The expression of PD-1 ligand 1 on macrophages and its clinical impacts and mechanisms in lung adenocarcinoma.

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Running title: PD-L1 on macrophages

Abstract
Background; Programmed death-1 (PD-1) and PD-1 ligand 1 (PD-L1) are target molecules for immunotherapy in non-small cell lung cancer. PD-L1 is expressed not only in cancer cells, but also on macrophages, and has been suggested to contribute to macrophage-mediated immune suppression. Methods; Clinical significance of PD-L1 expression on macrophages in human lung adenocarcinoma was examined. The mechanisms of PD-L1 overexpression on macrophages were investigated by means of cell culture studies. Results; High PD-L1 expression on macrophages was correlated with the presence of *EGFR* mutation, a lower cancer grade, and a shorter cancer-specific overall survival. In an *in vitro* study using lung cancer cell lines and human monocyte-derived macrophages, the conditioned medium from cancer cells was found to up-regulate PD-L1 expression on macrophages via STAT3 activation, and a cytokine array revealed that granulocyte-macrophage colony-stimulating factor (GM-CSF) was a candidate factor that induced PD-L1 expression. Culture studies using recombinant GM-CSF, neutralizing antibody, and inhibitors indicated that PD-L1 overexpression was induced via STAT3 activation by GM-CSF derived from cancer cells. Conclusions; PD-L1 overexpression on macrophages via the GM-CSF/STAT3 pathway was suggested to promote cancer progression in lung adenocarcinoma. Cancer cell-derived GM-CSF might be a promising target for anti-cancer therapy.

Keywords: lung adenocarcinoma, macrophage, PD-L1, GM-CSF, STAT3

Simple summary:

Programmed death-1 (PD-1) and PD-1 ligand 1 (PD-L1) are target molecules for immunotherapy in non-small cell lung cancer. PD-L1 is expressed not only in cancer cells, but also on macrophages, and has been suggested to contribute to macrophage-mediated immune suppression. We found PD-L1 overexpression on macrophages was associated to worse clinical course in lung adenocarcinoma and was mediated by GM-CSF/STAT3 signal. Cancer cell-derived GM-CSF might be a promising target for anti-cancer therapy.

Introduction

Lung cancer is one of the most common causes of cancer death, and tobacco smoking is the most well-known risk factor for lung cancer.[1] The incidence of lung cancer among never-smokers has been increasing over the past decade, and exposure to secondhand tobacco smoke, radon, and/or air pollution have been considered as possible etiological factors.[2,3] Although recent advances in diagnostic tools, such as computed tomography scans, have increased the detection rate of early stage lung cancer, many cases are still diagnosed at advanced stages. In addition to conventional chemotherapy/radiotherapy, immunotherapy blocking immune checkpoint molecules, such as programmed cell death protein 1 (PD-1), is now becoming one of the standard therapies for lung cancer.[4]

PD-1 ligand 1 (PD-L1) expression in cancer cells is considered to be a potential predictive biomarker for anti-PD-1/PD-L1 therapy in several cancers, including lung cancer, and immunohistochemistry (IHC) using anti-PD-L1 antibodies has been performed in pathological specimens.[5,6] Several retrospective studies have shown that PD-L1 expression in cancer cells was associated with a poor clinical course in lung adenocarcinoma, but not in lung squamous cell carcinoma and small cell carcinoma.[7-10] Although researchers have noted that PD-L1 is expressed in stromal cells in lung cancer tissues, the significance of the PD-L1 expression in stromal cells remains unclear.

It is known that myeloid cells, such as dendritic cells and macrophages, also express PD-L1.[11] Macrophages that infiltrate cancer tissues are referred to as tumor-associated macrophages (TAMs).[12-14] TAMs have protumor functions related to angiogenesis, invasion, and immunosuppression, and a high density of TAMs has been shown to be associated with a poor clinical course in many cancers, including lung cancer.[15-18] TAMs are known to express several molecules related to immune suppression, such as

indoleamine 2,3-dioxygenase, interleukin (IL) 10, Siglec-15, and PD-1 ligands,[19-21] and the immunosuppressive effect of the PD-1 ligands on TAMs has been demonstrated in a murine cancer model.[22,23] PD-L1 expression on TAMs is correlated with the efficacy of immunotherapy in ovarian cancer and melanoma patients.[24] In the present study, we tried to accurately evaluate the expression status of PD-L1 on TAMs in pathological specimens by double IHC with anti-macrophage antibodies. The mechanisms related to PD-L1 overexpression on TAMs were also investigated in cell culture studies.

Materials and methods

Samples

Paraffin-embedded samples were prepared from specimens obtained from 231 patients diagnosed with lung adenocarcinoma and 103 patients diagnosed with lung squamous cell carcinoma between 2010 and 2013 at Kumamoto University Hospital. Two pathologists reviewed all tissue specimens, and the most representative area of a 5-mm diameter core containing viable lung adenocarcinoma cells was carefully selected for tissue microarrays.

IHC

The DAKO automation system (Autostainer Link 48; DAKO, Glostrup, Denmark) was used for the immunohistochemical analysis of human PD-L1 (clone 22C3; DAKO). Anti-human CD8 antibody (clone C8/144B; Nichirei), anti-PU.1 antibody (clone EPR3158Y; Abcam, Cambridge, UK) and anti-Iba-1 antibody (Wako, Tokyo, Japan) were used as the primary antibodies to label the macrophages and lymphocytes. Horseradish peroxidase (HRP)-labeled anti-rabbit immunoglobulin antibody (Nichirei, Tokyo, Japan) was used as the secondary antibody. 3,3′ -Diaminobenzidine was used for the visualization of positive signals in the first step of double IHC. Subsequently, sections were treated by heating in 1 mM ethylenediaminetetraacetic acid (pH 8.0) buffer. Then, the sections were treated with HRP-labeled anti-rabbit immunoglobulin antibody, and positive signals were visualized with HistoGreen substrate (#AYS-E109; Linaris, Dossenheim, Germany) as the second step of double IHC. Two investigators (Y.K. and Y.S.), who were blinded to information about the samples, evaluated the PD-L1 and PU.1 expression. We also determined the macrophage proportion score (MPS), which is based on the tumor

proportion score (TPS). Images of ten randomly selected 400× fields were obtained under microscopy, and the image files were analyzed for cell counting and the evaluation of the stained areas by Image J software.

Cell culture of macrophages and cancer cell lines

Monocytes were isolated using RosetteSep Human Monocyte Enrichment Cocktail (STEMCELL Technologies, Vancouver, Canada). These monocytes were plated on UpCELL 6‐well plates (2×10^5 cells/well; CellSeed, Tokyo, Japan) and cultured in AIM‐V medium (Thermo Fisher, Waltham, MA, USA) supplemented with 2% human serum macrophage‐colony stimulating factor (M‐CSF; 100 ng/mL; Wako) for 7 days to induce the differentiation of macrophages.

Three human lung adenocarcinoma cell lines (NCI-H23, H358, and H1975) were obtained from Tomoya Yamaguchi (Kumamoto University, Kumamoto, Japan). All cells were cultured in RPMI1640 (Wako) supplemented with 10% fetal bovine serum. The conditioned medium (CM) of the cell lines was collected as previously described.[21] For the cell culture study using mouse macrophages, bone marrow cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum and M-CSF (100 ng/mL) for 5 days, and adherent cells were used as macrophages.

Cell enzyme-linked immunosorbent assay (ELISA)

Macrophages were cultured in a 96-well microplate and stimulated with the CM of the adenocarcinoma cell lines for 1 day. After fixation with 1% paraformaldehyde, cells were reacted with anti-PD-L1 antibody (clone 29E.2A3; BioLegend, San Diego, CA, USA) or isotype-matched control antibody (BioLegend). After the cells were washed with phosphate-buffered saline, HRP-labeled anti-mouse immunoglobulin antibody (Nichirei) was added. Then, the plate was washed with phosphate-buffered saline, and tetramethylbenzidine developing solution (BioLegend) was used to visualize the positive signals.

Phospho-receptor tyrosine kinase (RTK) array

Phospho-RTK array analysis was performed using the Human Phospho-RTK Array Kit

(ARY 001; R&D Systems) according to the manufacturer's instructions.

Cytokine array

Cytokine array analysis was performed using the Human XL Cytokine Array Kit (ARY 022; R&D Systems) according to the manufacturer's instructions.

ELISA for granulocyte-macrophage colony-stimulating factor (GM-CSF)

ELISA for GM-CSF was performed using the Human GM-CSF ELISA Kit (Cat. No. 432007; BioLegend) according to the manufacturer's instructions.

Recombinant proteins and inhibitors

IL-6 and GM-CSF recombinant proteins were purchased from Wako. The following inhibitors were used at a final concentration of 10 nM: Stat1 (Fludarabine; Wako), Stat3 (WP1066; Santa Cruz, Dallas, TX, USA), Stat5 (573108; Merck KGaA, Darmstadt, Germany), JNK (SP600125; Santa Cruz), ERK (FR180204; Santa Cruz), and JAK (Ruxolitinib; ChemScene LLC, Monmouth Junction, NJ, USA).

Western blot analysis

The macrophages were stimulated with the CM of the lung adenocarcinoma cell lines (concentration: 50%) for 10 min, 30 min, 1 h, 3 h, or 1 day. Then, the macrophages were collected, and the cellular proteins were solubilized in Tris buffer containing 2% sodium dodecyl sulfate and 10% glycerol. The amount of protein was quantified using the bicinchoninic acid assay. Equal amounts of protein were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene fluoride membrane. The following rabbit antibodies were used for western blotting: anti‐PD-L1 (clone E1L3N; Cell Signaling Technology), anti‐STAT3 (clone 124H6; Cell Signaling Technology, Danvers, MA), and anti‐pSTAT3 (clone Y705; Cell Signaling Technology) antibodies.

Flow cytometry

Human monocyte-derived macrophages were treated with human FcR-blocking reagent

(BioLegend), then reacted with phycoerythrin-labeled anti-human PD-L1 antibody (BioLegend) or isotype-matched control antibody (BioLegend). The stained cell samples were analyzed on a FACSverse (Becton Dickinson, Franklin Lake, NJ) flow cytometer with FACSuite (Becton Dickinson) software.

Statistical analysis

Statistical analysis was carried out using StatMate V (ATOMS, Tokyo, Japan) and JMP7 (SAS Institute, Chicago, IL, USA) software. Differences were considered to be statistically significant at $p < 0.05$.

Results

PD-L1 overexpression on TAMs was associated with a worse cancer-specific survival (CSS).

We tried to evaluate the PD-L1 that is specifically expressed on TAMs in tumor tissues by double IHC using macrophage-specific markers and anti-PD-L1 antibody. Since PU.1 is a critical molecule involved in macrophage differentiation and is expressed in the nucleus of Iba-1 (a pan-macrophage marker)-positive cells (Figure 1A), double IHC of PD-L1 and PU.1 was performed to accurately determine the expression on TAMs (Figure 1B). PD-L1 expression was scored based on the percentage of positive staining, and referred to as the MPS, as follows: low, <50% positive cells; high, ≥50% positive cells. Of the 231 cases, PD-L1 expression was low in 114 cases (49.4%) and high in 117 cases (50.6%). Although a high MPS was associated with female, the presence of epidermal growth factor receptor (*EGFR*) mutation, and a lower grade of cancer cells, the MPS was not correlated with age and smoking (Table 1). The MPS did not affect the progression-free survival or CSS in the total cases (Figure 1C, Table 2 and 3). The cases were divided into two groups according to the cancer stage, and statistical analysis was performed. Log-rank analysis showed that among the patients with a higher stage, the CSS was significantly shorter in the high MPS group than in the low MPS group ($p = 0.0409$; Figure 1C). There was no significant relationship between the MPS and the number of CD8-positive T cells in cancer tissues (Figure 1D).

Cancer cell-derived factors induced PD-L1 overexpression on cultured macrophages.

From the above results, PD-L1 expression in the TAMs in lung adenocarcinoma tissues was more likely to be affected by cancer cells than lymphocytes. Therefore, we hypothesized that cancer cell-derived factors influenced the PD-L1 expression on TAMs. To test this hypothesis, the CM of lung adenocarcinoma cell lines was added to human monocyte-derived macrophages, and PD-L1 expression was tested by immunocytostaining and cell ELISA. We confirmed that the expression intensity of PD-L1 was increased by the CM of the PC9, H23, H358, and H1975 cell lines (Figure 2A, 2B). The phosphorylation kinase array was then performed to elucidate the PD-L1 expression-inducing mechanism of the cancer cell-derived factors. The levels of some phosphorylation kinases were elevated; in particular, the levels of STAT3, STAT5, and c-Jun were significantly elevated (Figure 2C).

PD-L1 overexpression on macrophages was dependent on Stat3 activation.

The above results of the phosphorylation kinase array suggested that STAT3, STAT5, and c-Jun increased the PD-L1 expression on macrophages. Therefore, we investigated which pathway contributes to PD-L1 expression by using inhibitors against these molecules. No direct inhibitor was available for c-Jun, so inhibitors of its upstream kinases, JNK and ERK, were used instead. In addition, since it has been reported that STAT1 induced PD-L1 expression in cancer cells,[25] we added a STAT1 inhibitor. The results showed that PD-L1 expression was strongly suppressed by the STAT3 inhibitor, and was slightly suppressed by the STAT1 inhibitor and JNK inhibitor (Figure 3A). From western blotting, STAT3 activation was observed in the macrophages within 30 min after CM stimulation, and the PD-L1 expression level was increased after 1 h after CM stimulation (Figure 3B). Since JAK signals are located upstream of STAT3, we additionally tested whether the JAK inhibitor suppressed PD-L1 overexpression. The results showed that the PD-L1 protein expression level was significantly suppressed by the JAK inhibitor as well as the STAT3 inhibitor (Figure 3C). Similar results were confirmed by flow cytometry (Figure 3D, 3E).

GM-CSF induced PD-L1 expression.

Although it has been revealed that STAT3 is involved in PD-L1 expression on macrophages, the cancer cell-derived factors that induce STAT3 activation are unclear.

Therefore, we used a cytokine array to try to identify the cytokines that activate the STAT3 pathway. The CM of NCI-H358 and NCI-H1975, which increased PD-L1 expression, and the CM of A549, which did not increase PD-L1 expression, were used. GM-CSF and IL-6 were identified as cytokines that were abundantly contained in the CM of NCI-H358 and NCI-H1975, and were scarce in the CM of A549 (Figure 4A, 4B). Cell ELISA analysis (Figure 4C) and flow cytometry (Figure 4D) results showed that PD-L1 expression was increased by GM-CSF, but not by IL-6. No synergistic effect of IL-6 and GM-CSF was observed.

Among the CM of the macrophages and cell lines, GM-CSF production was detected in the CM of PC9, NCI-H358, and NCI-H1975 (Figure 5A). The CM-induced PD-L1 overexpression was significantly suppressed by the STAT3 inhibitor and anti-GM-CSF antibody (Figure 5B, 5C). Gene expression data from a lung adenocarcinoma cohort in The Cancer Genome Atlas (https://www.proteinatlas.org/) showed that PD-L1 expression was significantly associated with the expression of GM-CSF and IL-6, and a more significant correlation was seen between GM-CSF and PD-L1 (Figure 5D).

Discussion

PD-L1 is highly expressed not only in cancer cells, but also in immune cells, mostly TAMs, in lung cancer tissues. A previous study reported that the PD-L1 expression in tumor cells (the PD-L1 TPS) was strongly associated with the effects of anti-PD-1 therapy in non-small cell lung cancer (NSCLC).[26] On the other hand, it has been reported that PD-L1 expression in tumor cells and immune cells independently predicts the efficacy of anti-PD-L1 therapy.[27] In any case, there have been no reports evaluating PD-L1 expression in TAMs using the clone 22C3 antibody, which is the most commonly used antibody for lung cancer in clinical practice. Therefore, in the present study, PD-L1 expression on TAMs in lung adenocarcinoma tissue was evaluated by double-IHC using 22C3 antibody and anti-PU.1 antibody (as a marker for TAMs). In a previous study, we reported that the PD-L1 TPS could be accurately determined by double IHC using clone 22C3 and anti-Iba-1 antibodies.[7] Since positive signals of PD-L1 and Iba-1 were both observed in the cell membrane and cytoplasm, we could not accurately determine the MPS by double IHC of PD-L1 and Iba-1. PU.1 is expressed on the nucleus of macrophages; therefore, we

newly performed double IHC of PD-L1 and PU.1. The present study results suggested that double IHC using anti-PD-L1 and PU.1 antibodies is an adequate method for accurately determining the MPS in human samples.

In this study, PD-L1 expression on TAMs in the primary lesion of lung adenocarcinoma was seen in 90.0% of the cases, whereas in a previous study, PD-L1 expression in tumor cells was seen in 26.8% of the cases.[7] Consistent with the present study, it was reported that PD-L1 expression was observed in TAMs more frequently than in cancer cells in ovarian cancer and gastric cancer.[28,29] Sumitomo et al. reported that high PD-L1 expression in tumor-infiltrating immune cells was associated with a high density of M2-like TAMs and a shorter progression-free survival and overall survival in NSCLC.[30] Since TAMs are the main component of PD-L1-expressing stromal cells, the "PD-L1 expression in immune cells" is considered to be very similar to the "MPS". In our cases, although the CSS in the lung adenocarcinoma patients with pathological stage II cancer or higher was significantly shorter in the high MPS group, there was no association between the MPS and relapse-free survival. This discrepancy might be due to the differences in the histological subtypes of lung cancer. Since the significance of PD-L1 expression is potentially different between lung adenocarcinoma and squamous cell carcinoma,[31,32] studies on PD-L1 in NSCLC should be separated by histological subtypes.

PD-L1 expression on macrophages has been shown to be regulated by STAT1 and STAT3 signals.[33,34] PD-L1 overexpression in cancer cells may be induced by several mechanisms. Copy number amplification of 9p24.1 and 3'-untranslated region disruption were associated with increased PD-L1 expression in several cancers.[35,36] Several oncogenic transcription factors, including MYC, RAS, and JAK2/STAT3, also contribute to PD-L1 overexpression in cancer cells.[37] Hypoxia inducible factor 1a is also known to mediate PD-L1 expression in both cancer cells and myeloid cells. In this study, we showed that GM-CSF derived from cancer cells increased PD-L1 expression in TAMs via the JAK/STAT3 pathway in macrophages. Chemotherapy stimulates GM-CSF production in cancer cells, which accelerates the infiltration of immunosuppressive myeloid cells into the microenvironment.[38] Blockade of GM-CSF improved the anti-cancer effect of chemotherapy by modulating the immunosuppressive tumor microenvironment.[39] The PD-L1 expression on TAMs induced by GM-CSF might be involved in the immunosuppressive microenvironment.

Conclusion

High PD-L1 expression on TAMs was associated with a poor clinical course in advanced cases of lung adenocarcinoma, and cancer cell-derived GM-CSF was suggested to induce PD-L1 overexpression on TAMs via the STAT3 pathway. Cancer cell-derived GM-CSF appears to be a promising target for anti-cancer therapy.

Author contributions:

Data curation, Yusuke Shinchi, Yoshihiro Komohara and Yukio Fujiwara; Formal analysis, Eri Matsubara; Funding acquisition, Yoshihiro Komohara; Investigation, Eri Matsubara; Methodology, Kimihiro Yonemitsu and Daiki Yoshii; Project administration, Yukio Fujiwara; Resources, Koei Ikeda, Koji Tamada and Makoto Suzuki; Supervision, Yoshihiro Komohara; Writing – original draft, Yusuke Shinchi; Writing – review & editing, Yoshihiro Komohara and Makoto Suzuki.

Funding:

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Nos. 16H05162 and 20H03459).

Institutional Review Board Statement:

The study design was approved by the Kumamoto University Review Board (approval #1174). Peripheral blood mononuclear cells were obtained from three healthy voluntary donors in accordance with protocols approved by the Kumamoto University Hospital Review Board (#1169).

Informed Consent Statement:

Written informed consent was obtained from all patients and healthy donors in accordance with the protocols of the Kumamoto University Review Board.

Acknowledgments

We thank Mr. Takenobu Nakagawa and Ms. Michiyo Tokunaga for technical assistance.

Conflict of Interest

None of the authors have any conflicts of interest in association with this manuscript.

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Figure legends:

Figure 1: Double immunohistochemistry (IHC) using anti‐programmed death ligand 1 (PD-L1) and macrophage-specific markers. (A) Double IHC of Iba-1 (a pan-macrophage marker) and PU.1 (a nuclear transcription factor in macrophages) in lung tumor tissue and non-tumor tissue. Iba-1 and PU.1 signals are labeled as brown and green, respectively. (B) Representative images of double IHC from a high PD-L1 case (left side) and a low PD-L1 case (right side). PD‐L1 and PU.1 signals are labeled as brown and green, respectively. (C) PD-L1 expression was divided into two groups according to the macrophage proportion score (MPS). Statistical analyses related to progression‐free survival, cancer-specific survival, and overall survival were performed. (D) The infiltration of CD8-positive T lymphocytes was determine as the CD8-positive area (%) by Image J software and compared between the high and low PD-L1 MPS groups.

Figure 2: PD-L1 expression on macrophages. Human monocyte-derived macrophages were stimulated with the conditioned medium of lung adenocarcinoma cell lines for 24 h, and PD-L1 expression was evaluated by immunocytochemistry (A) and cell enzyme-linked immunosorbent assay (n = 3) (B). (C) Upstream signals related to PD-L1 expression on macrophages were analyzed using a phospho-receptor tyrosine kinase array. Each of the signal densities was evaluated by Image J software.

Figure 3: PD-L1 expression on macrophages and related signaling pathways. (A) Human macrophages were stimulated with the conditioned medium derived from the NCI-H358 and H1975 cell lines, and the inhibitory effects of inhibitors on the up-regulation of PD-

L1 were tested by cell enzyme-linked immunosorbent assay (ELISA) (n = 3). (B) Western blot analysis of PD-L1, pSTAT3, and STAT3 performed using macrophages stimulated with the conditioned medium of the NCI-H358 cell line. The suppressive effects of inhibitors of STAT3 and JAK signals were tested by cell ELISA (C) and flow cytometry (D) using macrophages stimulated with the conditioned medium of the NCI-H358 cell line. The bar graph shows the mean fluorescence intensity (MFI) for PD-L1 (n = 4).

Figure 4: Cytokines produced from the cell lines. (A, B) Cancer cell-derived factors that promote PD-L1 expression on macrophages were tested using a cytokine array. The signal density was evaluated by Image J software. Human macrophages were stimulated with IL-6 (10 ng/mL) and GM-CSF (10 ng/mL), and PD-L1 expression was measured by cell enzyme-linked immunosorbent assay (C) and flow cytometry (D). *$p < 0.05$, as compared to the control (CT) macrophages.

Figure 5: The involvement of GM-CSF in PD-L1 overexpression. (A) GM-CSF in the conditioned medium from macrophages and each of the cancer cell lines was tested by enzyme-linked immunosorbent assay. (B) The inhibitory effect of the STAT3 inhibitor and anti-GM-CSF antibody (Ab) on PD-L1 overexpression was tested by flow cytometry. (C) Bar graph showing the mean fluorescence intensity (MFI) for PD-L1 (n = 3). (D) Gene expression data (fragments per kilobase of exon model per million reads mapped) of PD-L1, GM-CSF, and IL-6 in lung adenocarcinoma were obtained from Protein Atlas (TCGA) website, and Spearman's correlation tests were performed to examine the correlation of the expression of each gene.

Table 1: PD-L1 expression(MPS) and clinicopathological factors

		MPS		
		Low	High	p
Age				
	<65	37	31	0.3203
	≥65	77	86	
Gender				
	Male	65	50	0.0300
	Female	49	67	
Smoking				
	Ever	61	54	0.2637
	Never	53	63	
EGFR				
	Mutation	42	64	0.0155
	Wild type	61	48	
	Unknown	11	5	
Grade				
	1	49	72	0.0048
	2-3	65	45	
pStage				
	0-I	82	96	0.0674
	II-VI	32	21	

Chi-square test was performed. Underline indicates statistically significant.

Table 2: Univariate analyses for cancer-specific survival of advanced stage.

		Univariate		
		p-value	HR	95%CI
Age	≤65 vs 65<	0.7716	1.175	0.405-3.824
Gender	Male vs Female	0.3593	0.564	0.127-1.829
Smoking	never vs ever	0.2393	2.061	0.640-9.142
Grade	1 vs 2-3	0.1429	2.736	0.739-17.65
PD-L1	Negative vs Positive	0.0491	2.961	1.004-9.252

HR: hazard ratio, CI: confidential interval. Underline indicates statistically significant.

Table 3: Univariate and multivariate analyses for cancer-specific survival.

		Univariate			Multivariate		
		p-value	HR	95%CI	p-value	HR	95%CI
Age	≤65 vs 65<	0.6655	1.252	0.471-3.911			
Gender	Male vs Female	0.0060	0.244	0.069-0.681	0.7291	0.763	0.149-3.201
Smoking	never vs ever	0.0047	4.255	1.525-15.020	0.4554	1.776	0.427-9.200
EGFR	Wild Type vs Mutation	0.0003	0.117	0.018-0.410			
Grade	1 vs 2-3	0.0005	6.548	2.159-28.277	0.0753	2.941	0.904-13.27
pStage	pStage0-Ivs pStageII-IV	<.0001	14.155	5.068-49.999	<.0001	8.499	2.851-31.59
PD-L1	Low vs High	0.5783	0.861	0.504-1.463			

HR: hazard ratio, CI: confidential interval. Underline indicates statistically significant.

Figure 1

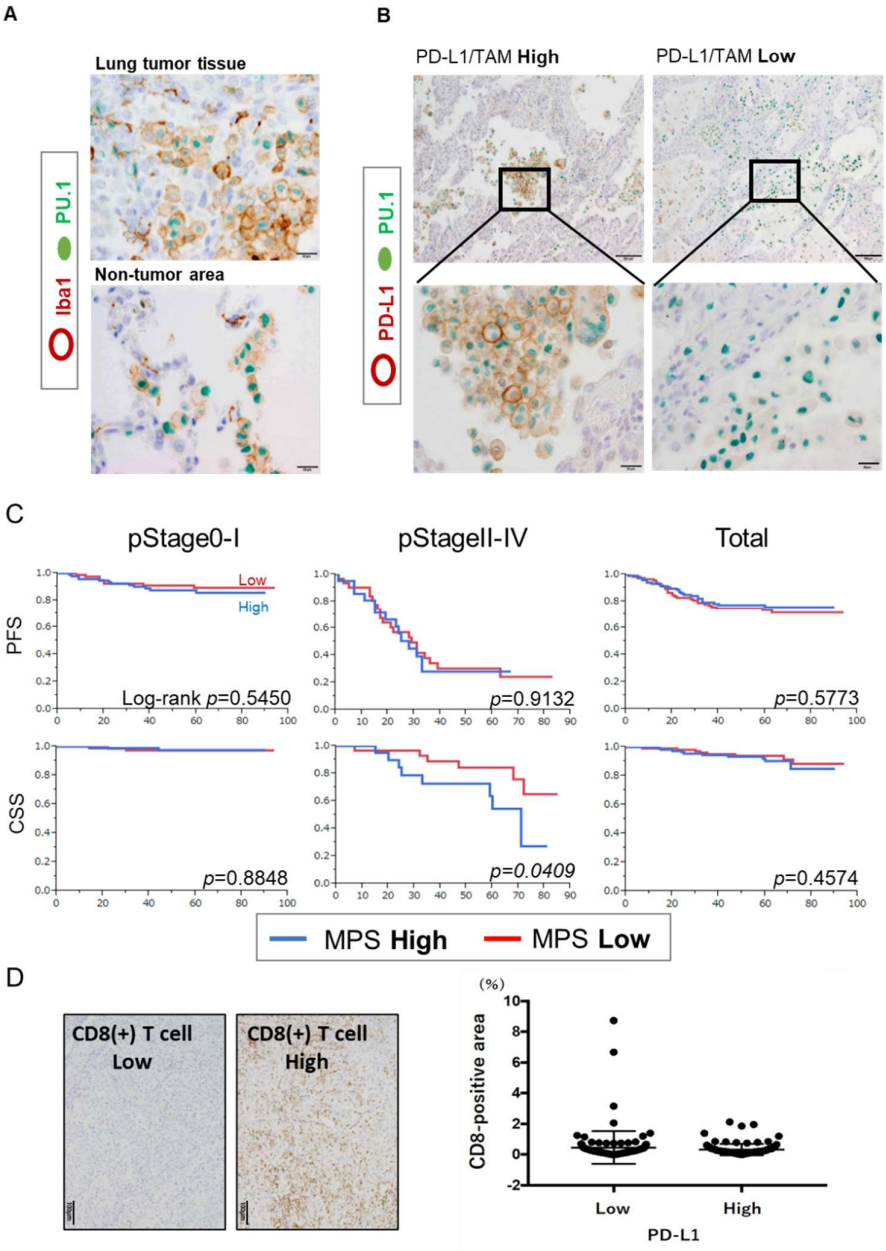

Figure 2

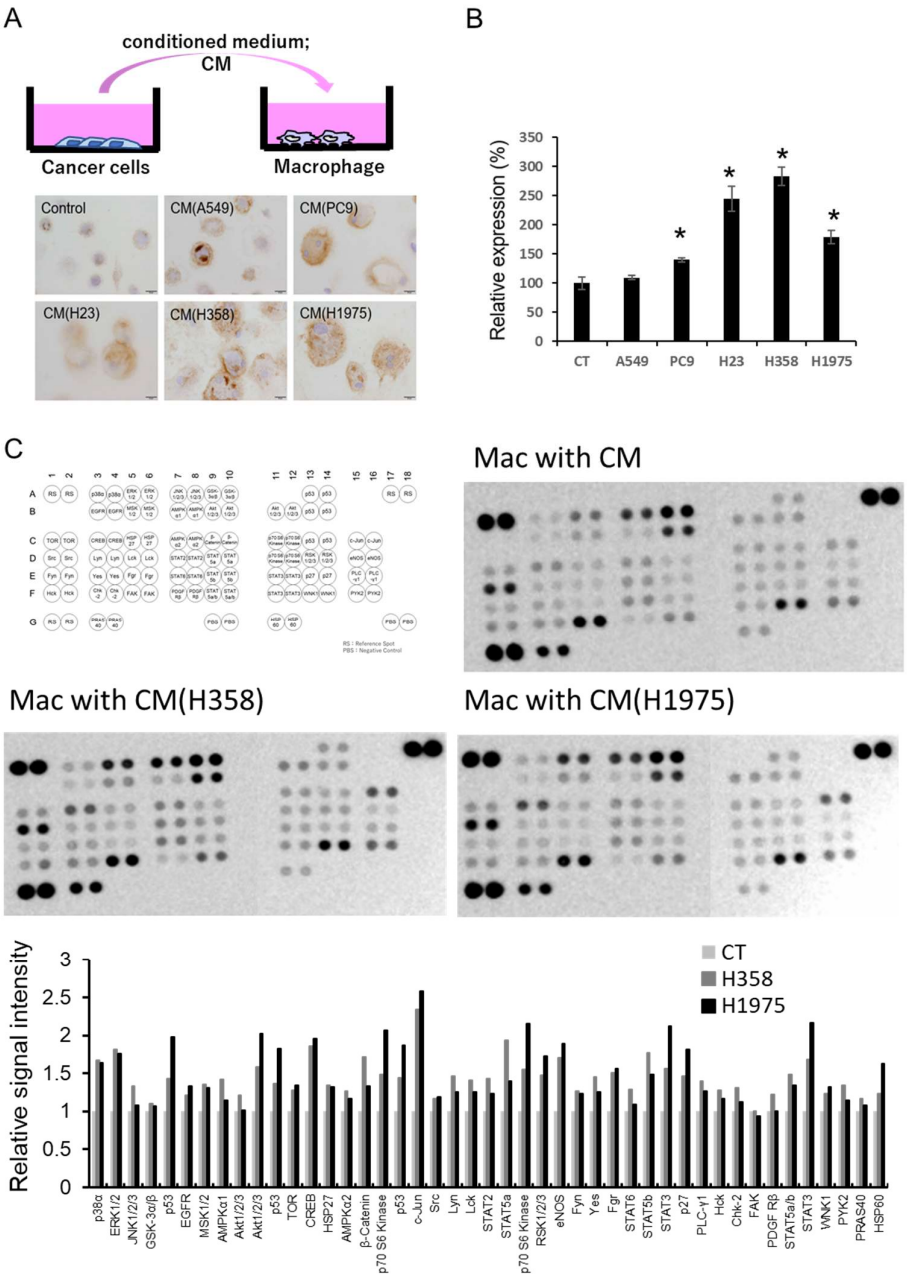

Figure 3

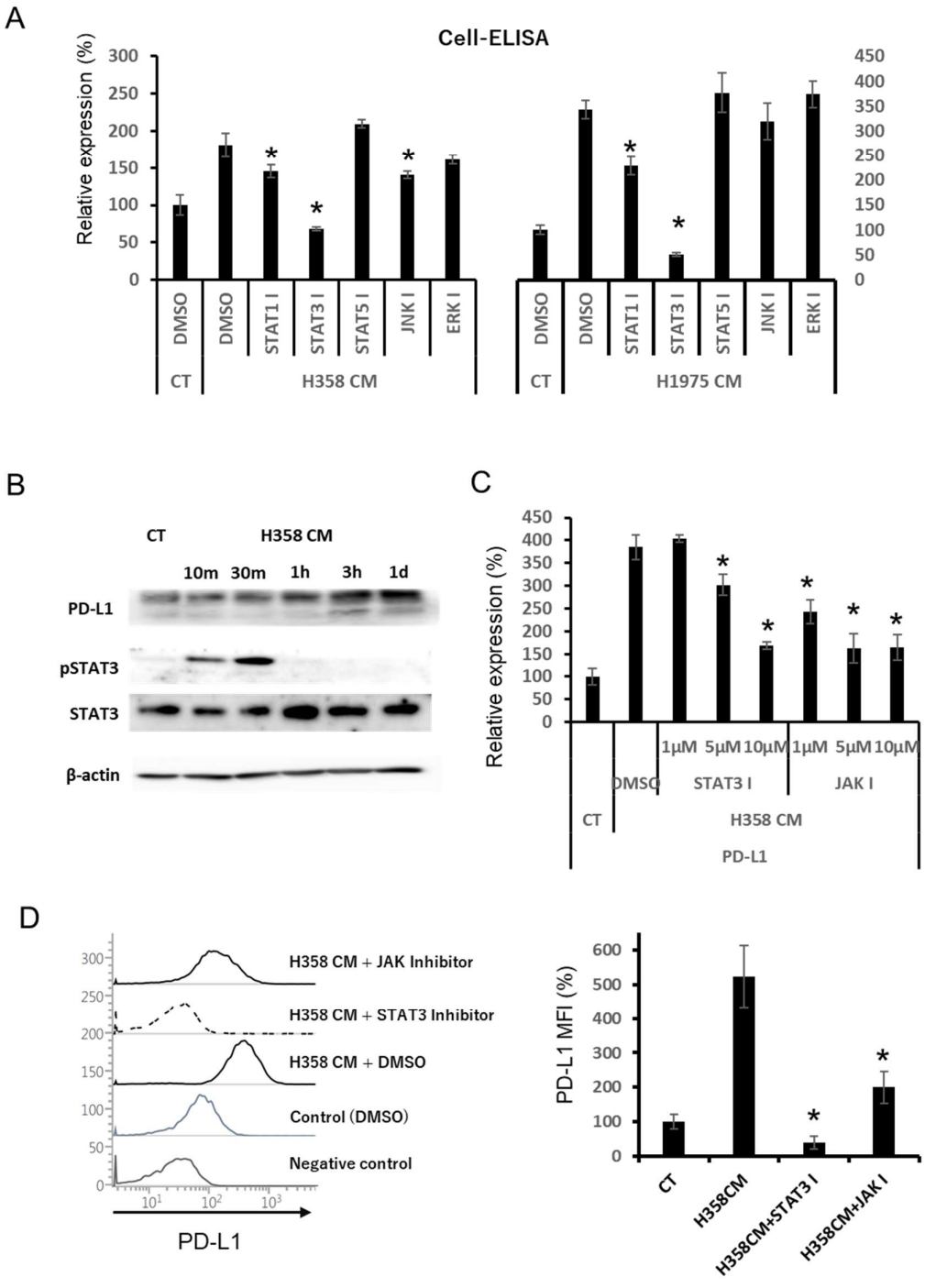

Figure 4

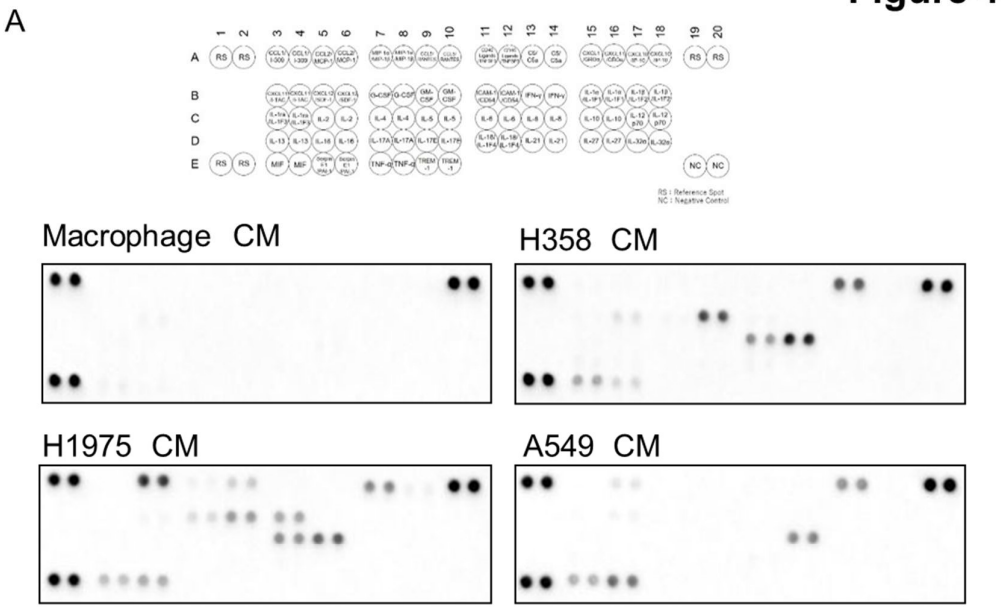

Figure 5