chemicals, Houston, TX, USA) stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), whereas IFNγ (PBL Assay Science, Piscataway, NJ, USA) stock solution was prepared in phosphate-buffered saline (PBS) (-).

Repeated exposure to cisplatin does not induce a cisplatin-resistant phenotype in NSCLC cell lines. Previously, the pharmacokinetics study showed that the platinum concentration in plasma ranged from 1.7 to 9.6 μg/ml in patients treated with 50-100 mg/m^2 of cisplatin (26), which was equivalent to cisplatin concentration of 8.7-49.2 μM. Based on this report, both A549 and PC-9 cells were treated with 10 μM cisplatin thrice every 1-2 weeks to establish the NSCLC cell models by repeated exposure to cisplatin. Second or third treatment with cisplatin was undergone after the cells reached 50% confluency in a cell culture dish.

WST-1 cell proliferation assay. The WST-1 assay was performed using the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Basel, Switzerland) as previously described (25). In brief, NSCLC cells were cultured in triplicate wells of 96-well flat-bottomed plates with 1-100 μM of cisplatin in culture medium for 48 h, then WST reagent were added to the wells according to the manufacturer's protocol. The colorimetric reaction was measured with the spectral scanning multimode reader Varioskan Flash (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cisplatin-mediated inhibition of

cell proliferation was calculated as described previously with the following formula: 100x (absorbance of the wells for the cells treated with cisplatin/absorbance of the wells for the cells treated with DMSO) (25).

Flow cytometry. Extracellular staining was performed with fluorochrome-conjugated antibodies as previously described (27). The following antibodies were used for staining: PE-labeled MICA (clone no. 159227; R&D Systems), allophycocyanin-labeled MICB (clone no. 236511; R&D Systems), PE-labeled MICA/B (clone no. 6D4; BioLegend, San Diego, CA, USA), Alexa Fluor 488-labeled ULBP-1 (clone no. 170818; R&D Systems), PE- and allophycocyanin-labeled ULBP-2/5/6 (clone no. 165903; R&D Systems), PE-labeled ULBP-3 (clone no. 1166510; R&D Systems), Alexa Fluor 488-labeled ULBP-4 (clone no. 709116; R&D Systems), PE-labeled PD-L1 (clone no. 29E.2A3; BioLegend), allophycocyanin-labeled HLA-A, B and C (clone no. G46-2.6; BioLegend), as well as PE-, allophycocyanin- and Alexa Fluor 488-labeled anti-mouse IgG1κ (clone no. MOPC-21; BioLegend) and IgG2bκ (clone no. MOPC-173; BioLegend) as isotype controls. The cells were assayed using a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using FlowJo software 6.4.7 (Tree Star, Inc., Ashland, OR, USA). The increase in mean fluorescence intensity (ΔMFI) was calculated as: (MFI with specific mAb-MFI with isotype control)/MFI with isotype control). The relative MFI (rMFI) values were calculated to compare the differences between ΔMFI of a specific treatment and control as: 100x (ΔMFI of a specific treatment/ΔMFI of the control treatment).

NK cell-mediated cytotoxicity assay. Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors by gradient centrifugation with Vacutainer CPT mononuclear cell preparation tubes (BD Biosciences, San Jose, CA, USA). NK cells were purified using a NK cell isolation kit (Stem Cell Technologies, Vancouver, BC, Canada) and incubated with 100 IU/ml IL-2 (Teceleukin; Shionogi & Co., Ltd., Osaka, Japan) for 24 h as previously described (25). The NSCLC cells, with or without repeated exposure to 10 μM cisplatin, were tested for sensitivity to NK cell-mediated cytotoxicity using an LDH release assay kit (CytoTox 96 Non-Radioactive Cytotoxicity assay; Promega) and a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Inc.) as previously described (25). To analyze the involvement of NKG2D in the cytotoxicity of NK cells, NK cells were co-incubated with 20 μg/ml of anti-NKG2D blocking antibody (clone no. 1D11; BioLegend) or an isotype-matched control antibody (clone no. 11711; R&D Systems). For the LDH release assay, blood samples were collected only from researchers who were involved with this study; therefore, written informed consent was not required. The Ethics Research Committee of the Kawasaki Medical School approved both the study and this consent procedure (no. 1217-5).

Statistical analysis. Differences in means were evaluated with the Student's t-test. All analyses were performed at a significance level of 5% ($P<0.05$) using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Tumor expression of NKG2D ligands MICA/B and ULBP-2/5/6 is attenuated by platinum-based chemotherapy in patients with NSCLC. We collected tissue samples from 10 patients who received more than two courses of platinum-based chemotherapy followed by surgery, and the expression levels of MICA/B and ULBP-2/5/6 were assessed by IHC. The patient characteristics are shown in Table I. Following chemotherapy, MICA/B expression was downregulated in 2 of 10 patients (Patient #1 and #3) (Fig. S1), whereas ULBP-2/5/6 expression was downregulated in 4 of 10 patients (Patient #1, #8, #9 and #10) (Fig. S2 and Table II), which could lead to tumor immunoescape from NK cells. Surprisingly, these findings contrasted the results of our previous *in vitro* study, which showed that cisplatin enhances NKG2D ligand expression in NSCLC cell lines (14). One important difference between our present and previous studies (14) is the number of exposures to chemotherapeutic reagents: Repeated vs. single, respectively. For this reason, in the subsequent *in vitro* experiments, we further investigated the effect of repeated exposure to cisplatin on NKG2D ligand expression.

Tumor expression of PD-L1 is enhanced while that of HLA-class I is attenuated by platinum-based chemotherapy in NSCLC patients. Through IHC, we also evaluated the expression of both PD-L1 and HLA class I, which are important for tumor recognition by T cells. Notably, the expression of PD-L1 was upregulated in 3 of the 10 patients (Patient #4, #5 and #8) (Fig. S3) and that of HLA class I was downregulated in 5 of the 10 patients (Patient #1, #4, #7, #8 and #10) (Fig. S4 and Table II), supporting tumor immunoescape from T cells.

Repeated exposure to cisplatin does not induce cisplatin-resistant phenotype in NSCLC cell lines. We previously reported that MICA/B was overexpressed in ~30% of patients with NSCLC (14) and all the tested NSCLC cell lines (25). Although no study has evaluated ULBP-2 expression in tumor tissues from patients with NSCLC, soluble ULBP-2 was detected in the serum of patients with lung cancer (28), and this ligand was expressed in all the tested NSCLC cell lines (25). To investigate whether repeated exposure to cisplatin alters the expression of NKG2D ligands in NSCLC cell lines, NSCLC cells were treated with 10 μM cisplatin thrice, once every 1-2 weeks. First of all, cisplatin resistance was assessed by a WST-1 cell proliferation assay in both the original cells (-original) and cells repeatedly exposed to cisplatin (-C1, -C2, -C3). A549-C cells showed marginal resistance, whereas PC-9-C cells showed marginal sensitivity to cisplatin (Fig. 1).

Repeated exposure to cisplatin enhances NK cell-mediated cytotoxicity via the upregulation of NKG2D ligands in NSCLC cell lines. Next, the expression levels of NKG2D ligands (MICA, MICB and ULBP-1, ULBP-2/5/6, ULBP-3, ULBP-4), PD-L1 and HLA class I in NSCLC cell lines were evaluated by flow cytometry. After exposure to 10 μM cisplatin thrice, the expression of MICA in A549 cells and one of ULBP-2/5/6 in PC-9 cells was significantly upregulated. Additionally, the

Table I. Clinicopathological characteristics of the NSCLC patients who were treated with platinum-based chemotherapy followed by surgery.

Patient no.	Age (years)	Sex	T (c/yp)	N (c/yp)	M (c/yp)	yp Stage	Histology	Regimen	Cycles	OR	pR
#1	69	Male	2/3	2/X	0	IIIA	pleo	CDDP+GEM	2	SD	Ef1a
#2	66	Male	2/2	2/2	0	IIIA	Sq	CDDP+GEM	2	SD	Ef1a
#3	70	Male	3/2b	0/1	0	IIB	Sq	CDDP+GEM	2	PR#	Ef1a
#4	54	Male	2/2b	2/2	1 (BRA)	IV	La	CDDP+DTX	2	SD	Ef1b
#5	68	Male	4/4	0/1	0	IIIA	Sq	CDDP+DTX	2	PR	Ef1a
#6	72	Male	4/3	1/2	0	IIIA	Sq	CDDP+DTX	2	PR	Ef2
#7	47	Male	4/4	0/0	0	IIIB	Ad	CDDP+PTX	4	SD	Ef1a
#8	61	Male	2/2	2/2	0	IIIA	Ad	CDDP+PTX	2	PR	Ef1b
#9	57	Male	1b/1b	0/2	1 (BRA)	IV	Ad	CBDCA+PTX	4	SD	Ef1a
#10	70	Female	2a/2a	2/2	0	IIIA	AdSq	CDDP+VNR	2	SD	Ef1a

NSCLC, non-small cell lung cancer; OR, objective response; BRA, brain; Pleo, pleomorphic carcinoma; Sq, squamous cell carcinoma; La, large cell carcinoma; Ad, adenocarcinoma; AdSq, adeno-squamous cell carcinoma; CDDP, cisplatin; GEM, gemcitabine; DTX, docetaxel; PTX, paclitaxel; CBDCA, carboplatin; VNR, vinorelbine; SD, stable disease; pR, pathological response; PR, partial response; yp, pathological; c, clinical.

expression of PD-L1 was significantly upregulated in both the cell lines, and the expression of one of the HLA class I was significantly enhanced in the PC-9 cells. The basal expression of MICA in the PC-9 cell line and the expression levels of MICB, ULBP-1, ULB-3 and ULB-4 in both the cell lines were too weak (ΔMFI <0.5) to analyze (Fig. 2).

To analyze the effect of repeated exposure to cisplatin on NK cell-mediated cytotoxicity, the sensitivity of A549 and PC-9 cells to NK cells was evaluated by an LDH release assay. As expected, NK cell-mediated death increased in the A549 and PC-9 cells after exposure to cisplatin thrice (Fig. 3A). To verify that an NKG2D receptor was involved in the repeated exposure to cisplatin-induced sensitivity to NK cell-mediated death, purified NK cells were pretreated with an anti-NKG2D blocking antibody prior to the assay. In this assay, we selected A549-C2 and PC-9-C3 cells since these cells showed the highest cisplatin-resistance phenotype among the three established phenotypes. The anti-NKG2D blocking antibody inhibited NK cell-mediated lysis of the cisplatin-treated tumor cells, whereas an isotype control antibody had no effect on both cell lines (Fig. 3B). These findings indicated that the NK cell-mediated cytotoxicity of repeated cisplatin exposure was dependent on NKG2D-NKG2D ligand interaction.

IFNγ downregulates NKG2D ligands but upregulates PD-L1 in NSCLC cell lines. To determine the reason for the difference in NKG2D ligand expression *in vivo* and *in vitro*, we focused on the immune response of NSCLC cells as another major difference between the two environments is the presence of host immunity. IFNγ is an important cytokine secreted by NK cells to attack tumor cells (29). Previous reports have shown that IFNγ increases PD-L1 expression (15) and decreases NKG2D ligand expression (30) in cancer cells. Thus, we evaluated the effect of IFNγ stimuli on the expression of NKG2D ligands, PD-L1, and HLA class I in NSCLC cell lines. We found that IFNγ significantly downregulated MICA/B in A549 and ULBP-2/5/6 in PC-9 cells, respectively. We previously reported that IFNγ enhanced PD-L1 expression in A549 cells, which was blocked by the JAK inhibitor tofacitinib (31). In line with our previous report (31), the present study demonstrated that IFNγ significantly upregulated PD-L1 in A549 cells and marginally but significantly enhanced PD-L1 expression in PC-9 cells. On the other hand, IFNγ upregulated HLA class I in both cell lines. Moreover, the JAK-STAT inhibitor tofacitinib significantly blocked the IFNγ-induced decrease in MICA/B and blocked the IFNγ-induced increase in PD-L1 in both cell lines (Fig. 4). These findings suggest that both IFNγ-induced decrease in MICA/B and IFNγ-induced increase in PD-L1 are mainly regulated by the JAK/STAT pathway in NSCLC cells. Notably, the IFNγ-treated NSCLC cell lines showed a similar trend for the expression of NKG2D ligands and PD-L1 to that of patients with NSCLC who received platinum-based chemotherapy (Figs. S1-S3), suggesting that the expression levels of NKG2D ligands and PD-L1 after platinum-based chemotherapy are determined not by the chemotherapeutic drug but by IFNγ. This process is one reasonable mechanism that may explain the effect of chemotherapy combined with a PD-1/PD-L1 axis-targeting

Table II. Immunohistochemical staining scores in NSCLC tissues before and after induction chemotherapy.

	MICA/B		ULBP-2/5/6		PD-L1		HLA-A, B, C	
Patient no.	pre-CTx	post-CTx	pre-CTx	post-CTx	pre-CTx	post-CTx	pre-CTx	post-CTx
#1	1	0	3	2	0	0	3	1
#2	1	1	2	2	0	0	1	1
#3	3	0	1	2	0	0	0	2
#4	0	0	1	2	1	2	2	0
#5	0	0	1	2	0	3	2	2
#6	0	0	2	2	0	0	2	2
#7	0	0	1	2	1	1	3	2
#8	0	0	3	2	1	2	3	2
#9	0	0	2	1	0	0	1	1
#10	0	0	3	2	1	0	3	1

NSCLC, non-small cell lung cancer; MICA/B, major histocompatibility complex (MHC) class I-related chains A and B; ULBP-2/5/6, UL16-binding proteins 2/5/6; PD-L1, programmed cell death-1 ligand 1; HLA-A, B, C, human leucocyte antigen; CTx, chemotherapy.

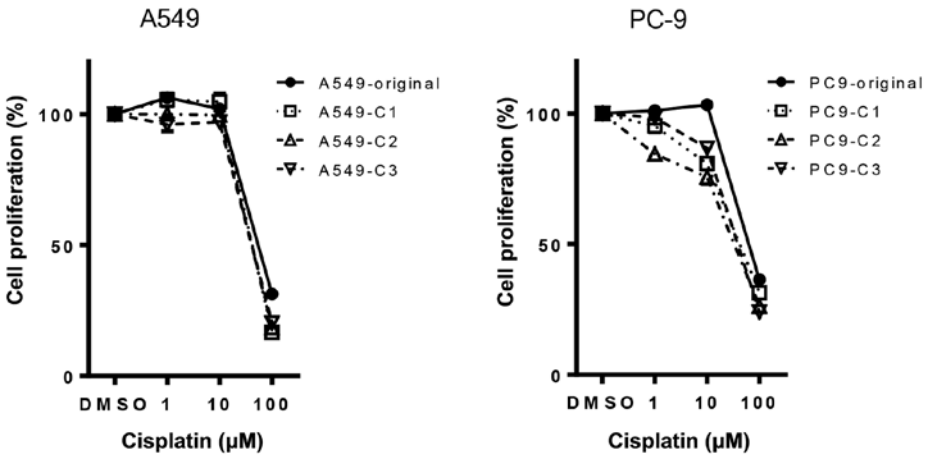

Figure 1. Repeated exposure to cisplatin minimally affects cisplatin resistance in non-small cell lung cancer cell lines. The cancer cells were treated with 10 μM cisplatin thrice, once every 1-2 weeks, in three independent experiments (A549- or PC-9-C1-3). Cell proliferation was evaluated by WST-1 assay. -original, untreated cells; -C1,C2,C3, three independent samples of cells thrice exposed to cisplatin. Bars indicate the standard error of the mean (SEM). DMSO, dimethyl sulfoxide.

drug on the improvement of clinical outcome of patients with NSCLC (21).

Discussion

The expression of NKG2D ligands is regulated by DNA stress-induced ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) protein kinase signaling. The ATM-ATR pathway is frequently activated in tumor cells and is found to regulate NKG2D ligands via transcriptional regulation (12). An increase in NKG2D ligand expression by chemotherapeutic reagents could represent an important antitumor mechanism whereby tumor cells are eradicated by the innate immune system (5). We previously reported that a single exposure to cisplatin enhanced NKG2D ligand expression and NK cell-mediated cytotoxicity in NSCLC cells *in vitro* (14). However, the present study showed that the expression of both MICA/B and ULBP-2/5/6 in the tissue samples was attenuated by cisplatin-based chemotherapy, which were not in line with the results of our previous *in vitro* study (14).

A primary difference between our previous and present studies concerns the numbers of exposure to cisplatin: Single vs. repeated, respectively. In the present study, the effect of repeated exposure to cisplatin on NKG2D ligand expression was first evaluated. In agreement with our previous study, repeated exposure to cisplatin enhanced the expression of MICA in A549 and the expression of one of ULBP-2/5/6 in PC-9 cells resulting in enhanced NK cell-mediated cytotoxicity via NKG2D-NKG2D ligand interaction in both cell lines.

Another important difference between our previous and present study is the study setting: *In vitro* vs. in patients, respectively. The *in vitro* study showed that the direct response of cancer cells to cisplatin can be assessed without the influence

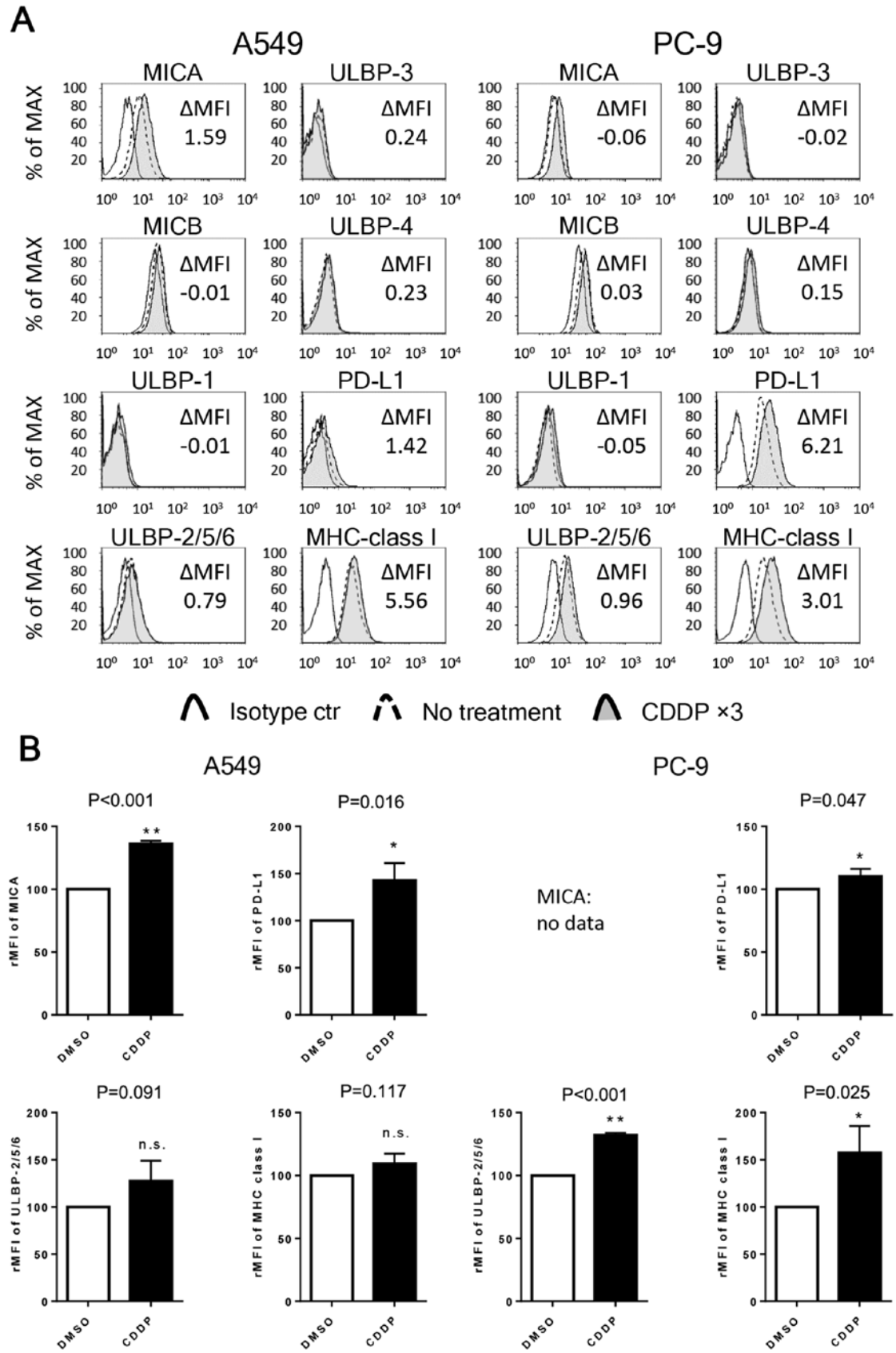

Figure 2. Repeated exposure to cisplatin enhances the expression of NKG2D ligands, PD-L1 and HLA class I in NSCLC cell lines. (A) The expression of MICA, MICB, ULBP-1, ULBP-2/5/6, ULBP-3, ULBP-4, PD-L1 and HLA class I in NSCLC cell lines. The expression of each cell surface molecule was assessed by flow cytometry in both untreated cells and cells thrice exposed to 10 μM cisplatin (CDDP x3). The cells were stained with an isotype control antibody or antibody specific for the indicated molecule. The results represent three independent experiments. (B) The relative MFI (rMFI) of MICA, ULBP-2/5/6, PD-L1 and MHC class I molecules were calculated based on three independent experiments and evaluated with the Student's t-test. Bars indicate SEM. *P<0.05 and **P<0.01. NKG2D, natural killer group 2 member D; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death-1 ligand 1; HLA-class I, human leucocyte antigen; MICA, MHC class I-related chain molecule A; MICB, MHC class I-related chain molecule B; ULBP-1, UL16 binding protein 1; rMFI, relative mean fluorescence intensity; MHC, major histocompatibility complex; SEM, standard error of the mean; n.s., not significant.

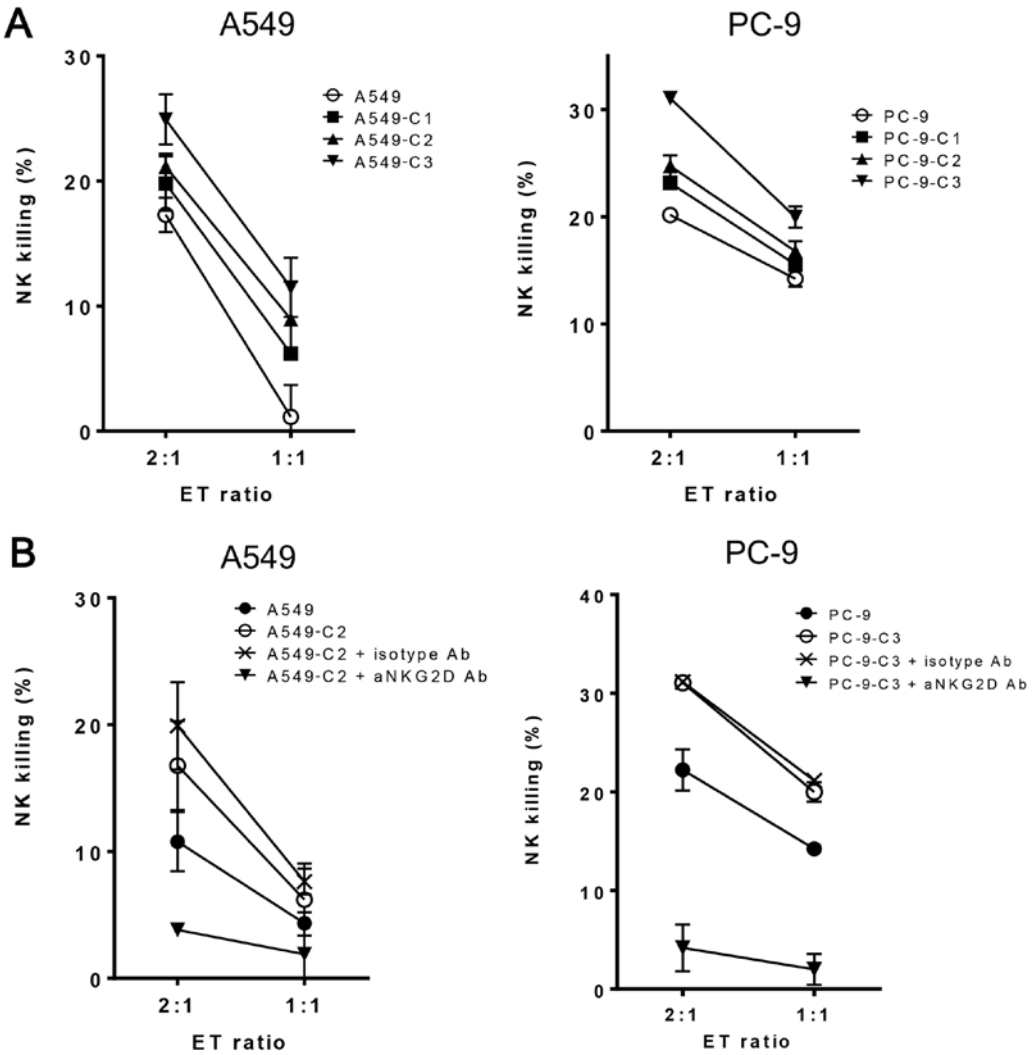

Figure 3. Cisplatin-induced NKG2D ligands enhance NK cell-mediated cytotoxicity via NKG2D-NKG2D ligand interaction in NSCLC cells. (A) A549 and PC-9 cells repeatedly exposed to 10 μM cisplatin (-C1, -2 and -3) and untreated cells (A549 or PC-9) were subjected to an LDH release assay for 4 h using IL-2-activated NK cells as effector cells. Data are presented as the means of triplicate experiments. (B) The IL-2-activated NK cells were pretreated with an NKG2D-blocking antibody (aNKG2D Ab) or isotype control antibody (isotype Ab) for 30 min prior to the cytotoxicity assay. A549-C2 and PC9-C3 cells were co-incubated with NK cells and NK cell-mediated cytotoxicity was evaluated by the LDH release assay. E:T ratio, effector/target ratio. Bars indicate SEM. NKG2D, natural killer group 2 member D, NK, natural killer; NSCLC, non-small cell lung cancer; LDH, lactate dehydrogenase; SEM, standard error of the mean.

of host immune cells. However, the residual tumors in patients were caused by both chemotherapy and antitumor immunity resistance. Our conflicting findings between the *in vitro* and in patient studyies support the hypothesis that repeated exposure to cisplatin upregulates the expression of NKG2D ligands in cancer cells and then NK cells eradicate tumor cells via NKG2D-NKG2D ligand interaction. Tumor heterogeneity is expected in the expression levels of both basal and chemotherapeutic reagent-induced increase in NKG2D ligand expression. Tumor cells expressing low levels of NKG2D ligands may escape from NK cell-mediated immunosurveillance and be immunoselected as residual tumor. Recently, chemotherapy was recognized as a PD-L1 inducer in cancer cells (18). Our findings suggested that repeated exposure to cisplatin and host immunoreaction with IFNγ enhanced PD-L1 expression, leading to the inhibition of T cell- or NK cell-mediated cytotoxicity in patients and resulting in tumor escape from host immunity. It is well known that NSCLC cells have different genetic backgrounds, such as EGFR driver mutation and EML4-ALK fusion gene (32). Both EGFR and ALK signaling regulate PD-L1 in NSCLC cells (33,34), and we have also reported that EGFR signaling regulates the expression of NKG2D ligand in PC-9 cells having EGFR driver mutation (25). These findings suggest genetic effects on the escape from host immunity via regulation of immune-related molecules.

One major limitation of the present study was that we could evaluate only the effect of NK cell-mediated immunoselection on NSCLC cells *in vitro*. Antitumor immunity involves not only NK cells but also several phenotypes of T cells, antibody-producing B cells, antigen-presenting cells and cytokines. Since host immunity against tumor cells is a complicated system, it is difficult to be reproduced in an *in vitro* assay with several types of immune cells. Another limitation was the very low number of patients who were evaluated using IHC for NKG2D ligand and PD-L1 expression

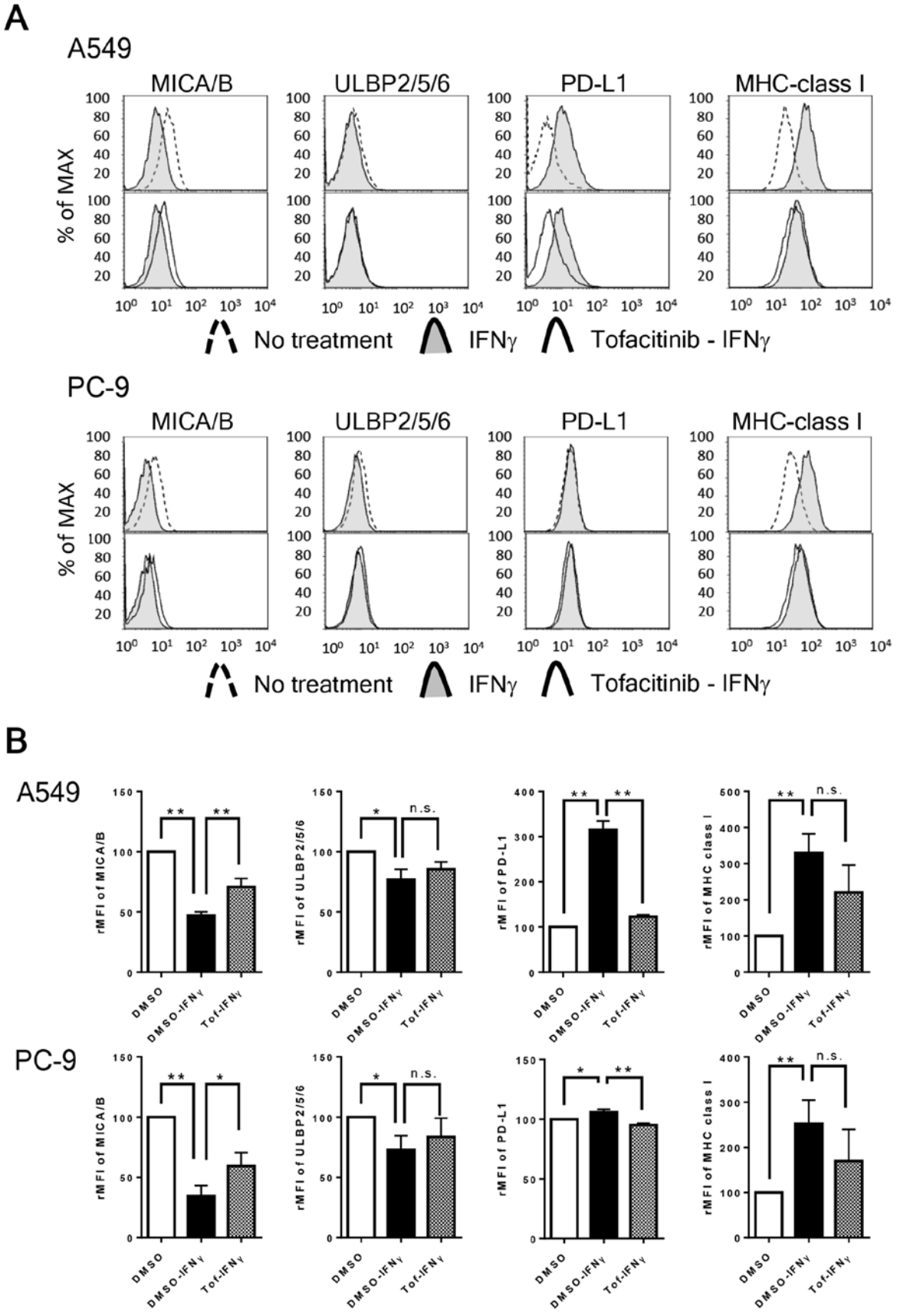

Figure 4. IFNγ downregulates NKG2D ligands and upregulates PD-L1 and HLA class I in NSCLC cells, and is blocked by tofacitinib. (A) The panels show representative images of the expression of MICA/B, ULBP-2/5/6, PD-L1 and HLA class I in NSCLC cell lines pretreated with or without 1 μM of tofacitinib (Tof) followed by 0.5 ng/ml of IFNγ. (B) The relative MFI (rMFI) of MICA/B, ULBP-2/5/6, PD-L1 and HLA class I were calculated based on three independent experiments and evaluated with the Student's t-test. The bars represent the standard deviations. *P<0.05 and **P<0.01. Bars indicate SEM. IFNγ, interferon γ; NKG2D, natural killer group 2 member D; PD-L1, programmed cell death-1 ligand 1; HLA class I, human leucocyte antigen class I; NSCLC, non-small cell lung cancer; rMFI, relative mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chains A and B; ULBP-2/5/6, UL16-binding proteins 2/5/6; SEM, standard error of the mean; DMSO, dimethyl sulfoxide; n.s., not significant.

as induction chemotherapy is not a standard therapy for locally advanced NSCLC.

In summary, the present clinical study suggests that residual tumor following chemotherapy is the result of tumor immunoescape via the downregulation of NKG2D ligands and/or upregulation of PD-L1 in tumor cells as tumor cells with this phenotype can easily escape from host immunity, even though cisplatin-based chemotherapy works in concert with NK cell-mediated cytotoxicity via the upregulation of NKG2D ligands *in vitro*. Although a recent clinical study showed that cisplatin-based chemotherapy combined with a PD-1/PD-L1 inhibitor is the optimal therapeutic approach for patients with advanced NSCLC (21), our current findings suggest that addition of the JAK-STAT inhibitor tofacitinib to chemo-immunocheckpoint therapy is a promising strategy as it may block IFNγ-induced downregulation of NKG2D ligands and upregulation of PD-L1 in NSCLC cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RO substantially contributed to the conception and design of the study. RO and AM substantially contributed to the acquisition of data, the analysis and interpretation of the data. KS, SS, YN and MN contributed to the acquisition of the data, the analysis and interpretation of the data. RO and MN drafted the article and all authors revised it critically for important intellectual content, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Kawasaki Medical School Ethics Committee (nos. 1217-5 and 1673-3). Written informed consent was obtained from all patients for the use of specimens.

Patient consent for publication

Not applicable.

Competing interests

Dr Masao Nakata received research funding from Kyowa Hakko Kirin, Taiho Pharma, Ono Pharma, and Nihon Medi-Physics Co. The sponsors had no control over the interpretation, writing, and publication of this study. All other authors declare that they have no competing interests.

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