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Potential of fluoropyrimidine to be an immunologically optimal partner of immune checkpoint inhibitors through inducing immunogenic cell death for thoracic malignancies

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Abstract

Background: Immune checkpoint inhibitors (ICIs) are a revolutionary paradigm in the treatment of thoracic malignancies and chemoimmunotherapy is a current standard care in this field. Chemotherapeutic agents are known to induce not only direct cytotoxic effects on tumor cells but also immune modulating effects, such as stimulating immunogenic cell death (ICD). Currently, either pemetrexed (PEM) or taxane plus platinum are combined with ICIs for patients with non-small cell lung cancer (NSCLC); however, it is still unknown whether these agents are immunologically optimal partners for ICIs.

Methods: To determine the immunologically optimal chemotherapeutic agent, we first evaluated the ability of several chemotherapeutic agents, including platinum, PEM, taxane, and 5-fluorouracil (5-FU) to induce ICD using several thoracic tumor cell lines in vitro. ICD was evaluated by the cell surface expression of calreticulin (CRT) and adenosine-triphosphate (ATP) secretion. We further performed an antitumor vaccination assay in vivo.

Results: 5-FU induced cell surface expression of CRT and ATP secretion most efficiently among the several chemotherapeutic agents. This effect was enhanced when it was combined with platinum. In the antitumor vaccination assay in vivo, we found that vaccination with dying-AB1-HA (a murine malignant mesothelioma cell line) cells treated with 5-FU, but neither PEM nor PTX, reduced the tumor growth of living-AB1-HA cells inoculated 1 week after vaccination by recruiting $CD3^{+}CD8^{+}$ T cells into the tumor microenvironment.

Conclusion: Our findings indicate that fluoropyrimidine can be an immunologically optimal partner of ICIs through the induction of ICD for thoracic malignancies.

KEYWORDS
5-fluorouracil, cancer immunotherapy, fluoropyrimidine, immunogenic cell death, thoracic malignancy

INTRODUCTION

Cancer immunotherapy is a revolutionary paradigm in the treatment of malignancies, and especially immune checkpoint inhibitors (ICIs), such as anti-programmed cell death-1 (PD-1), programmed cell death ligand-1 (PD-L1), and cytotoxic T lymphocyte antigen-4 (CTLA-4) antibodies are now widely used globally.[1] ICIs are known to show prolonged therapeutic efficacy in some patients, and the 5-year overall survival (OS) rate of pretreated advanced non-small

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cell lung cancer (NSCLC) patients who receive nivolumab, an anti PD-1 antibody, has been reported to be 16%, which has had a huge impact in the field of cancer therapy.[2] At the same time, however, the response rate (RR) of ICI monotherapy is not necessarily high and only a limited number of patients can benefit from it. To overcome this challenge, combined cancer immunotherapy, the combination of ICI with chemotherapy, radiotherapy, or another immunotherapy, has been actively investigated in recent years, and some combinations have already been applied in the clinical setting.

Thoracic malignant tumors, such as lung cancer or malignant pleural mesothelioma (MPM), are the leading cause of cancer death worldwide.[3] However, the prognosis of these tumors, especially NSCLC, is improving with the development of new chemotherapeutic agents, angiogenesis inhibitors, molecular targeted therapies, and immunotherapies, including combined cancer immunotherapies.[4] Currently, for patients with advanced NSCLC who have no oncogenic driver mutations, ICIs combined with platinum doublet chemotherapy are regarded as standard regimens in the first-line setting. Specifically, platinum plus either PEM or taxane (paclitaxel or nanoparticle albumin-bound (nab)-paclitaxel) for nonsquamous NSCLC, and platinum plus taxane for squamous NSCLC are generally selected.[5–7] These combination regimens were established as these platinum doublet chemotherapies had been standard for patients with NSCLC before the approval of ICIs.[8,9] However, whether these chemotherapeutic agents are the best partners for ICIs, from an immunological viewpoint, has not yet been fully considered.

Chemotherapeutic agents are known to show a favorable impact on the antitumor immune response.[10] Reducing the number of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs),[11–13] or stimulating immunogenic cell death (ICD)[14] are the fundamental mechanisms and rationale of combination therapy with ICIs. ICD is one of several cell death processes and is characterized by the release of damage-associated molecular patterns (DAMPs) on the surface of dying cells.[15–17] The essential feature of ICD is to induce the translocation of calreticulin (CRT), an endoplasmic reticulum (ER) chaperone, to the cell membrane, which enables it to provide a potent "eat me" signal for phagocytic cells and thereby initiate adaptive immunity.[18] In addition to CRT exposure, ICD is characterized by adenosine-triphosphate (ATP) secretion from dying cells, which can accumulate DCs and macrophages ("find me" signal).[19] Several chemotherapeutic agents have shown their potential to induce ICD; however, it is known that their abilities are not equivalent. Therefore, a study to determine the best inducer of ICD, especially in thoracic malignant tumor cells, is warranted.

We recently reported that fluoropyrimidine suppressed the infiltration of MDSCs into the tumor microenvironment by regulating tumor-derived Bv8 and S100A8 in both MPM and a lung cancer model, and that S-1 enhanced the therapeutic efficacy of ICI treatment more efficiently than PEM.[20] Based on this background, we investigated the potential of several chemotherapeutic agents, including fluoropyrimidine, which are currently administered for patients with thoracic malignancies, to induce ICD in NSCLC and MPM cells to determine the immunologically optimal partner of ICIs for the treatment of thoracic malignancies. In addition, we evaluated the antitumor effects triggered by chemotherapy-induced-ICD in mice.

METHODS

Cell lines

A549 cells (a human lung adenocarcinoma cell line) and H2170 cells (squamous cell carcinoma cell lines) were purchased from American Type Culture Collection (USA) and PC9 cells (a human lung adenocarcinoma cell line) was purchased from Immuno-Biological Laboratories Co. (Japan). H226 cells (human lung squamous cell carcinoma cell lines) were obtained from Dr J. D. Minna (University of Texas Southwestern Medical School, USA) and Dr M. Akiyama (Radiation Effects Research Foundation, Japan), respectively. AB1-HA cells (a murine malignant mesothelioma cell line), a transfectant with the gene encoding influenza HA into an AB1 cells, were purchased from Public Health England (UK). Tumor cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (50 μg/mL). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Chemotherapeutic agents

PEM, 5-FU, vinorelbine (VNR), paclitaxel (PTX), docetaxel (DTX), gemcitabine (GEM), cisplatin (CDDP), and carboplatin (CBDCA) were purchased from Selleck Chemicals.

Animals

Six-week-old male BALB/c mice were obtained from Charles River Japan Inc. (Japan). All experiments were performed in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use. At the end of each in vivo experiment, the mice were anesthetized with isoflurane and euthanized humanely by cutting the subclavian artery. All experimental protocols were reviewed and approved by the animal research committee of Tokushima University, Tokushima, Japan (approved no. T30-130 and T2021-110).

Cell proliferation assay

Human and murine thoracic tumor cells (2×10^3 cells per 100 μL) were plated into each well of a 96-well plate (BD Biosciences) in 10% FBS containing Dulbecco's

modified Eagle medium (DMEM) and incubated for 24 h. Chemotherapeutic agents were then added, and the cells were incubated for an additional 72 h. The proliferation of tumor cells was measured using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium) dye reduction method. The absorbance was measured with a SUNRISE Remote R microplate reader (Tecan). The median inhibitory concentration (IC_{50}) for each agent was calculated from survival curves.

Flow cytometry

The human and murine thoracic tumor cells (5×10^5 cells/mL) were plated into each well of a six-well plate (BD Biosciences) in 10% fetal bovine serum (FBS) containing DMEM and incubated for 24 h. Chemotherapeutic agents (10^{-2} to $10 \times IC_{50}$) were then added, and the cells were incubated for an additional 24 or 48 h. To examine the surface expression of CRT on human and murine cells, PE-conjugated mouse monoclonal antibody to CRT (1:50, FMC75, Abcam) and Alexa 488-conjugated rabbit monoclonal antibody to CRT (1:50, EPR3924, Abcam) were used, respectively. To examine the expression PD-L1 on human cells, PE-conjugated mouse monoclonal antibody to PD-L1 (1:100, W6/32, Thermo Fisher Scientific) was used. The stained cells were analyzed by flow cytometry using a BD LSRFortessa (BD Bioscience) for acquisition and the FlowJo software program (Treestar Inc.) for the analysis. Data were also expressed as mean fluorescence intensity (MFI).

ATP assay

The secretion of ATP from tumor cells treated with or without chemotherapeutic agents were measured with a RealTime-Glo extracellular ATP assay kit (Promega) according to the manufacturer's protocol. Briefly, the tumor cells (1×10^4 cells) were plated into each well of a 96-well white plate (136 101, Thermo Fisher Scientific) and incubated for 24 h. Chemotherapeutic agents (10^{-2} to $10 \times IC_{50}$) followed by RealTime-Glo extracellular ATP assay reagents were then added, and the cells were incubated for an additional 36 h. The luminescence was measured with a GloMax Discover system plate reader.

Western blotting

The tumor cells treated with or without chemotherapeutic agents (IC_{50}) for 6 h were homogenized in M-PER reagents (Thermo Fisher Scientific) containing phosphatase and protease inhibitor cocktails (Roche). The protein lysates were transferred to polyvinylidene fluoride membrane and blocked by blocking one solution for 1 h (Nacalai Tesque) The membranes were incubated overnight at 4°C with primary antibodies (dilution: 1:1000) to eIF-2α (9722, Cell Signaling Technology) phospho-eIF-2α (9721, Cell Signaling Technology), and β-actin (sc-47 778, Santa Cruz Biotechnology). After washing, membranes were applied using horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) and visualized through a chemiluminescent Amersham Imager 600 machine (Amersham). The intensity was quantitated with ImageJ software (National Institute of Health).

Antitumor vaccination assay in the animal model

AB1-HA cells (5×10^5 cells per well) were treated with 5-FU, PEM, or PTX ($10 \times IC_{50}$) for 48 h in six-well plate (BD Bioscience). For vaccination, tumor cells treated with chemotherapeutic agents were subcutaneously injected into the left flank of immunocompetent BALB/c mice (1×10^6 cells in 100 μL PBS per mouse; 7 mice per group). Saline or freeze-thawing-based necrotic AB1-HA cells (freeze-thawing for three times, which is not highly immunogenic) were injected as a control. One week later, the living AB1-HA cells (1×10^6 cells in 100 μL PBS per mouse) were injected into the right flank of vaccinated mice. The tumor size was measured using vernier calipers twice a week, where volume = $a \times b^2/2$ (a, long diameter; b, short diameter). The animal experiments were repeated twice.

Immunofluorescence of mouse tumor tissues

Excised tumor tissues from AB1-HA-bearing mice were placed into Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co.) and snap-frozen. Frozen tissue sections (8 μm thick) were fixed with 4% paraformaldehyde solution in phosphate buffered saline (PBS) and used for the identification of $CD3^+CD8^+$ T cells using a rabbit anti-CD3 monoclonal antibody (1:150, SP7; Abcam) and a rat anti-CD8a monoclonal antibody (1:150, 53–6.7; BD Pharmingen). Alexa 488-labeled goat anti-rabbit and Alexa 594-labeled goat anti-rat secondary antibody (1:250; Invitrogen) was used for immunofluorescent detection. Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). In each slide, the number of double-positive cells was counted under a fluorescent microscope at ×200 magnification. The images were acquired using an Olympus BX61 fluorescence light microscope (Olympus).

Statistical analysis

The data are presented as the mean ± standard error of the mean. The statistical analyses were performed using a one-way analysis of variance, followed by Dunnett's multiple comparison post hoc test, as appropriate. p-values of <0.05 were considered to be statistically significant.

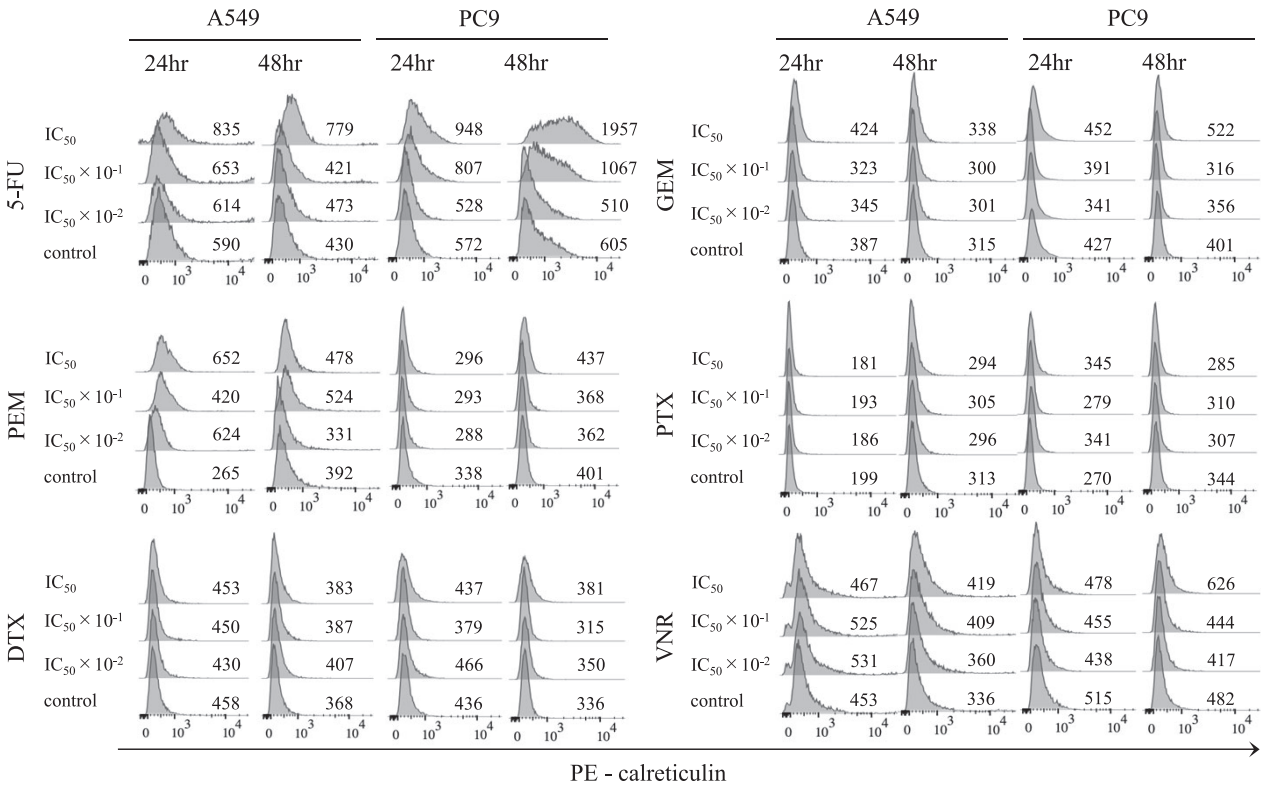

FIGURE 1 5-fluorouracil (5-FU) induced the cell surface expression of calreticulin (CRT) in human nonsquamous non-small cell lung cancer (NSCLC) cell lines. A549 and PC9 cells were incubated with the chemotherapeutic agents at their 10^{-2} to 1 × IC_{50} values for 24 and 48 h. The cell surface expression of calreticulin (CRT) was evaluated by flow cytometry. The number indicates the mean fluorescence intensity.

RESULTS

Efficiency of 5-FU in the induction of CRT was higher than that of PEM or taxane in human NSCLC cell lines

We initially performed the cell proliferation assay with several human NSCLC cell lines and determined the IC_{50} of several chemotherapeutic agents that are generally administered in the treatment of patients with NSCLC. Six third-generation chemotherapeutic agents (e.g., 5-FU [a representative fluoropyrimidine], PEM, DTX, GEM, PTX, and VNR) and two platinum agents (CDDP and CBDCA) were included in this analysis. The IC_{50} for each agent was calculated from survival curves after 72 h incubation (Table S1). We next analyzed the cell surface expression of CRT on NSCLC cells after treatment with each chemotherapeutic agent at their 10^{-2} to 1 × IC_{50} values to evaluate the effects in a unified scale by flow cytometry. Regarding lung adenocarcinoma, 5-FU notably induced the cell-surface expression of CRT in both A549 and PC9 cells, even after 48 h of incubation (Figure 1). On the other hand, PEM, a current standard partner of ICIs, induced the cell surface expression of CRT on A549 cells, but not PC9 cells. Furthermore, the effects of PEM last only for 24 h, but not 48 h. Other chemotherapeutic agents, including taxane had little effect on the cell surface expression of CRT. Regarding lung squamous cell carcinoma, 5-FU also induced the cell surface expression of CRT more efficiently than taxane (another current standard partner of ICIs) in both cell lines tested (Figure 2). We also observed that a higher concentration of 5-FU showed inferior efficacy in the induction of ICD to middle or lower concentration in some cells (H2170) (Figure 2), suggesting that the optimal concentration would be different in each cell line.

5-FU enhanced the secretion of ATP from human NSCLC cells

We next evaluated the secretion of ATP from NSCLC cells after treatment with each chemotherapeutic agent at their 10^{-2} to 1 × IC_{50} values by extracellular ATP assay based on bioluminescence detection chemistry. 5-FU enhanced the secretion of ATP from both PC9 (adenocarcinoma) and H226 (squamous cell carcinoma) cells in a dose-dependent manner (Figure 3a). Furthermore, the efficiency of 5-FU in the secretion of ATP from PC9 cells was superior to PEM (Figure 3b). To address whether 5-FU influenced the expression of other determinant factors for efficacy of ICI, we analyzed the expression MHC class I on PC9 and H226 cells after treatment with 5-FU at 10^{-2} to 1 × IC_{50} values. 5-FU did not upregulate the expression of MHC class I on both cells (Figure S1). These data suggest that 5-FU can

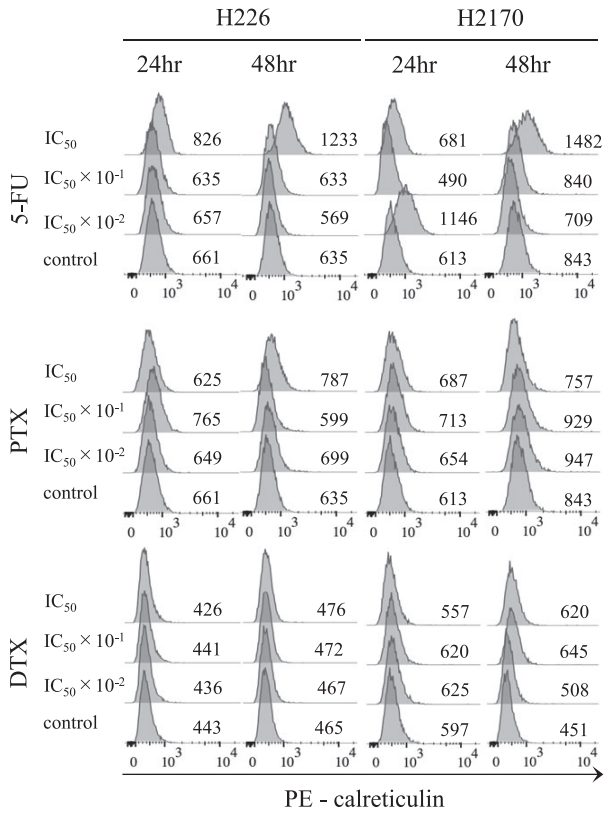

FIGURE 2 5-fluorouracil (5-FU) induced the cell surface expression of calreticulin (CRT) in human squamous non-small cell lung cancer (NSCLC) cell lines. H226 and H2170 cells were incubated with the chemotherapeutic agents at their 10^{-2} to 1 × IC_{50} values for 24 and 48 h. The cell surface expression of CRT was evaluated by flow cytometry. The number indicates the mean fluorescence intensity.

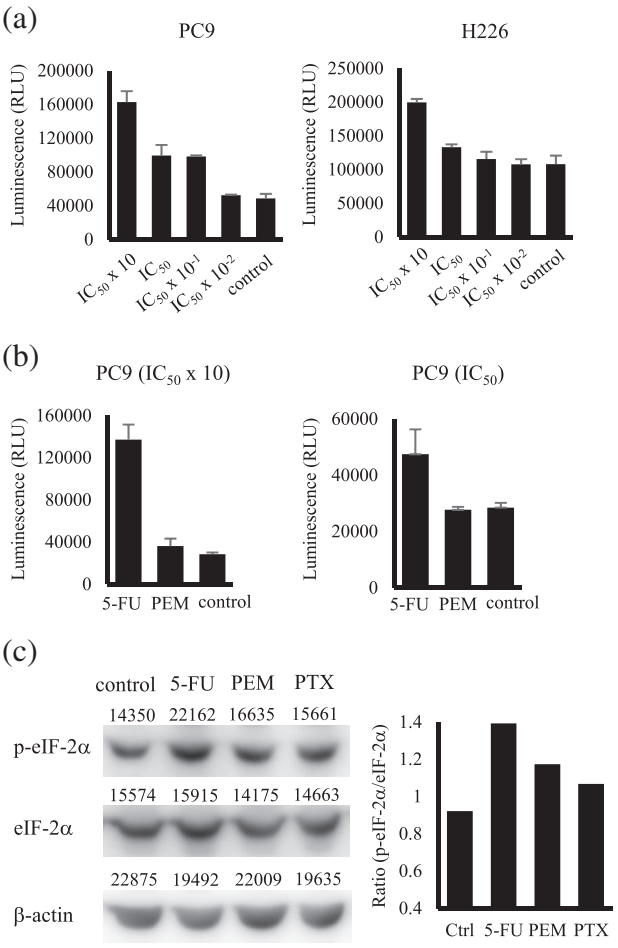

FIGURE 3 5-fluorouracil (5-FU) enhanced the secretion of adenosine-triphosphate (ATP) from human non-small cell lung cancer (NSCLC) cells. (a) PC9 and H226 cells were plated into a 96-well white plate and incubated for 24 h. 5-FU (10^{-2} to 10 × IC_{50}) followed by RealTime-Glo extracellular ATP assay reagents were then added, and the cells were incubated for an additional 36 h. The luminescence was measured with a GloMax Discover system plate reader. (b) PC9 cells were treated with 5-FU or pemetrexed (PEM) at their 1 to 10 × IC_{50} values for 36 h, and the luminescence was measured. (c) PC9 cells were treated with or without 5-FU, PEM, or paclitaxel (PTX) for 6 h. The cells were then homogenized and used for western blotting to evaluate the expression and phosphorylation of eIF-2α. The expression of β-actin was also evaluated as an internal control. The membrane was visualized through a chemiluminescent Amersham Imager 600 machine, and the intensity was quantitated with ImageJ software.

induce ICD more efficiently than other chemotherapeutic agents in NSCLC cells.

5-FU efficiently enhanced the phosphorylation of eIF-2α in human NSCLC cells

We next analyzed the mechanisms in which 5-FU induced the cell surface expression of CRT on NSCLC cells. As previously reported that CRT expression on the cell surface membrane is triggered by ER stress and the phosphorylation of eIF-2α is essential in this process,[21] we evaluated the effects of 5-FU on this signaling pathway. The efficacy of 5-FU in the induction of eIF-2α phosphorylation in PC9 cells was superior to that of PEM or PTX (Figure 3c), suggesting that this signaling pathway may play an important role in the promotion of CRT expression on NSCLC cells.

Ability of 5-FU in the induction of ICD in human NSCLC cells was enhanced when combined with platinum

The current standard regimens of chemotherapy, which are combined with ICI, are platinum-doublet (platinum plus either PEM or taxane) for the first-line treatment of advanced stage NSCLC. Either PEM or taxane is chosen based on the histology or molecular phenotypes of tumor cells and comorbidities of the patients. Therefore, we next investigated the effects of combination therapy of 5-FU and platinum in the induction of ICD in NSCLC cells. Platinum agents, such as CBDCA or CDDP, also induced the cell surface expression of CRT on NSCLC cells (Figure 4). Furthermore, while low-dose monotherapy with 5-FU or platinum (10^{-2} to 10^{-1} × IC_{50}) did not induce the cell surface expression of CRT, combination therapy with low-dose 5-FU and platinum (10^{-2} × IC_{50}) notably induced the cell surface expression of CRT (Figure 5). Taken together, the present results indicate that fluoropyrimidine

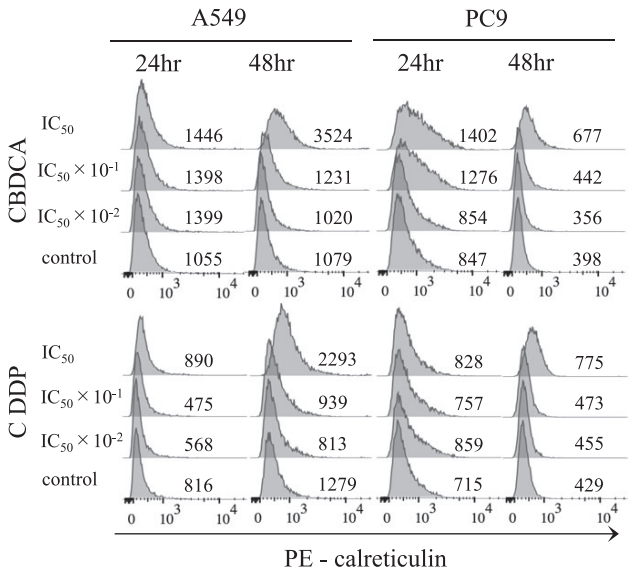

FIGURE 4 Platinum cytotoxic agents induced the cell surface expression of calreticulin (CRT) in human non-small cell lung cancer (NSCLC) cell lines. A549 and PC9 cells were incubated with platinum cytotoxic agents (e.g., cisplatin [CDDP] and carboplatin [CBDCA]) at their 10^{-2} to 1 × IC_{50} values for 24 and 48 h. The cell surface expression of CRT was evaluated by flow cytometry. The number indicates the mean fluorescence intensity.

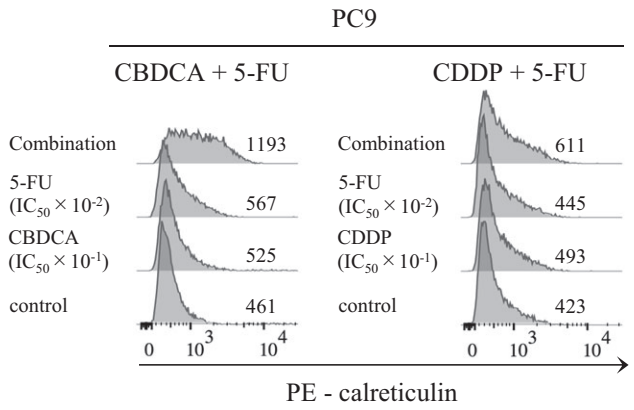

FIGURE 5 The ability of 5-fluorouracil (5-FU) to induce immunogenic cell death (ICD) in human non-small cell lung cancer (NSCLC) cell lines was enhanced by combination with platinum. PC9 cells were incubated with 5-FU (10^{-2} × IC_{50} value) and/or carboplatin (CBDCA) (10^{-1} × IC_{50} value) for 48 h. The cell surface expression of calreticulin (CRT) was evaluated by flow cytometry. The number indicates the mean fluorescence intensity.

analog-containing platinum doublet chemotherapy has the potential to enhance ICD and improve the antitumor effects of ICI therapy.

Tumor cells treated with 5-FU, but not PEM, induced antitumor immunogenicity in vivo

We next performed an antitumor vaccination assay to determine whether 5-FU-induced ICD enhanced the antitumor immune response in vivo. We used our syngeneic mouse model using a murine mesothelioma cell line (AB1-HA) for this analysis.[22] First, we treated AB1-HA cells with 5-FU, PEM, or PTX at their 10^{-2} to 10 × IC_{50} values in vitro, and found that high dose (10 × IC_{50}) of 5-FU, but neither PEM nor PTX, induced the cell surface expression of CRT (Figure 6a). We next vaccinated the immunocompetent BALB/c mice subcutaneously with dying-AB1-HA cells treated with 5-FU, PEM, or PTX at their 10 × IC_{50} values. We vaccinated the control mice with saline or dying-AB1-HA cells induced by freeze-thawing. At 1 week after vaccination, we rechallenged the living AB1-HA cells in the opposite side of each mouse and measured the rechallenged tumor volume twice a week for an additional 2-week period (Figure 6b). The growth rate of rechallenged tumor in necrosis group (the mice vaccinated with dying-AB1-HA cells induced by freeze-thawing) was comparable to that in saline group, suggesting that dying-AB1-HA cells induced by freeze-thawing were less immunogenic. The mice vaccinated with dying-AB1-HA cells by 5-FU, but neither PEM nor PTX, reduced the growth of rechallenged tumor growth (Figure 6c). In addition, the number of tumor-infiltrating cytotoxic T lymphocytes, defined as $CD3^+CD8^+$ T cells, was significantly increased in rechallenged tumor tissue from mice vaccinated with 5-FU-treated tumor cells (Figure 6d,e). These results indicate that, among the chemotherapeutic agents used to treat thoracic tumors, 5-FU would be a potent inducer of ICD and an optimal partner for ICI agents in combined cancer immunotherapy.

DISCUSSION

Fluoropyrimidines, such as 5-FU, are an essential component of antimetabolites, and form the foundation of a wide variety of chemotherapeutic regimens.[23] In fact, 5-FU is the third most commonly used chemotherapeutic agent for solid malignancies.[24] S-1 is an oral fluoropyrimidine formulation that consists of tegafur, a prodrug of 5-FU, 5-chloro-2,-4-dihydroxypyridine, and potassium oxonate.[25] Fluoropyrimidine is known to induce not only direct cytotoxic effects on cancer cells but also antitumor immune response by affecting multiple cell types.[15,26] For example, fluoropyrimidine showed the potential to deplete MDSCs in a MPM and lung cancer model,[20] thymoma model[13] and to facilitate antigen presentation by dendritic cells in a gastric cancer model.[13,27] Another study showed that fluoropyrimidine was an inducer of ICD in colorectal cancer cells.[28] Furthermore, in recent phase III trials, combination therapy of fluoropyrimidine based chemotherapy with ICI showed favorable therapeutic efficacy in comparison to chemotherapy alone in gastric and esophageal cancer.[29–31] However, although S-1 is a standard regimen for patients with NSCLC,[32,33] the immunological effects of fluoropyrimidine in NSCLC cells remain unclear.

PEM, an antifolate, is one of the key chemotherapeutic agents in the treatment of nonsquamous NSCLC and MPM. It exerts the function by inhibiting the enzymes involved in

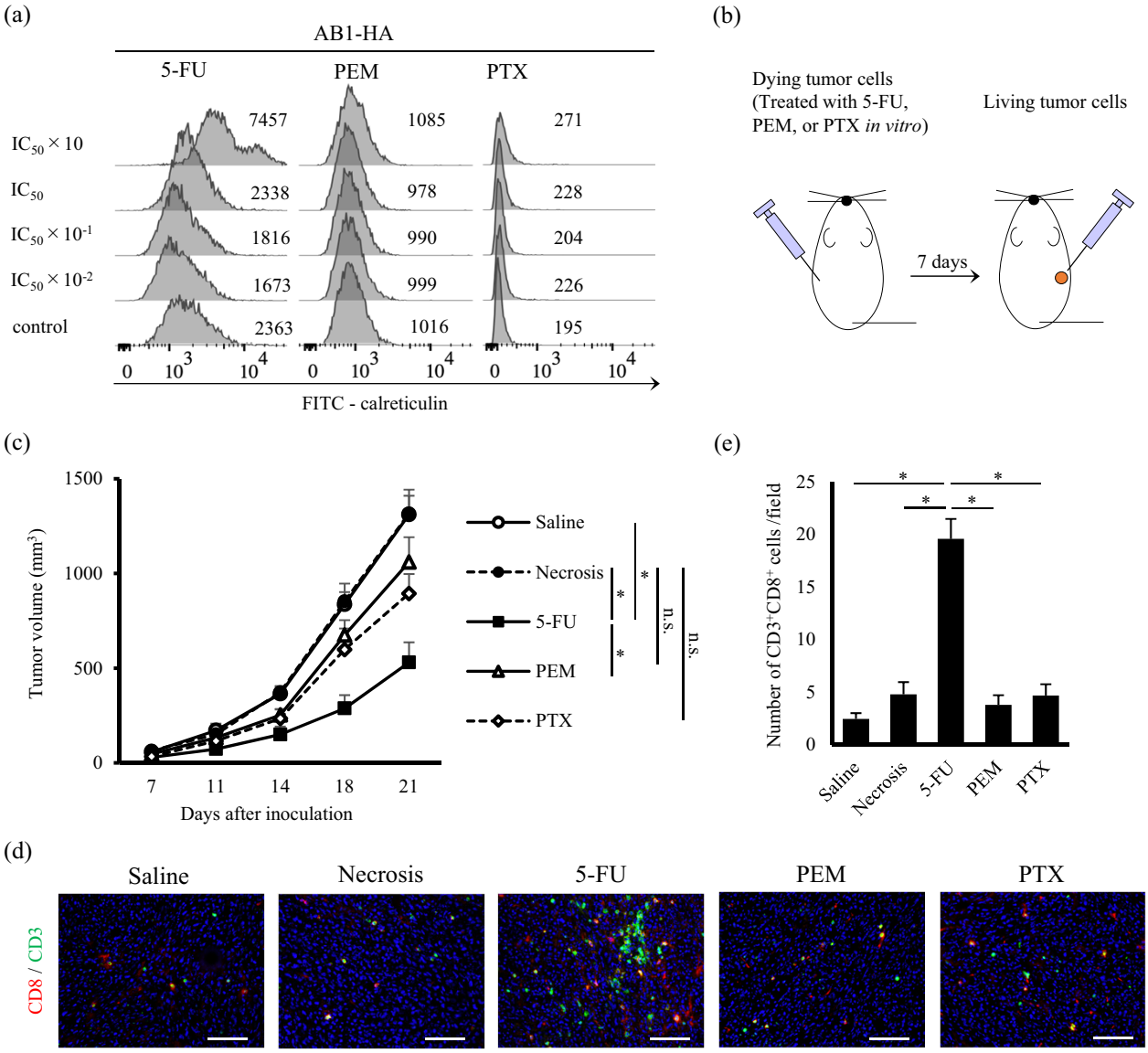

FIGURE 6 Dying tumor cells treated with 5-fluorouracil (5-FU) induced antitumor immunogenicity in vivo. (a) AB1-HA cells were incubated with 5-FU, pemetrexed (PEM), or paclitaxel (PTX) at their 10^{-2} to 10 × IC_{50} values for 48 h. The cell surface expression of calreticulin (CRT) was evaluated by flow cytometry. The number indicates the mean fluorescence intensity. (b) The schematic diagram illustrates the tumor vaccination model in vivo. Briefly, we vaccinated immunocompetent BALB/c mice subcutaneously in their left flank with dying AB1-HA cells treated with 5-FU or PEM at their 10 × IC_{50} values. We vaccinated the control mice with dying AB1-HA cells induced by freeze-thawing. At 1 week after vaccination, we rechallenged the living AB1-HA cells in the right flank of each mouse and measured the rechallenged tumor volume twice a week for an additional 2 weeks. (c) The quantitative evaluation of the rechallenged tumor growth ($n = 7$ per group). (d) Representative images of the sections from AB1-HA tumors at the rechallenge site double-stained for CD3 and CD8. Tumors were harvested on day 21 from each group in (c). Scale bar, 200 μm. (e) The quantitative evaluation of the total number of $CD3^+CD8^+$ cells per field in each tumor of mice (9 fields from 3 mice per group) studied in (d). The data are shown as mean ± SEM. *$p < 0.05$ by one-way analysis of variance, followed by Dunnett's multiple comparison post hoc test.

the folate pathway, predominantly thymidylate synthase, but also dihydrofolate reductase and glycinamide ribonucleotide formyltransferase.[34–36] PEM is also known to impact the antitumor immune response by increasing T cell infiltration,[37] and activating $CD45RO^+$ memory T cells.[14] Interestingly, one report demonstrated that PEM—but not CDDP, PTX, or VNR—induced the expression of PD-L1 in NSCLC cells.[38] Furthermore, another report demonstrated that PEM induced ICD more efficiently than platinum, taxane, or GEM in NSCLC cells;[39] however, fluoropyrimidines were not evaluated in the study. These results indicate that PEM may be a good partner for combination with ICIs. Actually, PEM plus CBDCA combined with pembrolizumab, an anti-PD-1 antibody, showed favorable antitumor efficacy in comparison to PTX plus CBDCA combined with pembrolizumab in patients with advanced stage nonsquamous NSCLC.[40] However, to the best of our knowledge, no report has demonstrated the impact of PEM on the antitumor immune response in comparison to fluoropyrimidine, even in preclinical studies.

In the present study, we demonstrated that 5-FU induced cell surface CRT and ATP secretion more efficiently than PEM or taxane in both non- and squamous NSCLC, and MPM cells. 5-FU-induced ICD, but neither PEM nor PTX-induced ICD, showed favorable antitumor immune responses in vivo by recruiting $CD3^+CD8^+$ T cells in the tumor microenvironment. Our previous and current results indicate that fluoropyrimidine is superior not only in suppressing the infiltration of MDSCs into tumor microenvironment but also in inducing the ICD on thoracic malignant tumor cells to other chemotherapeutic agents. Based on these results, we consider that fluoropyrimidine would have the potential to show synergistic antitumor immune responses and be a superior agent to PEM or taxane in combination with ICIs for the treatment of thoracic cancer. A clinical trial should be organized to validate the therapeutic efficacy of fluoropyrimidine in combination with ICI in thoracic cancer patients.

The current study was associated with several limitations. First, we mainly analyzed the immunological effects of 5-FU on ICD but not on other molecules which regulate the immune response against tumor including PD-L1 on tumor cells and IFN-γ from immune cells. To understand the tumor immunity totally, the effects of 5-FU on these factors should be addressed in the future. Second, we defined ICD by mainly cell-surface exposure of CRT and partially secretion of ATP in the current analyses. In addition to these biological reactions, ICD is characterized by the release of high-mobility group box 1 (HMGB1) from dying cells, which can provoke an anti-tumor immune response through interaction with Toll-like receptor 4 on DCs ("danger" signal).[41] We evaluated the secretion of HMGB1 from NSCLC cells treated with either 5-FU, PEM, taxane, or control; however, it was below the limit of detection even after high-dose treatment (data not shown). Nevertheless, the translocation of CRT to the plasma membrane is regarded as a key determinant of ICD, as blockade of CRT suppressed the phagocytosis of tumor cells by DCs and abolished their immunogenicity,[42] supporting that the expression of CRT represents the general status of ICD. Third, limited thoracic cancer cell lines were used in the analyses, suggesting that our results do not necessarily generalize NSCLC and MPM. Furthermore, the cell line used in the in vivo analyses was HA antigen-transduced malignant mesothelioma cells (AB1-HA). We consider that AB1-HA cells are suitable to evaluate the efficacy of immunotherapy for thoracic tumor as AB1 (the parental cell line) was poorly immunogenic and not suitable for the experiments with immunotherapy.[43] Nevertheless, additional analyses are needed to apply our current findings in the clinical setting. Fourth, although we evaluated the efficacy of chemotherapeutic agents in the induction of CRT at $1 \times IC_{50}$ in the analyses with human NSCLC cells, we treated the AB1-HA cells with $10 \times IC_{50}$ of chemotherapeutic agents to generate vaccine as $1 \times IC_{50}$ of agents did not induce the cell-surface expression of CRT on AB1-HA cells. Fifth, we did not evaluate the synergistic effect of 5-FU and platinum in the induction of CRT in vivo.

In addition to the preclinical studies, Inoue et al. recently demonstrated that systemic chemotherapy increased plasma levels of DAMPs, such as HMGB1 and CRT, in NSCLC patients and the maximum fold changes in concentration of these DAMPs tended to be associated with the clinical response, suggesting the clinical importance of ICD in patients.[44] In the era of combined cancer immunotherapy, an understanding of ICD in the treatment of thoracic cancer patients will be more important in the future.

In conclusion, in this study we demonstrated that 5-FU induced ICD—as determined by the cell surface expression of CRT and ATP secretion—more efficiently than other chemotherapeutic agents in several thoracic cancer cell lines. Furthermore, 5-FU-induced ICD, but neither PEM nor PTX-induced ICD reduced tumor growth in vivo. These results indicate the possibility that 5-FU may be the optimal partner in combination therapy with ICIs, and that additional analyses and validation studies could change the current standard regimens used in combined cancer immunotherapy for thoracic malignancies.

AUTHOR CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Conceptualization, Yasuhiko Nishioka. Methodology, Hiroyuki Kozai, Hirokazu Ogino and Atsushi Mitsuhashi. Investigation, Hiroyuki Kozai, Hirokazu Ogino, Atsushi Mitsuhashi, Na Thi Nguyen, Yuki Tsukazaki, Yohei Yabuki, Ryohiko Ozaki, Hiroto Yoneda, Seidai Sato and Hiroshi Nokihara. Formal analysis, Hiroyuki Kozai, Hirokazu Ogino and Atsushi Mitsuhashi. Writing – original draft, Hiroyuki Kozai and Hirokazu Ogino. Writing – review and editing, Hirokazu Ogino, Atsushi Mitsuhashi, Seidai Sato, Masaki Hanibuchi, Tsutomu Shinohara, Hiroshi Nokihara and Yasuhiko Nishioka. Visualization, Hiroyuki Kozai, Hirokazu Ogino and Atsushi Mitsuhashi. Supervision, Yasuhiko Nishioka. Funding acquisition, Yasuhiko Nishioka.

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CONFLICT OF INTEREST STATEMENT

Yasuhiko Nishioka reports research fees paid to his institution and personal fees from Taiho Pharmaceutical Co., Ltd. Hirokazu Ogino and Atsushi Mitsuhashi also report research fees paid to their institution from Taiho Pharmaceutical Co., Ltd.

DATA AVAILABILITY STATEMENT

The data generated in the present study may be requested from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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