

## ORIGINAL ARTICLE

# Pemetrexed sensitizes human lung cancer cells to cytotoxic immune cells

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## Abstract

Pemetrexed (PEM) is a useful drug that can be combined with immune checkpoint blockade therapy for treatment of patients with advanced non-small-cell lung cancer (NSCLC). However, its effects on anti-cancer immunity, especially the sensitivity of NSCLC cells to cytotoxic immune cells, have not been fully investigated. In this study, we examined the effects of PEM on the sensitivity of human NSCLC cells to two different types of cytotoxic immune cells. Pre-treatment with PEM increased the sensitivity of two NSCLC cell lines, PC9 and A549, to activated T cells and natural killer (NK) cells, and decreased the expression of anti-apoptotic proteins, including XIAP and Mcl-1. In addition, PEM treatment increased the cell surface expression of programmed death-ligand 1 (PD-L1) on PC9 cells. PEM-induced upregulation of PD-L1 on PC9 cells was at least partially ascribed to activation of ERK and the NFκB pathway. In contrast, PEM treatment increased the expression of UL16-binding proteins (ULBP), ligands for the NKG2D NK receptor, on PC9 and A549 cells, as well as the induction of senescence. Although the addition of anti-programmed cell death 1 antibody showed no effect on the sensitivity of PEM-treated PC9 and A549 cells to activated T cells, that of anti-NKG2D antibody decreased the enhanced sensitivity of PEM-treated A549 cells to NK cells. These results indicate that PEM can effectively sensitize human NSCLC cells to cytotoxic immune cells while modulating the expression of immune-regulatory molecules.

## KEYWORDS

natural killer cells, non-small-cell lung cancer, PD-L1, pemetrexed, T cells

## 1 | INTRODUCTION

Lung cancer is the leading cause of death due to cancer worldwide. However, the emergence of immune checkpoint blockade (ICB) therapy dramatically changed the treatment strategy for non-small-cell lung cancer (NSCLC). Compared with platinum-based chemotherapy,

anti-programmed cell death 1 (PD-1) antibody therapy can significantly prolong progression-free and overall survival of previously untreated advanced NSCLC patients with PD-L1 expression on at least 50% of cancer cells.<sup>1</sup> By contrast, in patients with previously untreated stage IV or recurrent NSCLC with PD-L1 expression on 5% or more of cancer cells, anti-PD-1 antibody therapy was not associated with significantly longer progression-free survival and

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overall survival compared with chemotherapy.<sup>2</sup> To improve the therapeutic efficacy of ICB therapy, combinations with other types of anti-cancer therapies have been used. Anti-PD-1 antibody therapy in combination with chemotherapeutic drugs resulted in better overall survival of patients with nonsquamous NSCLC with any level of PD-L1 expression compared with chemotherapeutic drugs alone.<sup>3</sup> In addition, a combination of anti-PD-L1 antibody, anti-vascular endothelial growth factor antibody and chemotherapy improved progression-free survival and overall survival of patients with metastatic nonsquamous NSCLC regardless of the level of PD-L1 expression.<sup>4</sup> Moreover, compared with chemotherapy alone, the addition of anti-PD-1 antibody therapy to chemotherapy led to longer overall survival and progression-free survival of patients with previously untreated metastatic squamous NSCLC.<sup>5</sup>

Pemetrexed (PEM) is an anti-folate drug<sup>6</sup> that exerts anti-cancer effects on nonsquamous NSCLC.<sup>7</sup> PEM has therapeutic advantages over gemcitabine when used with platinum-based chemotherapy to treat nonsquamous NSCLC.<sup>8</sup> Platinum-based chemotherapy is repeated in four cycles, followed by continuous administration of PEM alone. This continuous maintenance therapy has been conducted to prevent recurrence after platinum-based chemotherapy and to improve the overall survival of advanced nonsquamous NSCLC patients.<sup>9</sup> Therefore, PEM appears to be a promising drug to improve the effects of ICB therapy. Indeed, combination therapy consisting of anti-PD-1 antibody, carboplatin/cisplatin, and PEM improved survival compared to placebo combination therapy.<sup>3</sup> However, although ICB therapy induces anti-cancer effects via restoration of exhausted anti-cancer T cells,<sup>10</sup> the means by which PEM affects the susceptibility of NSCLC cells to cytotoxic immune cells has not been elucidated.

In this study, we examined the effects of PEM on the sensitivity of human NSCLC cells to two different types of immune cytotoxicity. Our results reveal that PEM can sensitize two human NSCLC cell lines, PC9 and A549, to activated T cells and natural killer (NK) cells. We also found that PEM decreased the expression of anti-apoptotic proteins and increased the cell surface expression of programmed death-ligand 1 (PD-L1) on PC9 cells and UL16-binding proteins (ULBP), ligands for the NKG2D NK receptor,<sup>11</sup> on PC9 and A549 cells. In addition, experiments with blocking antibodies revealed that, although augmented expression of PD-L1 on PEM-treated PC9 cells showed no effect on their sensitivity to activated T cells and NK cells, augmented expression of ULBP on PEM-treated A549 cells affected their sensitivity to NK cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and reagents

Two human lung adenocarcinoma cell lines, PC9 and A549, were obtained from the ATCC. PC9 has an epidermal growth factor receptor (EGFR) mutation and A549 has a KRAS mutation. PC9-RP and PC9-ER are PEM-resistant and erlotinib (ERLO)-resistant PC9 cell lines, respectively,<sup>12</sup> and A549-RP is a PEM-resistant A549 cell line.<sup>13</sup>

These cell lines were maintained in RPMI-1640 medium (Wako) supplemented with 10% (v/v) FCS (Invitrogen) and 20 µg/mL gentamicin (Sigma-Aldrich), and grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. PEM was purchased from Eli Lilly Japan KK and diluted with PBS. ERLO was purchased from SA Bioscience and diluted with DMSO. Anti-CD3 antibody (clone UCHT1) was purchased from Ancell. Inhibitors against JAK1/2 (Ruxolitinib, Enzo Life Sciences), ERK (U0126, Calbiochem), JNK (SP600125, ALEXS Biochemicals), p38 (SB203580, ALEXS Biochemicals) and NFκB (PDTc, Calbiochem) were used.

### 2.2 | Preparation of effector immune cells

Anti-EGFR chimeric antigen receptor (CAR)-T cells were purchased from ProMab. After initial thawing, these cells were cultured in RPMI-1640 supplemented with 2% human serum and 300 U/mL interleukin (IL)-2 for 10 days. The cultured cells were aliquoted in vials and kept at -70°C. For each experiment, the T cells were expanded in anti-CD3 antibody-coated plates with 300 U/mL IL-2 for 7-10 days.

### 2.3 | Cell viability assay

Cell viability was measured using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay (Nacalai Tesque). Cells were seeded in flat-bottomed 96-well plates, and PEM or ERLO was added at the indicated doses. Two days later, 10 µL of WST-8 solution was added to each well after removal of half of the medium, and the plates were incubated for an additional 3 hours. The plates were then read at a wavelength of 450 nm using a microplate reader (Beckman Counter).

### 2.4 | Flow cytometric analysis

Apoptosis was measured using an annexin V-FITC Apoptosis Detection Kit (BioVision). To examine the expression of human EGFR on cancer cells, cells were stained with either FITC-conjugated anti-EGFR antibody (NeoMarkers) or isotype-matched control antibody (Beckman Coulter). To examine the expression of PD-1 and NKG2D on NK cells, cells were stained with anti-PD-1-FITC (BioLegend) and anti-CD56-PE (BioLegend) antibodies. All analyses were performed by flow cytometry (FACSCalibur, Becton Dickinson).

### 2.5 | Preparation of human natural killer cells

Natural killer cells were prepared from the peripheral blood of healthy donors. Peripheral blood mononuclear cells were prepared from blood using Lymphocyte Separation Solution (Nacalai Tesque). NK cells were enriched using an NK Cell Purification Kit (Invitrogen). The study protocol was approved by the Ethics Review Board of the Shimane University Faculty of Medicine (approval No. 20140918-2).

## 2.6 | Cytotoxicity assay using flow cytometry

To examine apoptotic cancer cells, cancer cells were co-cultured with activated T cells or purified NK cells with the indicated antibodies in 96-well plates for 6 or 12 hours. After harvesting, whole cells were stained with anti-CD45-APC followed by annexin V-FITC. To evaluate the percentage of apoptotic cancer cells, annexin V-FITC<sup>+</sup> cells among CD45-APC<sup>-</sup> cells were calculated by flow cytometry. In some experiments, anti-human PD-1 (nivolumab) or anti-CD20 (rituximab) antibody, as a control, was added 30 minutes before the initiation of culture at a dose of 10 µg/mL. In other experiments, anti-human NKG2D (BioLegend) or mouse IgG, as a control, was added 1 hour before the initiation of culture at a dose of 10 µg/mL.

## 2.7 | Detection of senescence-associated β-galactosidase

Senescence-associated β-galactosidase (SA β-gal) was detected by confocal imaging. Cancer cells were cultured on round coverslips in 24-well plates with or without PEM for 48 hours. After incubation with SPiDER β-gal (Dojindo Molecular Technologies) and Hoechst 33342 (5 µg/mL) for 30 minutes, cells were fixed with 4% paraformaldehyde and placed on slide glasses with 4 µL of mounting medium for fluorescence analysis (Vectashield, Vector Laboratories). Confocal imaging was performed using a laser-scanning microscope (FV1000-D, Olympus).

## 2.8 | ELISA

The levels of human IL-6 and IL-8 in the supernatants were determined using an ELISA kit (PeproTech).

## 2.9 | Immunoblotting assay

Cancer cells were lysed with RIPA Buffer (Fujifilm Wako Pure Chemical) containing a protease inhibitor cocktail (Nacalai Tesque) and a phosphatase inhibitor cocktail (Nacalai Tesque). Equal amounts of protein were resolved on 4%-12% gradient or 12% SDS-PAGE gels, followed by transfer onto polyvinylidene difluoride membranes. After blocking the membranes, the blots were incubated with the indicated primary antibodies: anti-p21<sup>Cip1/Waf1</sup> (#2947, Cell Signaling Technology), anti-p16<sup>Ink4a</sup> (SPC-1280, StressMarq Biosciences), anti-STAT1 (#14994, Cell Signaling Technology), anti-pSTAT1<sup>Ser727</sup> (#8826, Cell Signaling Technology), anti-pSTAT1<sup>Tyr701</sup> (#9167, Cell Signaling Technology), anti-STAT3 (#4904, Cell Signaling Technology), anti-pSTAT3<sup>Ser727</sup> (#9134, Cell Signaling Technology), anti-pSTAT3<sup>Tyr705</sup> (#9145, Cell Signaling Technology), anti-ERK (#4695, Cell Signaling Technology), anti-pERK (#4370, Cell Signaling Technology), anti-p38 (sc-81621, Santa Cruz Biotechnology), anti-pp38 (sc-166182, Santa Cruz Biotechnology), anti-NFκB (#3987, Cell Signaling Technology), anti-IκB (#4812, Cell Signaling Technology), anti-PD-L1 (#13684,

Cell Signaling Technology), anti-cFLIP (ALX-804-428, Enzo Life Sciences), anti-survivin (#2803, Cell Signaling Technology), anti-XIAP (#2042, Cell Signaling Technology), anti-Bcl-2 (#658701, BioLegend), anti-Bcl-xL (#2764, Cell Signaling Technology), anti-Mcl-1 (sc-819, Santa Cruz Biotechnology), and anti-β-tubulin (sc-5286, Santa Cruz Biotechnology) and anti-β-actin (#622102, BioLegend). After washing, the membranes were incubated at room temperature for 60 minutes with either goat anti-rabbit or horse anti-mouse HRP-conjugated secondary antibody (#7074 or #7076, respectively, Cell Signaling Technology) to detect the primary antibodies. Protein bands were visualized using an ImageQuant LAS-4000 system (FujiFilm).

## 2.10 | Quantitative RT-PCR

Cells at around 80% confluence were washed with PBS. The total RNA from these cells were extracted using a PureLink RNA Mini Kit (Thermo Scientific) in accordance with the manufacturer's instructions. cDNA were generated from the RNA via reverse transcription using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO) in accordance with the manufacturer's instructions. Quantitative PCR (qPCR) was performed using KOD SYBR qPCR Mix (TOYOBO). The primers used for qPCR were as follows: ULBP2 primers 5'-CGCCGCTACCAAGATCCTTC-3' and 5'-ATCCAC CTGGCCTTGAACCG-3'; ULBP5 primers 5'-ACCACCCTCATCCTTTG CTG-3' and 5'-CCATGGCTTTGGGTCAGACT-3'; ULBP6 primers 5'-GG GCTAGGCGAGACGACC-3' and 5'-ACTGACGGGTGTGACTGTCT-3'; and GAPDH primers 5'-AGGTGAAGGTCCGAGTCA-3' and 5'-GGTC ATTGATGGCAACA-3'. The levels of mRNA expression were subsequently normalized relative to GAPDH mRNA levels and calculated according to the delta-delta Ct method.

## 2.11 | Statistical analysis

Data were statistically evaluated using the unpaired two-tailed Student's *t* test. In all analyses, *P* < 0.05 was taken to indicate statistical significance.

# 3 | RESULTS

## 3.1 | Pemetrexed decreases the cell viability of non-small-cell lung cancer cell lines

First, we examined the effects of PEM on two human NSCLC cell lines, PC9 and A549. In this assay, we included PEM-resistant PC9 (PC9-RP), ERLO-resistant PC9 (PC9-RE) and PEM-resistant A549 (A549-RP) cell lines, which were established previously.<sup>12,13</sup> PEM decreased the viability of PC9 and PC9-RE cells in a dose-dependent manner, whereas PC9-RP cells showed apparent resistance to PEM (Figure 1). Similarly, PEM decreased the viability of A549 cells in a dose-dependent manner, whereas A549-RP cells showed clear

resistance to PEM. The PEM-induced decrease in the viability of PC9 and A549 cells was due to both growth arrest and cell death.<sup>13</sup>

### 3.2 | Pemetrexed sensitizes PC9 and A549 cells to cytotoxic immune cells

We next tested whether PEM could influence the sensitivity of their lung cancer cell lines to cytotoxic immune cells. We attempted to use anti-EGFR CAR-T cells as antigen-specific cytotoxic immune cells because the two NSCLC cell lines express EGFR on their cell surfaces (Figure S1A). Before the assays, T cells were *in vitro* expanded after 2 days of culture in anti-CD3 antibody-coated wells with 300 U/mL IL-2 and then with IL-2 alone for 7-10 days. Although the *in vitro* expanded CAR-T cells were unexpectedly positive for CD4,<sup>14</sup> we performed experiments using these activated T cells. The percentages of apoptotic cancer cells were examined by flow cytometry by gating CD45-negative cells. As a result, PEM significantly increased the susceptibility of PC9 and A549 cells to activated T cells (Figure 2A and B). These data are summarized in Figure 2C. We also determined whether PEM treatment could influence the sensitivity of these cancer cells to NK cells. First, we performed a 6-hour cytotoxicity assay, but no difference in sensitivity was observed (Figure S2). Therefore, we performed a 12-hour assay. The results showed that PEM significantly increased the susceptibility of PC9 and A549 cells to NK cells (Figure 2D and E). These data are summarized in Figure 2F. These results indicate that PEM treatment can increase the sensitivity of PC9 and A549 cells to different types of cytotoxic immune cells.

### 3.3 | Effects of pemetrexed on the expression of anti-apoptotic proteins in PC9 and A549 cells

Next, we searched for the mechanisms underlying the increased sensitivity of PEM-treated PC9 and A549 cells to activated T cells and NK cells. Given that the expression of intracellular anti-apoptotic proteins influences the sensitivity of cancer cells to cytotoxic immune cells,<sup>15</sup> we compared their expression in untreated and PEM-treated cancer cells (Figure 3). PEM treatment failed to affect the expression of cFLIP<sub>L</sub> and cFLIP<sub>S</sub>, both of which are inhibitors of caspase-8.<sup>16</sup>

Although PEM increased the expression of survivin in PC9 cells and Bcl-2 in PC9 and A549 cells, the expression of XIAP in PEM-treated PC9 cells and Mcl-1 in PEM-treated A549 cells was decreased.

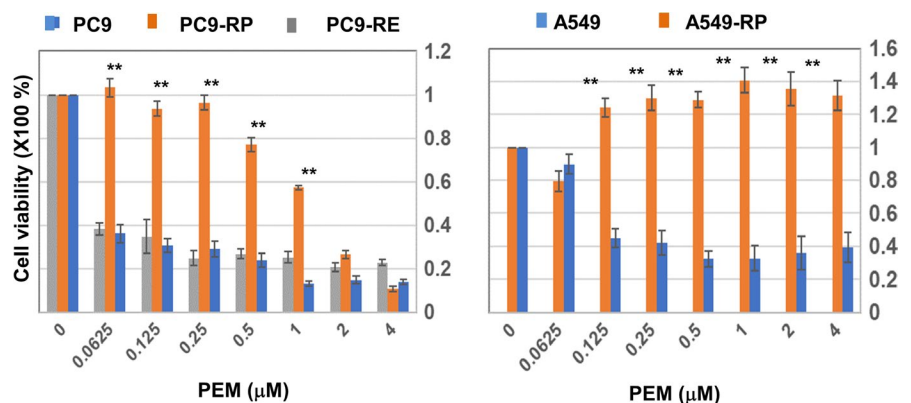
### 3.4 | Pemetrexed upregulates the expression of programmed death-ligand 1 on PC9 cells

Next, we examined the effects of PEM treatment on the expression of PD-L1 on PC9 and A549 cells. PEM treatment significantly increased the expression of PD-L1 on PC9 cells, whereas no increase in PD-L1 expression was observed on A549 cells (Figure 4A and B). PEM treatment increased the expression of PC9 and PC9-RE but not on PC9-RP cells. Furthermore, ERLO did not increase the expression of PD-L1 on PC9 cells but, rather, decreased it (Figure 4C and D).

Several signaling pathways, including the JAK-STAT, MAPK and NFκB pathways, participate in the expression of PD-L1 on human cancer cells.<sup>17,18</sup> To identify the pathways involved in the increased expression of PD-L1 on PEM-treated PC9 cells, we performed immunoblot analysis (Figure 4E). PEM treatment decreased the expression of STAT3, pSTAT3, p38, NFκB and IκB in PC9 cells, but increased the expression of pERK in PC9 cells. A decrease in IκB allows NFκB to enter the nucleus.<sup>19</sup> PEM treatment apparently increased the phosphorylation of ERK in PC9 and A549 cells; however, this change had no effect on the expression of PD-L1 on A549 cells (Figure 4A and B). In addition, inhibitors of ERK or NFκB inhibited the PEM-induced increase in the expression of PD-L1 on PC9 cells (Figure 4F). Taken together, these results indicate that PEM-induced upregulation of PD-L1 expression is partially dependent on activation of ERK and the NFκB pathway.

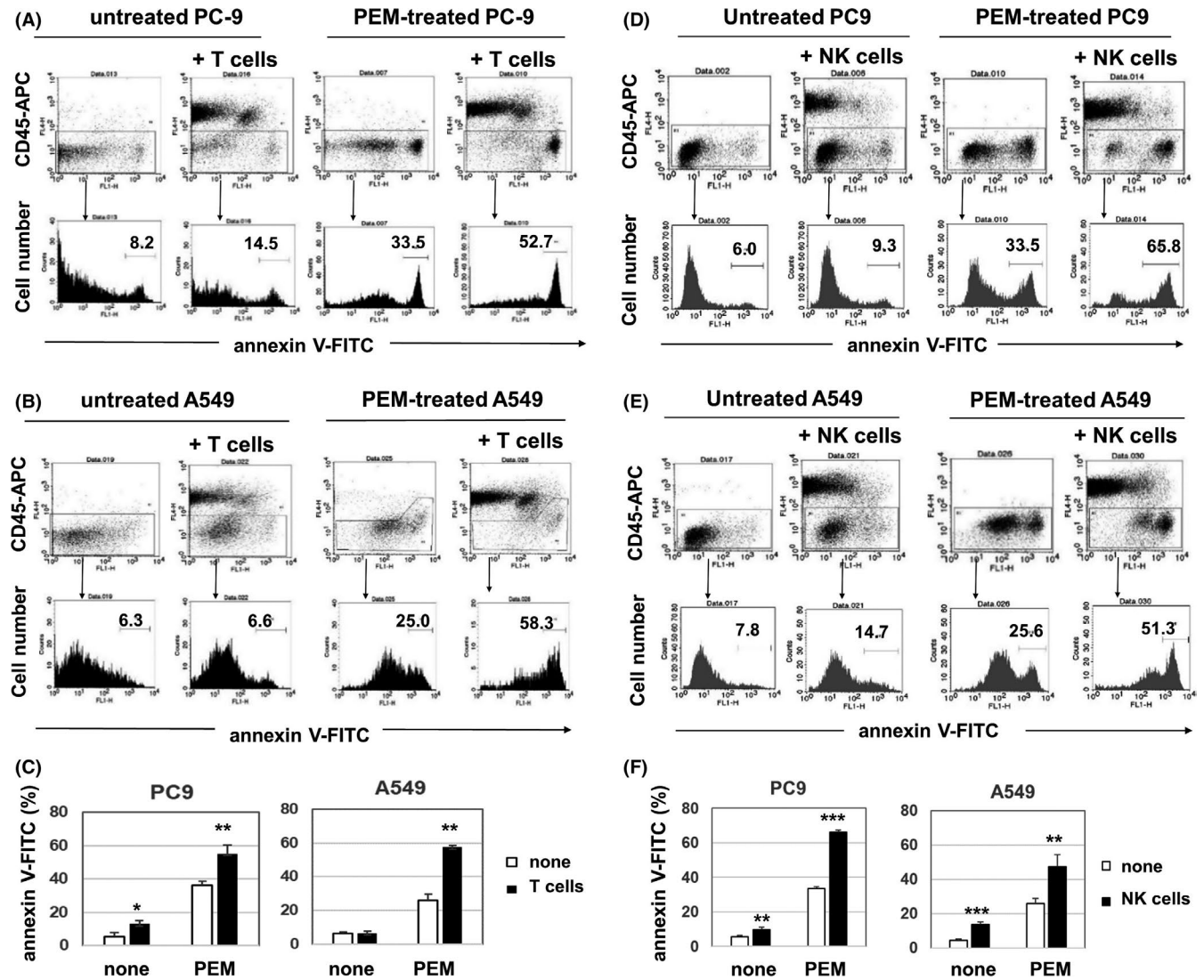
### 3.5 | Pemetrexed increases the expression of natural killer receptor ligands on PC9 and A549 cells

We next investigated the expression of NK receptor ligands, including MICA/B and ULBP1/2/5/6, on PC9 and A549 cells after PEM treatment. The results showed that although ULBP2/5/6 expression on both cell lines was upregulated by PEM treatment, PEM treatment significantly increased the expression of ULBP2/5/6 on PC9 cells; the increased expression of ULBP2/5/6 on A549 cells was not significant



**FIGURE 1** Pemetrexed (PEM) decreases the viability of non-small-cell lung cancer (NSCLC) cells. Cancer cells were cultured in the presence of the indicated doses of PEM for 2 d. The percent cell viability was determined by WST8 assay. \*\* $P < 0.01$





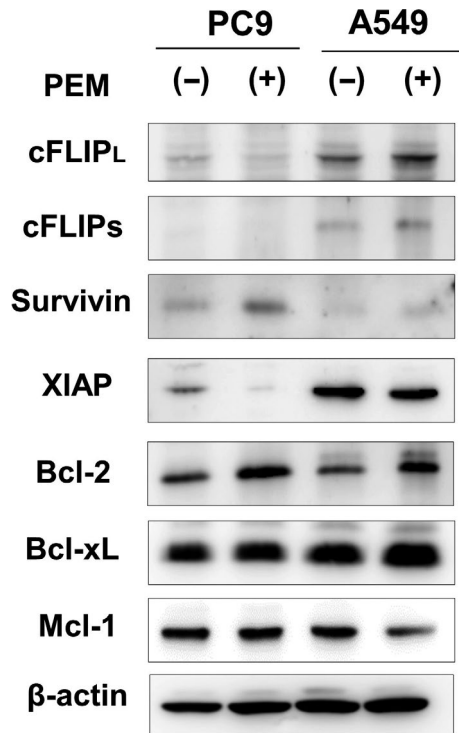
**FIGURE 2** Pemetrexed (PEM) sensitizes PC9 and A549 cells to activated T cells or natural killer (NK) cells. A and B, PC9 or A549 cells were cultured with PEM (2  $\mu\text{mol/L}$ ) for 2 d. Thereafter, untreated or PEM-treated PC9 or A549 cells ( $5 \times 10^4$  cells) were cultured with activated T cells ( $1 \times 10^5$  cells) in 96-well round plates for 6 h. After harvesting, whole cells were stained with anti-CD45-APC, followed by annexin V-FITC. A representative result from flow cytometry is shown. The numbers represent the percentages of annexin V<sup>+</sup> cells. C, The results from three wells are shown. Similar results were obtained in two separate experiments. \* $P < 0.05$ . \*\* $P < 0.01$ . D and E, Similarly, untreated or PEM-treated PC9 or A549 cells ( $5 \times 10^4$  cells) were cultured with purified NK cells ( $1 \times 10^5$  cells) for 12 h and analyzed by flow cytometry. A representative result from flow cytometry is shown. F, The results from three wells are shown. Similar results were obtained in two separate experiments. \*\* $P < 0.01$ . \*\*\* $P < 0.005$

(Figure 5A and B). Because the antibody against ULBP2/5/6 was unable to discriminate among ULBP molecules, we performed quantitative RT-PCR (RT-qPCR) and found that PEM treatment mainly increased the mRNA expression of ULBP2/5/6 in PC9 and A549 cells (Figure 5C). In addition, given that PEM can induce senescence in lung cancer cells<sup>13</sup> and that NK receptor ligands on senescent cells can be targets in immunosurveillance,<sup>20</sup> we next examined senescence in PEM-treated cancer cell lines. On confocal imaging, PEM treatment increased the expression of SA  $\beta$ -gal (Figure 5D). In addition, PEM treatment increased the expression of p21 in A549 cells (Figure 5E) and the production of IL-6 by PC9 cells and IL-8 by A549 cells (Figure 5F). The production of IL-8 by PC9 cells was slightly but significantly decreased by PEM treatment. The expression of SA  $\beta$ -gal,

growth arrest and production of inflammatory cytokines are features of senescent cells;<sup>21,22</sup> these results suggest that PEM can increase the expression of NK receptor ligands, ULBP, in association with the induction of senescence, and increase the sensitivity to NK cells.

### 3.6 | NKG2D ligands affect the sensitivity of pemetrexed-treated A549 cells to cytotoxic immune cells

Finally, we determined whether the PEM-induced changes in PD-L1 and NKG2D expression on lung cancer cells could influence their sensitivity to cytotoxic immune cells. The activated CD4<sup>+</sup> T cells



**FIGURE 3** Effect of pemetrexed (PEM) treatment on the expression of apoptosis-related molecules. PC9 and A549 cells were cultured with PEM (2  $\mu$ mol/L) for 2 d. After preparing cell lysates, the expression of the indicated proteins was examined by immunoblot.  $\beta$ -actin was measured as a control

used in this study had low positive expression of both PD-1 and NKG2D receptors (Figure 6A). The NK cells purified from PBMC from a healthy donor had low positive expression of PD-1 and clear positive expression of NKG2D (Figure 6B). Thus, we performed cytotoxicity assays in the presence of blocking antibodies against the PD-1 inhibitory receptor or the NKG2D-activating receptor. The results showed that the increase in the cytotoxicity of activated T cells against PEM-treated PC9 and A549 cells was not influenced by the addition of these antibodies (Figure 6C, Figure S3). In contrast, although the addition of anti-PD-1 antibody had no effect on the cytotoxicity of NK cells to PEM-treated A549 cells, that of anti-NKG2D antibody significantly decreased the cytotoxicity of NK cells against PEM-treated A549 cells (Figure 6D, Figure S4), suggesting a positive role for the PEM-induced increase in ULBP in NK cell-mediated cytotoxicity.

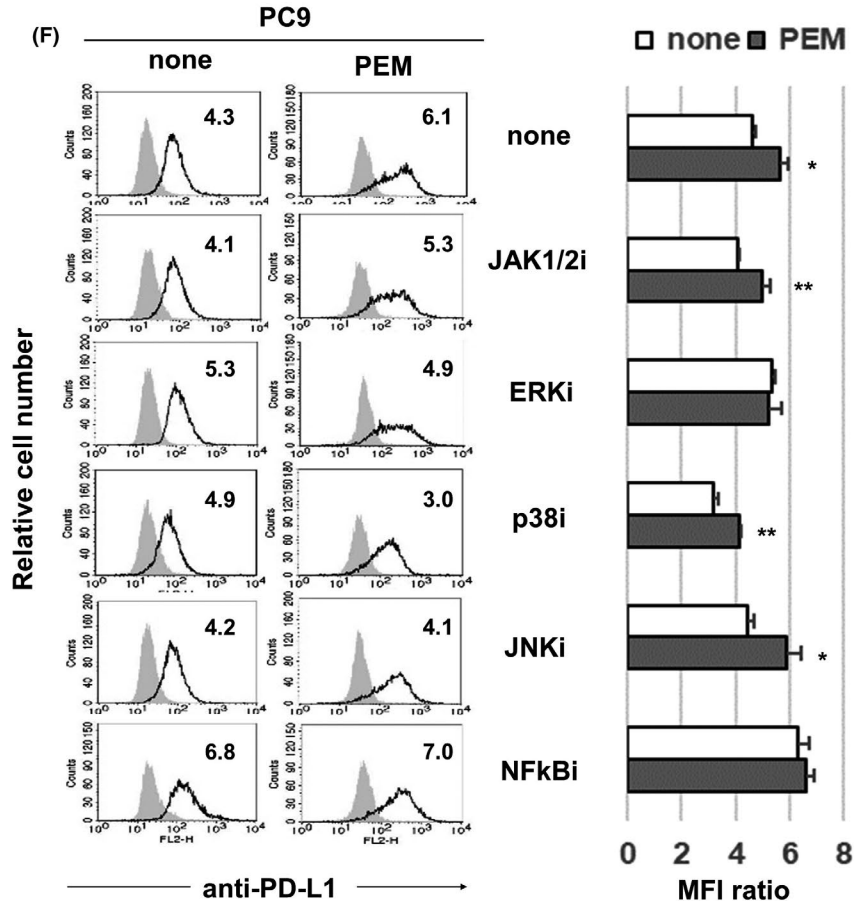
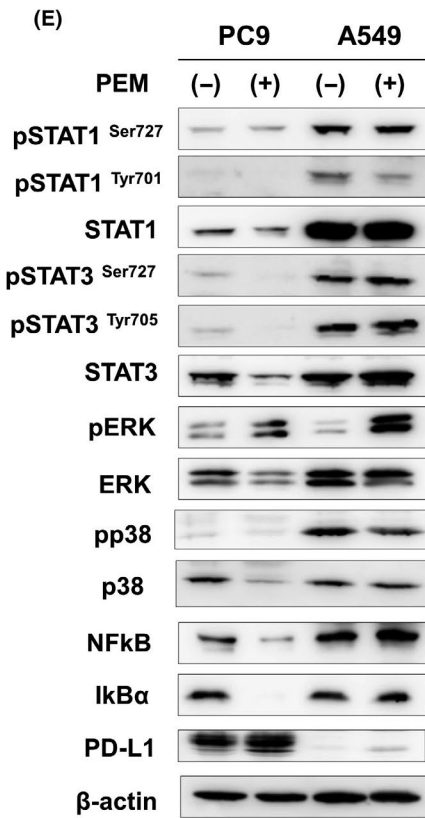
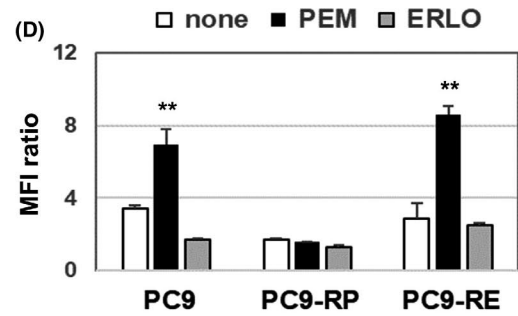
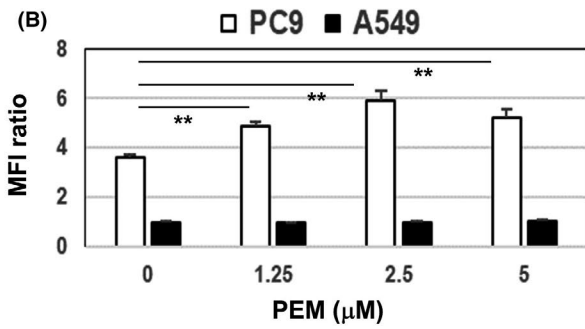
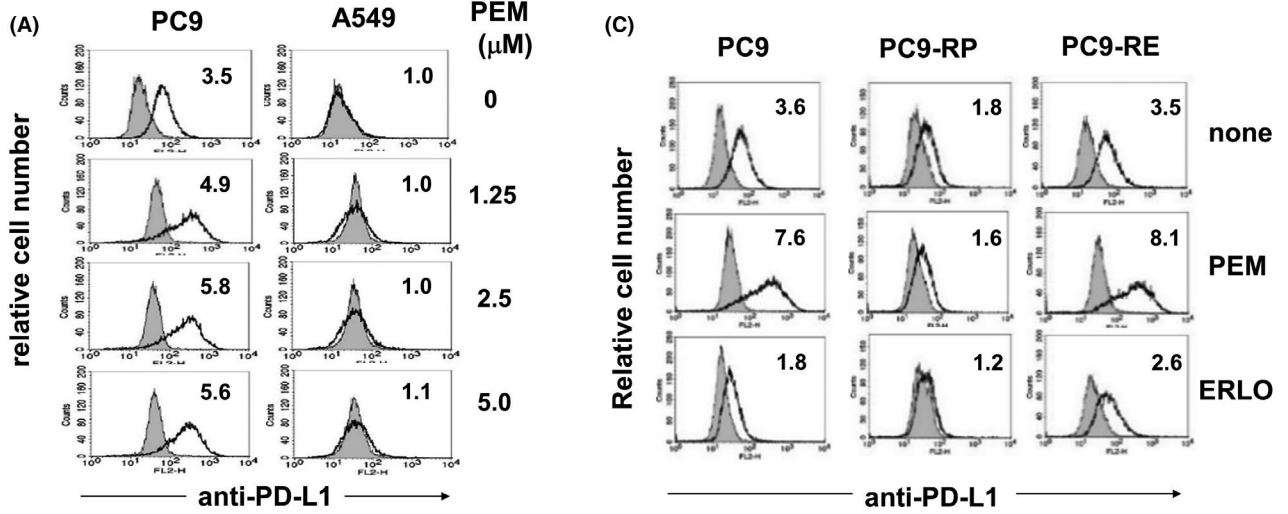
#### 4 | DISCUSSION

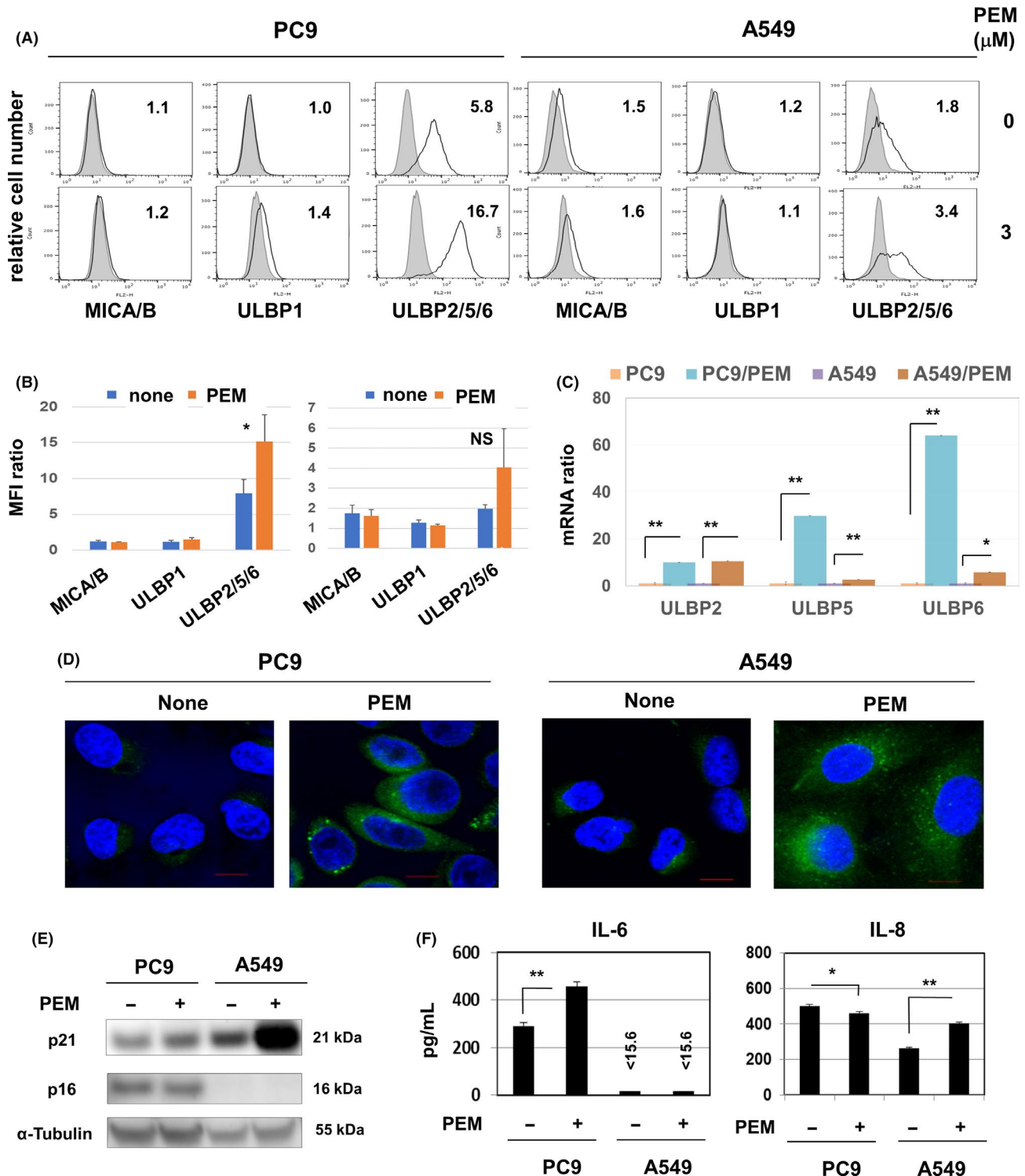
Pemetrexed has been frequently used as maintenance therapy for the treatment of NSCLC patients<sup>9</sup> and recently in combination with ICB therapy.<sup>3</sup> Although ICB therapy targeting the PD-1/PD-L1 interaction exerts anti-cancer effects via restoration of exhausted anti-cancer T cells,<sup>10</sup> the effects that PEM exerts on the sensitivity of NSCLC cells toward cytotoxic immune cells have not been elucidated. Here, we demonstrated that PEM can effectively augment the sensitivity of human NSCLC cells to cytotoxic immune cells.

Due to the unavailability of autologous lymphocytes for PC9 and A549 cell lines, we attempted to use allogeneic anti-EGFR CAR-T cells as antigen-specific cytotoxic immune cells. However, these T cells were found to be CD4<sup>+</sup> T cells after in vitro expansion. Nevertheless, they exerted higher levels of cytotoxicity against PEM-treated PC9 and A549 cells than against untreated cancer cells. In terms of the underlying mechanism, we recently reported that these activated CD4<sup>+</sup> T cells showed enhanced cytotoxicity against doxorubicin-treated human breast cancer cells partially through the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/death receptor system.<sup>14</sup> This apoptosis-inducing system may be involved in augmenting the sensitivity of PEM-treated NSCLC cells to cytotoxic immune cells. Both PC9 and A549 cell lines were positive for HLA class I but negative for HLA class II, and PEM treatment showed no effects on their expressions (Figure S1B). These results could exclude the possibility of the involvement of HLA class II-restricted killing of cancer cells by activated CD4<sup>+</sup> T cells. By contrast, PEM treatment decreased the expression of the anti-apoptotic proteins XIAP in PC9 cells and Mcl-1 in A549 cells (Figure 3). XIAP and Mcl-1 inhibit extrinsic and intrinsic apoptotic pathways<sup>23</sup> and are involved in resistance to immune cell-mediated cytotoxicity.<sup>24</sup> Therefore, decreased expression of these anti-apoptotic proteins might, at least in part, account for PEM-mediated sensitization of NSCLC cells to cytotoxic immune cells.

Pemetrexed increased the expression of PD-L1 on PC9 cells (Figure 4A). To identify signal pathways involved in this augmentation, we screened a panel of signaling molecules by immunoblot assay using specific inhibitors. Our results suggest that PEM-induced upregulation of PD-L1 is dependent on activation of ERK and the NF $\kappa$ B pathway. In terms of the mechanisms by which ERK activation led to increased expression of PD-L1 on PC9 cells, RAS/RAF/MEK, upstream molecules of ERK,<sup>25</sup> must be involved. Given that PC9 cells have an EGFR mutation and that RAS activation can

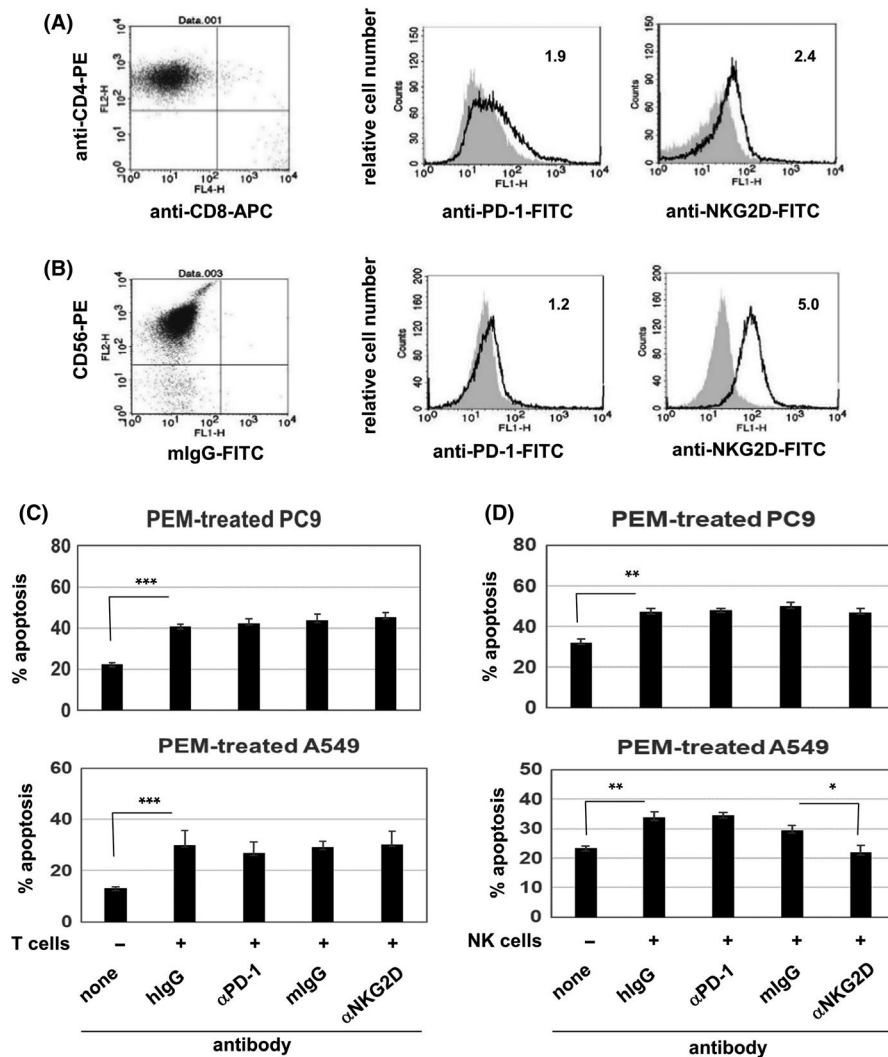
**FIGURE 4** Pemetrexed (PEM) increases the expression of programmed death-ligand 1 (PD-L1) on PC9 cells. A, Cancer cells were cultured with the indicated doses of PEM for 2 d. After harvesting, these cells were analyzed by flow cytometry. The bold line indicates staining with PE-conjugated anti-PD-L1 antibody, and the gray background indicates staining with isotype-matched PE-conjugated mouse IgG. The number represents the mean fluorescence intensity (MFI) ratio. B, The results from three samples are shown.  $**P < 0.01$ . C, PC9, PC9-RP and PC9-RE cells were cultured with PEM (3  $\mu$ mol/L) or ERLO (0.5  $\mu$ mol/L) for 2 d. After harvesting, these cells were analyzed by flow cytometry. The number represents the MFI ratio. D, The results from three samples are shown.  $**P < 0.01$ . E, Cancer cells were cultured with PEM (2  $\mu$ mol/L) for 2 d. Using lysates, the expressions of the indicated proteins and phosphorylated proteins were examined with the indicated antibodies.  $\beta$ -actin was measured as a control. F, PC9 cells were cultured with PEM (2  $\mu$ mol/L) for 2 d. The dose of the NF $\kappa$ B inhibitor was 0.5  $\mu$ mol/L, and the dose of other inhibitors was 4  $\mu$ mol/L. After harvesting, these cells were analyzed by flow cytometry. Left: The number represents the MFI ratio. Right: The results from three samples are shown.  $*P < 0.05$ .  $**P < 0.01$





**FIGURE 5** Pemetrexed (PEM) increases the expression of NKG2D ligands on PC9 and A549 cells and induces senescence. **A**, Cancer cells were cultured with PEM (3  $\mu\text{mol/L}$ ) for 2 d. The expression of the indicated proteins was examined by flow cytometry using the indicated antibodies. The bold line indicates staining with specific antibody, and the gray background indicates staining with isotype-matched control IgG. The number represents the mean fluorescence intensity (MFI) ratio. **B**, The results from three samples are shown. \* $P < 0.05$ . NS, not significant. **C**, Cancer cells were cultured with PEM (3  $\mu\text{mol/L}$ ) for 2 d, and quantitative RT-PCR was then performed. \* $P < 0.05$ . \*\* $P < 0.01$ . **D**, To examine the expression of senescence-associated  $\beta$ -galactosidase (SA  $\beta$ -gal), cancer cells were treated with or without PEM (2  $\mu\text{mol/L}$ ) for 2 d and stained with SPiDER  $\beta$ -gal. Scale bar: 10  $\mu\text{m}$ . **E**, Cancer cells were treated with PEM (2  $\mu\text{M}$ ) for 2 d, and the expressions of p21 and p16 in cell lysates were examined using the indicated antibodies.  $\alpha$ -tubulin was measured as a control. **F**, Cancer cells were treated with or without PEM (2  $\mu\text{mol/L}$ ) for 2 d. After harvesting, cancer cells were cultured without PEM for 2 d. Thereafter, ELISA was used to determine the levels of interleukin (IL)-6 and IL-8 in the supernatant. \* $P < 0.05$ , \*\* $P < 0.01$





**FIGURE 6** Role of programmed death-ligand 1 (PD-L1) and NKG2D ligands on pemetrexed (PEM)-treated cancer cells in mediating sensitivity to cytotoxic immune cells. (A) Activated T cells were stained with APC-conjugated anti-CD8, PE-conjugated anti-CD4, FITC-conjugated anti-PD-1 or FITC-conjugated anti-NKG2D antibody. As a control, these cells were stained with FITC-conjugated mouse IgG. The bold line indicates staining with FITC-conjugated anti-PD-L1 antibody, and the gray background indicates staining with isotype-matched FITC-conjugated mouse IgG. The number represents the mean fluorescence intensity (MFI) ratio. (B) Similarly, purified natural killer (NK) cells were stained with PE-conjugated anti-CD56 antibody and analyzed by flow cytometry. The number represents the MFI ratio. (C) Cancer cells that were cultured with PEM (2  $\mu$ mol/L) for 2 d were cultured with activated T cells in the presence of the indicated antibodies in 96-well plates for 6 h. After harvesting, annexin V-FITC positive cells were examined as shown in Figure 2. (D) Similarly, PEM-treated cancer cells were cultured with *in vitro* NK cells in the presence of the indicated antibodies in 96-well plates for 12 h. After harvesting, annexin V-FITC positive cells were examined as shown in Figure 2D and E. \* $P < 0.05$ . \*\* $P < 0.01$ . \*\*\* $P < 0.005$

be induced by signals via receptor tyrosine kinase, RAS activation could lead to ERK activation. Indeed, several studies have revealed that MEK/ERK signaling, as well as NF $\kappa$ B, can increase the expression of PD-L1 on cancer cells.<sup>26,27</sup> However, PEM treatment also increased the phosphorylation levels of ERK in A549 cells, but no change of their PD-L1 expression was observed. These results suggest that increased phosphorylation of ERK does not necessarily result in increased expression of PD-L1. In contrast, a recent report suggested that IL-6, which is involved in the JAK/STAT3 pathway, contributes to the expression of PD-L1 on NSCLC.<sup>28</sup> Thus, we examined the *in vitro* effect of IL-6 on PD-L1 expression on PC9 and A549 cells but no change was observed (Figure

S5). In addition, PEM treatment failed to change the expression of pSTAT3 in PC9 cells (Figure 4E).

Pemetrexed treatment increased the expression of NK receptor ligands, ULBP, on PC9 and A549 cells (Figure 5A). We also confirmed that the activating NK receptor NKG2D was expressed on both NK cells and T cells (Figure 6A and B). Of note, PEM-induced upregulation of ULBP on A549 cells seemed to contribute to their increased sensitivity to NK cells because the addition of anti-NKG2D antibody decreased the levels of cytotoxicity (Figure 6D). We also examined the expression of NKG2D on NK cells from four healthy donors and found that variation of the NKG2D expression is small (Figure S6). These findings suggest that PEM treatment may increase

the expression of NK receptor ligands on cancer cells, leading to enhancement of their sensitivity to NK cell-mediated cytotoxicity in NSCLC patients.

In cellular senescence, cells undergo irreversible cell cycle arrest in response to a variety of cellular stresses, including anti-cancer drugs.<sup>29</sup> Senescent cells acquire a unique feature, called a senescence-associated secretory phenotype (SASP), and they produce some inflammatory cytokines, such as IL-6 and IL-8, which lead to cancer promotion and recurrence.<sup>21,22</sup> Therefore, senescent cancer cells with SASP are a candidate therapeutic target in anti-cancer therapy.<sup>30</sup> Alternatively, senescent cancer cells upregulate NK receptor ligands, such as MICA/B and ULBP.<sup>31,32</sup> Such ligands activate NK cells and T cells, both of which play important roles in immunosurveillance and anti-cancer immunity. In a recent couple of years, NKG2D and its ligands have been given considerable attention as targets for anti-cancer immunotherapy.<sup>33-35</sup> These lines of evidence suggest that NK cell-mediated cytotoxicity could be involved in the therapeutic efficacy of ICB therapy combined with PEM in NSCLC patients.

Both PEM and methotrexate are anti-folate drugs, and methotrexate has the immunosuppressive properties and has been used in graft-versus-host disease, rheumatoid arthritis and other chronic inflammatory diseases.<sup>36</sup> Therefore, we examined the effects of PEM on the viability of PC9 cells and CAR-T cells in vitro. Both cells showed similar sensitivity toward PEM (Figure S7), indicating its immunosuppressive effect. However, PEM has been reported to enhance the effects of anti-cancer immunotherapy through the induction of immunogenic cancer cell death and the promotion of infiltration/activation of T cells in murine tumor models.<sup>37</sup> More importantly, PEM has been combined with ICB therapy for NSCLC patients.<sup>3</sup> Further studies are needed to elucidate the discrepant in vitro and in vivo results of PEM

In conclusion, we revealed that PEM can effectively sensitize human NSCLC cells to cytotoxic immune cells while modulating the expression of immune-regulatory molecules. We hope that these findings provide useful information to improve the efficacy of combination therapy with ICB therapy and PEM for NSCLC patients.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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