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Original Article

CD40 Agonists Alter the Pancreatic Cancer Microenvironment by Shifting the Macrophage Phenotype toward M1 and Suppress Human Pancreatic Cancer in Organotypic Slice Cultures

Chae Yoon Lim[1], Jae Hyuck Chang[2], Won Sun Lee[1], Jeana Kim[3], and Il Young Park[4]

[1]Institute of Clinical Medicine Research, Departments of [2]Internal Medicine, [3]Hospital Pathology, and [4]Surgery, College of Medicine, The Catholic University of Korea, Seoul, Korea

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Corresponding Author
Jae Hyuck Chang
ORCID https://orcid.org/0000-0003-1180-2693
E-mail wwjjaang@catholic.ac.kr

Background/Aims: CD40 agonists are thought to generate antitumor effects on pancreatic cancer via macrophages and T cells. We aimed to investigate the role of CD40 agonists in the differentiation of macrophages and treatment of human pancreatic adenocarcinoma.

Methods: Immunohistochemistry was performed on paraffin-embedded surgical blocks from patients with pancreatic cancers to evaluate macrophage phenotypes and their relationship with survival. The effects of CD40 agonists on macrophage phenotypes and human pancreatic cancer were evaluated utilizing cell cocultures and organotypic slice cultures.

Results: CD163$^+$ (predominant in M2 macrophages) and FOXP3$^+$ (predominant in regulatory T cells) expression levels in the tumors were significantly lower in patients with stage IB pancreatic cancer than in those with stage II or III disease (p=0.002 and p=0.003, respectively). Patients with high CD163$^+$ expression had shorter overall survival than those with low CD163$^+$ expression (p=0.002). *In vitro* treatment of THP-1 macrophages with a CD40 agonist led to an increase in HLA-DR$^+$ (predominant in M1 macrophages) and a decrease in CD163$^+$ expression in THP-1 cells. Cell cocultures showed that CD40 agonists facilitate the suppression of PANC-1 human pancreatic cancer cells by THP-1 macrophages. Organotypic slice cultures showed that CD40 agonists alter the pancreatic cancer microenvironment by shifting the macrophage phenotype toward M1 (increase HLA-DR$^+$ and decrease CD163$^+$ expression), decreasing the abundance of regulatory T cells, and increasing tumor cell apoptosis.

Conclusions: CD163 is related to advanced human pancreatic cancer stages and shorter overall survival. CD40 agonists alter macrophage phenotype polarization to favor the M1 phenotype and suppress human pancreatic cancer. **(Gut Liver 2022;16:645-659)**

Key Words: CD40 immunoglobulin; CD163 antigen; Macrophages; Pancreatic neoplasm; Tumor microenvironment

INTRODUCTION

Pancreatic cancer, with the fourth to fifth highest mortality rate among all cancers, presents with profound tumor stroma and a large number of macrophages.[1] Macrophages are a fundamental part of the innate immune system. However, macrophages within the tumor microenvironment have been associated with the promotion of cancer cell growth, migration, angiogenesis, and immunosuppression.[2] Macrophages within the tumor, called tumor-associated macrophages (TAMs) have functional plasticity, which may result in the polarized expression of either anti- or protumoral functions known as the M1 or M2 phenotype, respectively. Several studies have highlighted the relationships between TAMs and the prognosis of cancer patients. CD163 (an M2 macrophage marker) predicts the clinical prognosis of patients with intrahepatic cholangiocarcinoma,[3] and overexpression of CD68 (a pan-macrophage marker) and CD163 was associated with a worse outcome in patients with hepatocellular carcinoma.[4]

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Although there has been some research on TAMs in pancreatic cancer,[1,5-7] the role of M1 and M2 macrophages in the clinical aspects of pancreatic cancer patients still needs to be clarified. Since TAMs have functional plasticity and exhibit a mixed phenotype with properties of M1 and M2 macrophages,[4] identifying macrophage phenotypes and controlling phenotype polarization are important for understanding cancer pathogenesis and treating this disease.

CD40 is a cell surface molecule on immune cells and a member of the tumor necrosis factor (TNF) receptor superfamily.[8] As binding of CD40 to the CD40 ligand triggers activating signals to CD40$^+$ immune cells,[8] CD40 antibody agonists were found to mimic the signal of the CD40 ligand.[9] CD40 agonists can mediate T cell-independent and T cell-dependent immune mechanisms regarding cancer regression,[10,11] and macrophages are the main effectors in T cell-independent mechanisms. A previous study suggested that CD40 agonists are involved in the systemic activation of macrophages that infiltrate the tumor and become tumoricidal.[10] In a murine *in vitro* model, CD40 agonists generated cancer immunoediting by eliciting M1 macrophages in sarcoma,[12] and CD40-activated macrophages inhibited melanoma growth.[13,14]

Herein, we report M2 polarization in human pancreatic cancer and its association with cancer stages and overall survival in patients with pancreatic cancer. Coculture of macrophages and human pancreatic cancer cells showed that CD40 agonist-activated M1 macrophages suppress the proliferation of human pancreatic cancer cells, and organotypic slice culture demonstrated that CD40 agonists induce M1 differentiation in the tumor microenvironment and increase the apoptosis of human pancreatic cancer cells.

MATERIALS AND METHODS

1. Patients and cell lines

Paraffin-embedded pancreatic cancer blocks from 12 patients (#1–12) who underwent surgery between 2015 and 2018 were obtained from the tissue bank at our institute. For the treatment of pancreatic cancer, radical antegrade modular pancreatosplenectomy was performed in six patients, a Whipple procedure was performed in four patients, and pylorus-preserving pancreaticoduodenectomy was performed in two patients. The 8th edition American Joint Committee on Cancer stages of pancreatic cancer after surgery were IB for three patients, IIA for four, IIB for three, and III for two. In another eight patients (#13–20) with pancreatic cancer, pancreatic tumor specimens were procured during surgery for the production of organotypic slice cultures. Overall the characteristics of all 20 patients are listed in Table 1.

For the coculture experiment, a pancreatic adenocarcinoma cell line (PANC-1) and monocyte cell line (THP-1) were purchased from a Korean cell line bank. The passage numbers of the cell lines at the time of purchase were 70 for PANC-1 cells and 13 for THP-1 cells. The cell lines were stored at –196℃ prior to use.

Table 1. Patient Characteristics

Patient No.	Age, yr	Sex	Location	Tumor differentiation	Tumor size, cm	T stage	LN metastasis	AJCC stage, 8th	Surgical procedure
1	53	M	Body	Moderate	3.2×2.8	T2	3/38, N1	2B	RAMPS
2	55	M	Tail	Well	3.2×2.3	T2	3/37, N1	2B	RAMPS
3	65	M	Tail	Moderate	4.5×3.5×4.0	T3	0/7, N0	2A	RAMPS
4	55	M	Head	Moderate	3.0×2.8	T2	0/20, N0	1B	PPPD
5	53	M	Body	Poor	2.4×2.0	T2	2/16, N1	2B	RAMPS
6	65	F	Head	Moderate	2.3×1.7	T2	0/2, N0	1B	RAMPS
7	64	M	Tail	Moderate	9.5×4.5	T3	0/11, N0	2A	RAMPS
8	68	F	Tail	Moderate	4.2×3.0×4.5	T3	0/6, N0	2A	Whipple
9	55	M	Head	Poor	5.0×4.0	T3	0/2, N0	2A	PPPD
10	51	M	Head	Moderate	3.0×2.5×2.0	T2	6/35, N2	3	Whipple
11	77	M	Head	Well	5.0×4.2	T3	10/32, N2	3	Whipple
12	58	F	Head	Poor	2.4×2.2	T2	1/32, N1	2B	Whipple
13	66	F	Head	Adenosquamous	2.5×2.3	T2	0/27, N0	1B	Whipple
14	59	M	Head	Moderate	2.5×2.0×1.0	T2	0/6, N0	1B	Whipple
15	58	F	Tail	Poor	7.0×6.0×5.5	T3	4/14, N2	3	RAMPS
16	53	F	Body	Moderate	2.8×1.7	T2	2/30, N1	3	RAMPS
17	67	F	Head	Poor	4.1×4.0	T2	17/29, N2	3	Whipple
18	78	M	Head	Adenosquamous	1.7×1.6	T1	11/25, N2	3	Whipple
19	53	F	Body	Moderate	2.8×1.7	T2	2/30, N1	2B	RAMPS
20	54	F	Tail	Adenosquamous	7.5×5.5	T3	5/32, N2	3	RAMPS

LN, lymph node; AJCC, American Joint Committee on Cancer; M, male; F, female; RAMPS, radical antegrade modular pancreatosplenectomy; PPPD, pylorus-preserving pancreaticoduodenectomy.

2. Coculture of THP-1 and PANC-1 cells

Human monocytic THP-1 cells maintained in RPMI 1640/10% fetal bovine serum were seeded in 12-well transwell inserts (0.4 μm pore size) at a concentration of 1×10^5 cells/mL. Settled THP1 cells were treated with 10 ng/mL 12-O-tetradecanoylphorbol-13-acetate (PMA; Promega, Madison, WI, USA, #V1171) for 24 hours in an incubation system (37℃, 5% CO_2). The PMA containing media were aspirated gently from the inserts and replaced with media containing with 15 ng/mL lipopolysaccharide (Sigma, St. Louis, MO, USA, #L2630); the cells were incubated for 48 hours to allow differentiation into macrophages. Differentiated macrophages within the inserts were transferred onto preplated human PANC-1 cells (1×10^5/mL) and cocultured in medium supplemented with 2 μg/mL CD40 antibody (R&D System, Minneapolis, MN, USA, #MAB6321-500) or 2 μg/mL isotype monoclonal antibody control (R&D System, #MAB004) for 96 hours.

3. Cell viability test

Cell viability was evaluated by the CCK-8 assay using a Cell Counting Kit-8 (Dojindo, Incheon, Korea, #CK04). The viability of cocultured PANC-1 cells was measured according to the protocol provided by the manufacturer. CCK-8 solution was added at 1/10 volume into each well and incubated at 37℃ in humidified incubator with 5% CO_2 for 2 hours. The absorbance at 450 nm was measured and recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader (VersaMax; Molecular Devices, Silicon Valley, CA, USA).

4. Immunofluorescent staining

Cocultured THP1 cells were attached to slides with a cytospin, fixed in 4% paraformaldehyde and then rinsed with phosphate-buffered saline. To inhibit nonspecific signals, the slides were exposed to a blocking solution (2% normal goat serum) at room temperature for 2 hours. Then, the slides were incubated with antibodies against CD163 (1:300 dilution; Abcam Ltd., Cambridge, UK) and HLA-DR (an M1 macrophage marker) (1:300, Abcam Ltd.) overnight at 4℃. The slide was rinsed with phosphate-buffered saline three times and then incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG secondary antibody (1:600; Molecular Probes, Eugene, OR, USA) at room temperature for 2 hours. The samples were rinsed with phosphate-buffered saline three times before they were stained with 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and examined under a fluorescence microscope (BX50; Olympus, Tokyo, Japan).

5. Immunohistochemistry

Immunohistochemistry (IHC) was performed using an automated immunohistochemical stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's protocol. The sections were deparaffinized, pretreated with cell conditioning solution (CC1; Ventana), and subjected to ultraviolet radiation to abrogate endogenous peroxidase activity. The primary antibodies were diluted in Dako antibody diluent (DakoCytomation, Glostrup, Denmark) with background-reducing components and targeted the following proteins: CD68 (1:1,000; mouse, Dako), CD163 (1: 2,000; rabbit, Abcam, Franklin Lakes, NJ, USA), HLA-DR (1:10,000; mouse, Abcam), caspase-3 (1:500; rabbit, Cell Signaling Technology, Inc., Danvers, MA, USA) and FOXP3 (1:50; mouse, Abcam). The sections were incubated with primary antibodies at room temperature for 32 minutes and then hybridized with horseradish peroxidase-conjugated secondary antibody (Ventana) for 8 minutes. The reaction was developed with diaminobenzidine (DAB; Dako) for 5 minutes and the slides were counterstained with hematoxylin II (Ventana) for 4 minutes and a bluing reagent (Ventana) for 4 minutes. These sections were finally observed under a light microscope (BX50; Olympus).

6. Western blot

Cell lysates were extracted in RIPA lysis buffer containing protease inhibitor cocktail and protein phosphatase inhibitor cocktail for 20 minutes on ice. Total cell lysates were clarified by centrifugation. Protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Protein samples (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 4% to 20% gradient gels and transferred to a polyvinylidene fluoride membrane (Roche, Mannheim, Germany), which was blocked with 5% skim milk in tris-buffered saline with polysorbate 20 for 1 hour at room temperature. Primary antibodies against CD163 (ab189915; Abcam), HLA-DR (ab20181; Abcam), and β-actin (Cell Signaling Technology, Inc.) were diluted 1:1,000 in TBST. The membranes were incubated overnight at 4℃ with the primary antibodies and then washed three times with TBST before they were incubated in 1:5,000 horseradish peroxidase-conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody (Bio-Rad) for 1 hour at room temperature. The membrane was washed in TBST, and the hybridized protein bands were detected with an ECL detection kit (Bio-Rad) and processed with Image Lab software (Bio-Rad).

7. Enzyme-linked immunosorbent assay

The supernatants of cell cocultures and organotypic slice cultures were subjected to ELISA analysis to quantify the following cytokines: interleukin-10 (IL-10; Invitrogen, Carlsbad, CA, USA), TNF-α (R&D System), and tumor growth factor (TGF)-β (R&D System). The concentrations of cytokines were assessed using standard ELISA methods with a Quantikine kit according to the manufacturer's guidelines.

8. Organotypic slice culture and drug treatment

Freshly resected specimens were transferred from the operating room to the laboratory in a 50-mL conical tube containing ice-cold transport medium (HBSS (1X) supplemented with 1X Pen-Strep solution and 500 nmol/L diphenyl diselenide). Cores 6 mm in diameter were punched from fresh tumor tissues using a biopsy punch and sliced on a vibratome to a thickness of 250 μm. The detailed methods for slicing tumors and culture conditions are described in our previous study.[15] Pancreatic cancer slices were cultured in 6- or 24-well plates with culture media. Tumor slices were cultured for 4 days, and drug treatment was performed on the 2nd day. CD40 agonists (2 μg/mL or 10 μg/mL) or control isotype monoclonal antibodies (2 μg/mL) were added to pancreatic cancer slices for 48 hours in an incubation system (37℃, 5% CO_2). Gemcitabine (0.1 μM) was also administered under all conditions for 48 hours. Then, pancreatic cancer slices were fixed and embedded vertically to include both interfaces of tumor slices (the upper contacts with the air and the lower contacts with the membrane) in the final slides. Paraffin blocks containing three to five pieces of tumor slices were sectioned using a microtome into 3 μm thick sections. Hematoxylin and eosin staining, IHC staining (CD68, CD163, HLA-DR, FOXP3, and caspase-3), Western blotting (CD163 and HLA-DR), and ELISA (IL-10, TNF-α, and TGF-β) were performed using the sections or supernatants. Each slide had three to five pieces of tumor.

9. Image analysis and cell counting

For IHC evaluation of surgically resected human pancreatic cancers, we selected the most suitable tissue block which contains sufficient tumor and peritumor stroma together, from each patient. Six to eight high-power fields (HPFs, ×200 magnification) of IHC-stained slides that had the greatest staining intensity in each specimen per patient were selected for evaluation of IHC expression. For IHC evaluation of organotypic slice culture tissues, 6 to 12 HPFs (×200 magnification) of an IHC-stained slide that had the greatest staining intensity were selected and used for counting. All the stained slides including those processed for hematoxylin and eosin and IHC, were scanned using a slide scanner (Aperio CS2; Leica Microsystems, Wetzlar, Germany). Pictures from the scanned slides were captured with an Aperio ImagScope (version 12.3.2.5030; Leica Microsystems). In the captured images (×200 magnification), positive pixels corresponding to CD68, CD163, HLA-DR, and caspase-3 were counted automatically using an ImageJ (NIH, Bethesda, MD, USA), and $FOXP3^+$ cells were manually counted.

10. Ethics statement

This study was approved by the Institutional Research Board of the Catholic University of Korea (IRB numbers: HC16TNSI0100 and HC20SISI0043). Informed consent was obtained from all patients who had been enrolled. All study protocols were in complete compliance with the Declaration of Helsinki.

11. Statistical analysis

Quantitative data are described as the median and interquartile range. Error bars in the figures indicate standard errors. Quantitative variables in each group were compared using the Mann-Whitney U-test. The four cancer stage groups were compared using the Kruskal-Wallis test. Overall survival was plotted by the Kaplan-Meier method and compared by the log-rank test. A p-value <0.05 was considered to be significant. SPSS version 20 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA, USA) were used to conduct the statistical analysis and create the figures.

RESULTS

1. Expressions of M2 macrophage and regulatory T cell markers were related to advanced stages

IHCs of 12 human pancreatic cancers showed positive expression of macrophage markers (CD68, CD163, and HLA-DR) and regulatory T cell marker (FOXP3) (Fig. 1A). Macrophage markers were stained diffusely with high intensity, which indicated that there were many macrophages in human pancreatic cancer tissues. Since these macrophage markers were diffuse throughout the samples and the macrophages could not be distinguished clearly from surroundings, the positive expression of CD68, CD163, and HLA-DR was quantified by ImageJ with stained pixels. The median (interquartile range) positive pixels/HPF were 15,128 (9,402 to 31,583) for CD68, 6,118 (2,730 to 10,833) for CD163, and 4,623 (3,063 to 6,192) for HLA-DR. $CD163^+$ expression levels were significantly higher than HLA-DR expression levels (p<0.001), which indicates that M2 macrophages were

more predominant in human pancreatic cancer than M1 macrophages. In contrast to the macrophage-related expression, there were a small number of FOXP3⁺ cells in the tissues of pancreatic cancer. Because FOXP3 was detected in only small round cells (T lymphocytes), FOXP3⁺ small cells were manually counted. The median number (interquartile range) of FOXP3⁺ cells in human pancreatic cancer tissues was 7.5 (2.0 to 14.2) cells/HPF.

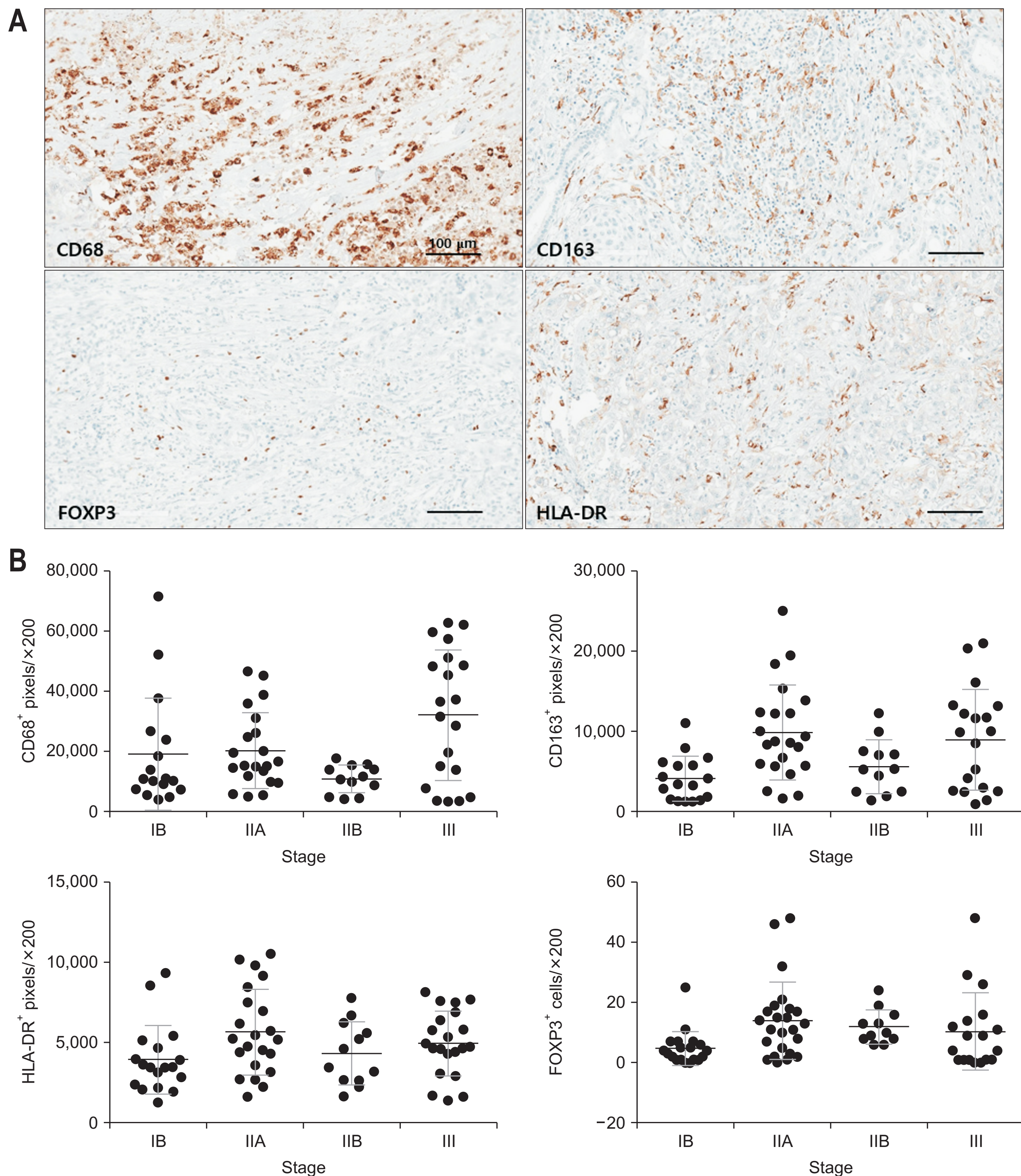

Fig. 1. Expression of macrophage and regulatory T cell markers in surgically resected human pancreatic cancers (n=12). (A) Representative immunohistochemical (IHC) staining (×200). (B) IHC expression levels according to cancer stages (Kruskal-Wallis test: p=0.051 for CD68, p=0.003 for CD163, p=0.118 for HLA-DR, and p=0.006 for FOXP3). (C) IHC expression levels according to cancer stage (stage IB vs stage II/III). *p<0.01.

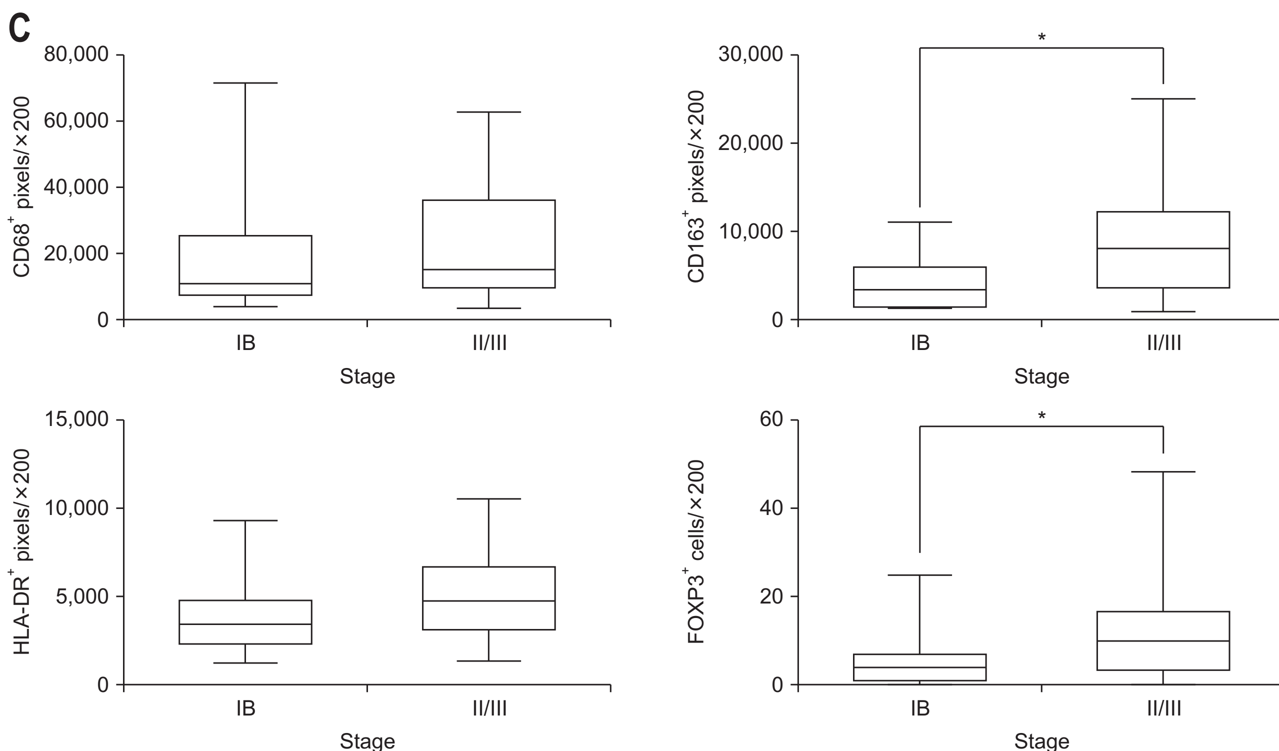

Fig. 1. Continued.

To evaluate the association between pancreatic cancer progression and macrophage phenotypes or regulatory T cells, the expression levels of CD68, CD163, HLA-DR, and FOXP3 were grouped according to cancer stage (Fig. 1B). Among the cancer stage groups, CD163$^+$ and FOXP3$^+$ expression levels significantly differed (p=0.003 and p=0.006, respectively). The patients were divided into groups based on stage (IB and II/III), and the analysis results indicated that CD163$^+$ and FOXP3$^+$ expression levels were significantly higher in patients with stage II/III pancreatic cancer than in those with stage IB disease (p=0.002 and p=0.003, respectively) (Fig. 1C). These findings suggest that M2 macrophages and suppressive regulatory T cells are significantly related to advanced pancreatic cancer.

2. Patients with high CD163$^+$ expression had shorter overall survival

We performed survival analysis with the four markers (CD68, CD163, HLA-DR, and FOXP3) to assess their influence on the prognosis of pancreatic cancer patients. The patients were divided into the high-expression group (more than the mean positive pixels/HPF) and low-expression group (less than the mean positive pixels/HPF). The mean positive pixels/HPF were 21,865 for CD68, 7,553 for CD163, and 4,823 for HLA-DR. The mean number of FOXP3$^+$ cells was 10.4 cells/HPF. Regarding CD163, patients with high CD163$^+$ expression levels (more than an average of 7,553 pixels/HPF) had significantly shorter overall survival than those with low CD163$^+$ expression levels (p=0.002) (Fig. 2). However, CD68$^+$, HLA-DR$^+$, and FOXP3$^+$ expression levels did not show significant relationships with survival in patients with pancreatic cancer. Thus, CD163 is considered to be more closely related to overall survival than HLA-DR, CD68, and FOXP3.

3. CD40 agonists facilitated the suppression of PANC-1 cells by shifting phenotypes of THP-1 cells

Macrophages pretreated with PMA and lipopolysaccharide showed strong expression of CD68 and CD163 and low expression of HLA-DR, which indicated that they are predominantly M2 macrophages. In cocultures of pretreated THP-1 macrophages and PANC-1 cells, immunofluorescence staining demonstrated that CD40 agonists (2 μg/mL) led to decreased CD163$^+$ expression (Fig. 3A) and increased HLA-DR$^+$ expression (Fig. 3B) in THP-1 macrophages cultured at ratios 1:1 and 1:10 (PANC-1:THP-1). Additionally, Western blot (Fig. 3C and D) showed that CD40 agonists deceased CD163 (p=0.034) and increased HLA-DR (p=0.046) expression at culture ratios of 1:1 and 1:10, respectively (n=4). These results suggested that CD40 agonists can promote macrophage polarization toward the M1 phenotype. THP-1 macrophages significantly reduced PANC-1 proliferation after coculture (p<0.001), and PANC-1 proliferation decreased according to the number

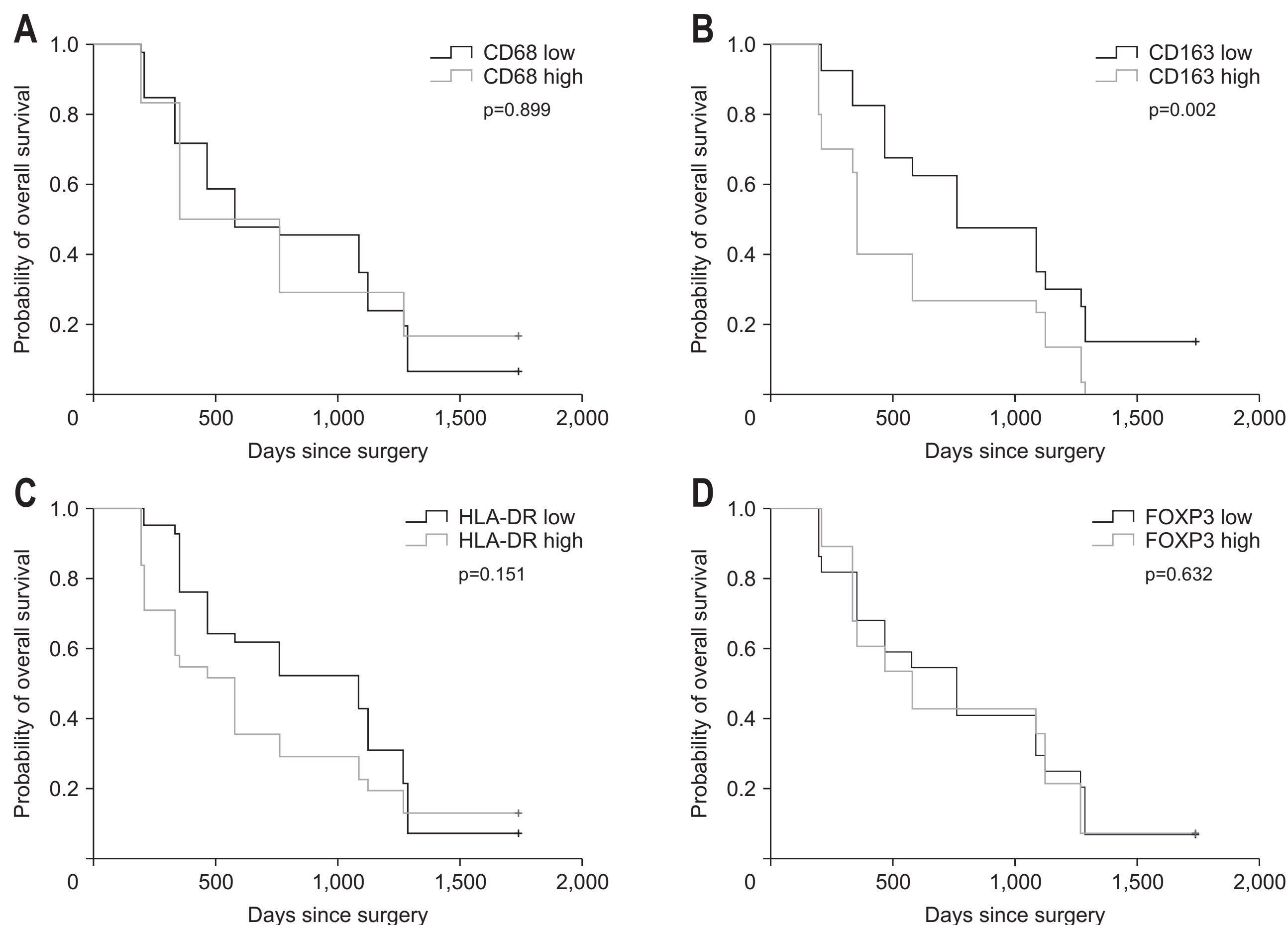

Fig. 2. Overall survival of patients with pancreatic cancer according to the expression of markers: CD68 (A), CD163 (B), HLA-DR (C), and FOXP3 (D).

of THP-1 cells and presence of CD40 agonist (n=4) (Fig. 4). PANC-1 proliferation was reduced more in the 1:10 ratio coculture (10 times that of THP-1 cells) than in the 1:1 coculture (p=0.026). Regarding CD40 agonists, the proliferation of PANC-1 cells was reduced significantly by CD40 agonists at 1:1 and 1:10 coculture ratios (p=0.003 and p=0.001, respectively). These results of the coculture experiments with CD40 agonists revealed that the suppressive effect of THP-1 macrophages on the proliferation of PANC-1 is proportional to the number of THP-1 cells, and CD40 agonists facilitate macrophages to suppress the proliferation of PANC-1 by promoting M1 polarization of THP-1 cells.

4. Organotypic slice cultures showed that CD40 agonists shifted macrophage polarization, decreased regulatory T cells, and increased tumor apoptosis

Tumor slices from eight patients with pancreatic cancer were cultured on membrane inserts in the presence or absence of CD40 agonists. The tumor slices were subjected to IHC staining (similar to surgical paraffin blocks) for CD68, CD163, HLA-DR, FOXP3, and caspase-3 (Fig. 5A). Macrophage markers (CD68, CD163, and HLA-DR) were diffusely stained, and there was a small number of FOXP3$^+$ regulatory T cells. CD40 agonists affected the expression of macrophage and regulatory T cell markers. IHC of tumor slices treated with CD40 agonists showed macrophage differentiation into the M1 phenotype with increased HLA-DR$^+$ expression and decreased CD163$^+$ expressions (Fig. 5B). Although a low dose of CD40 agonist (2 μg/mL) did not induce significant changes, a high dose (10 μg/mL) led to significant changes in HLA-DR$^+$ and CD163$^+$ expression. CD163$^+$ expression levels were significantly lower in the 10 μg/mL CD40 agonist group than in the isotype control (p=0.014) and 2 μg/mL CD40 agonist groups (p=0.012), and HLA-DR$^+$ expression levels were significantly higher in the 10 μg/mL CD40 agonist group than in the isotype control (p<0.001) and 2 μg/mL CD40 agonist groups (p<0.001). CD40 agonists reduced suppressive regulatory T cells, with significantly lower numbers in 10 μg/mL CD40 agonist group than in the 2 μg/mL CD40 agonist group (p=0.009). The M1/M2 (HLA-DR/CD163) ratio was also significantly increased in the 10 μg/mL

Fig. 3. Different expression of macrophage markers induced by CD40 agonists after coculture with PANC-1 cells and THP-1 macrophages (×200). Immunofluorescence staining of (A) CD163 and (B) HLA-DR expression in THP-1 cells; (a) 1:1 (PANC-1:THP-1) isotype control, (b) 1:1 CD40 agonist, (c) 1:10 isotype control, and (d) 1:10 CD40 agonist. Western blotting of (C) CD163 and (D) HLA-DR expression in THP-1 cells. *p<0.05.

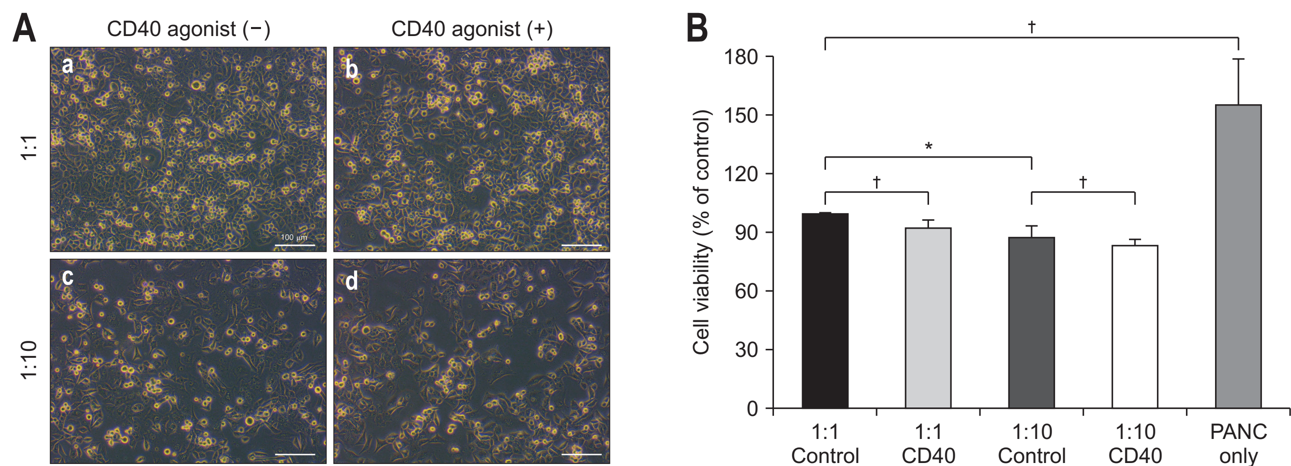

Fig. 4. Coculture of PANC-1 cells and THP-1 macrophages (×200). (A) PANC-1 cells after 4 days coculture; (a) 1:1 (PANC-1:THP-1) isotype control, (b) 1:1 CD40 agonist, (c) 1:10 isotype control, and (d) 1:10 CD40 agonist. (B) Viability (CCK8) of PANC-1 cells. *p<0.05 and †p<0.01.

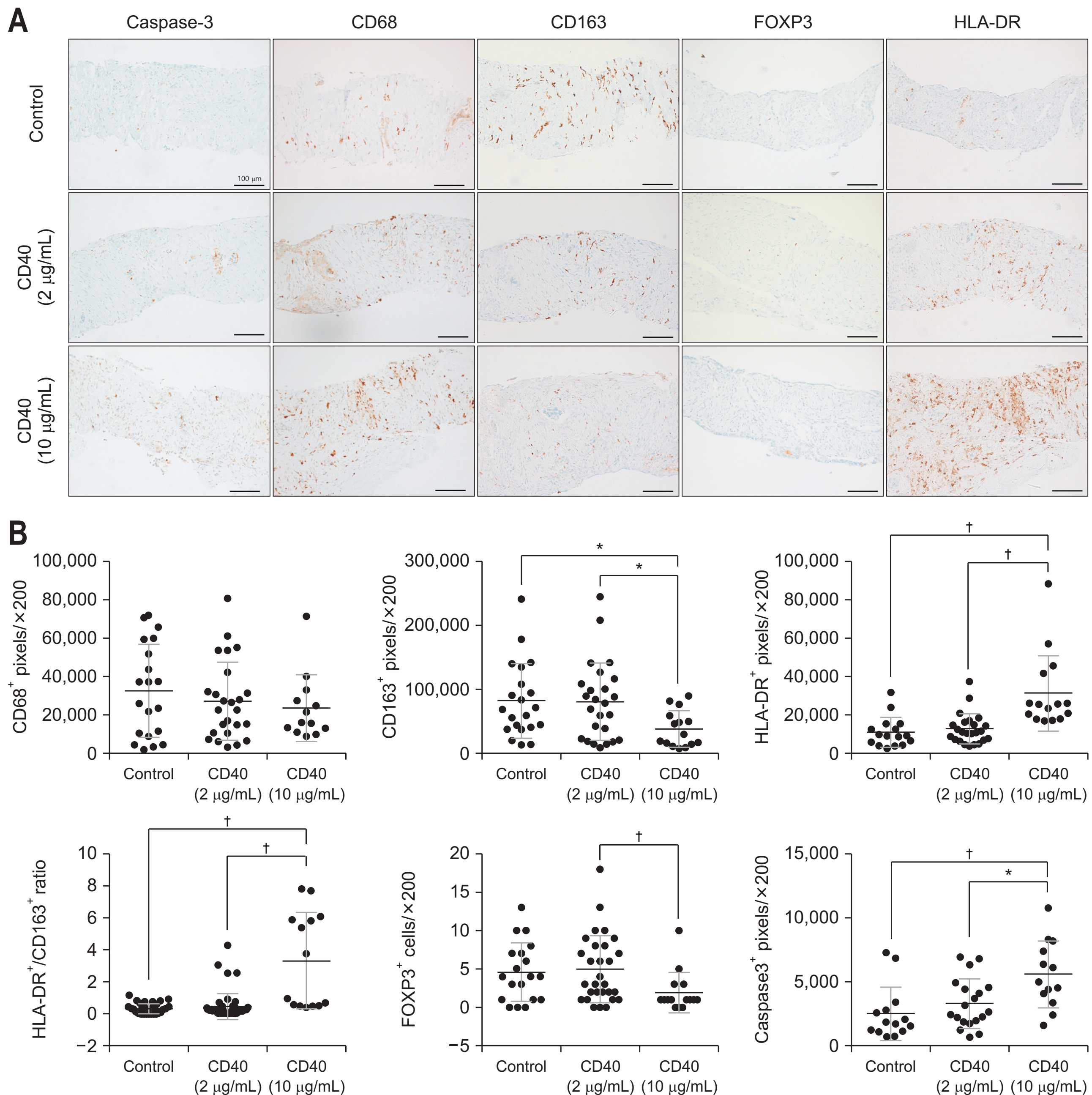

Fig. 5. Responses of human pancreatic cancer in organotypic slice culture to CD40 agonists. (A) Immunohistochemical staining of macrophage, regulatory T cells, and apoptosis markers. Rows are conditions, and columns are markers (×200). (B) Expression levels of five markers after CD40 agonist treatment. (C) Comparison of differences in immunohistochemical expression between adenosquamous carcinoma and adenocarcinoma. Western blot of (D) CD163 and (E) HLA-DR expression in tumor slices (n=4). *p<0.05 and †p<0.01.

CD40 agonist group compared to the control and 2 μg/mL CD40 agonist groups (p<0.001). In addition, CD40 agonists increased tumor apoptosis: caspase-3$^+$ expression was significantly higher in the 10 μg/mL CD40 agonist group than in the isotype control (p=0.002) and 2 μg/mL CD40 agonist groups (p=0.016). The expression levels of CD68$^+$, CD163$^+$, and FOXP3$^+$ were significantly higher in adenosquamous carcinoma specimens than those in adenocarcinoma specimens (p<0.001) (Fig. 5C). In addition, HLA-DR$^+$ expression tended to be lower in adenosquamous carcinoma specimens than in adenocarcinoma specimens. This indicated that adenosquamous carcinoma has M2 characteristics and elicits greater immune suppression, which might be related to the aggressive biologic behavior of adenosquamous carcinoma. Western blot also showed a decrease in CD163 expression and an increase in HLA-DR expression in the tumor slices treated with CD40 agonists (10 μg/mL), p=0.032 and p=0.041, respectively (n=4) (Fig.

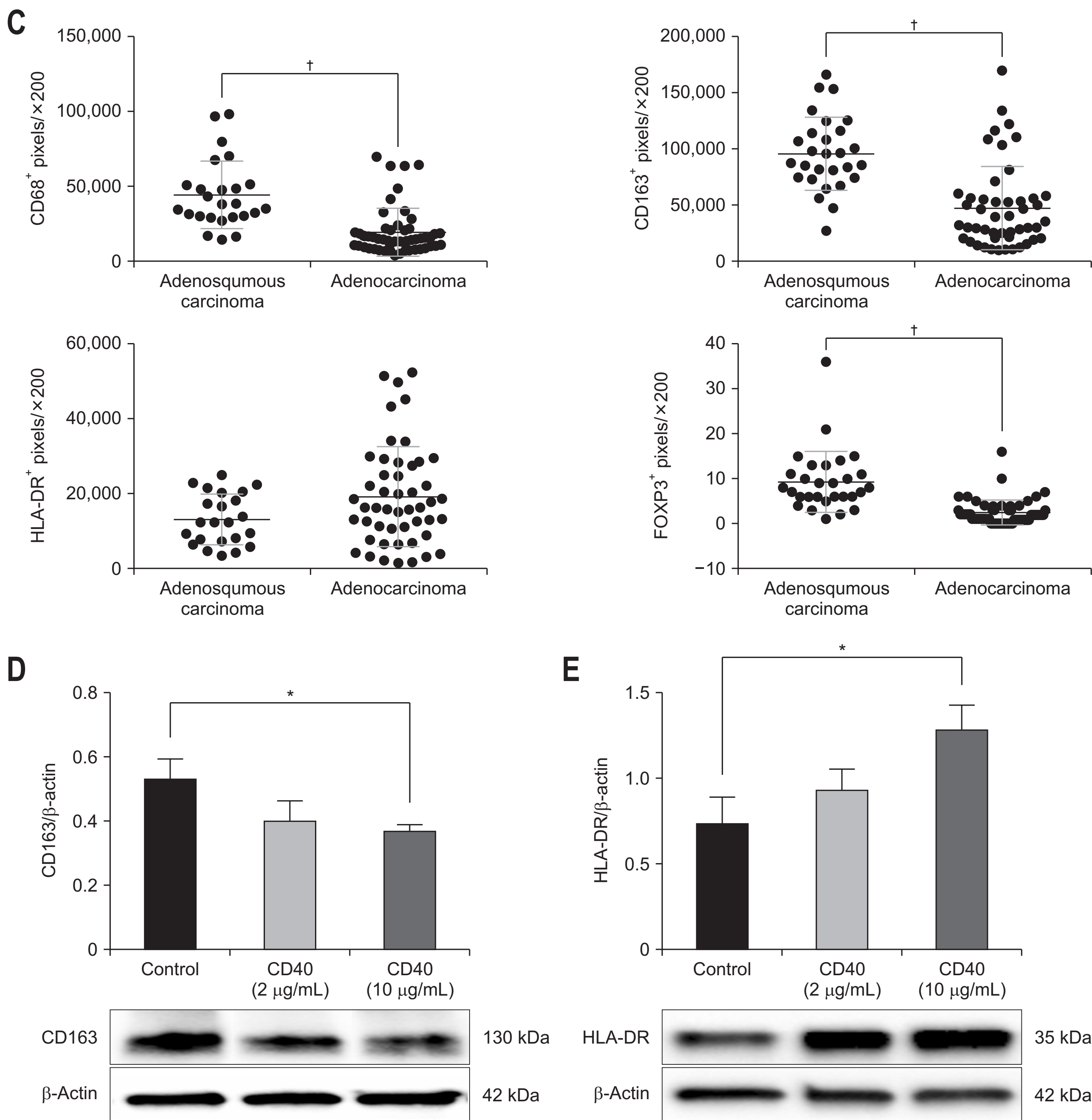

Fig. 5. Continued.

5D and E).

5. CD40 agonists increased TNF-α levels in the supernatant

The supernatants from cell cocultures (n=5) and organotypic slice cultures (n=4) were collected and analyzed by ELISA to evaluate the levels of macrophage cytokine, such as TNF-α, TGF-β1, and IL-10 (Fig. 6). TNF-α is considered an M1 cytokine, and TGF-β1 and IL-10 are considered M2 cytokines.[2] In 1:10 (PANC-1:THP-1) cell cocultures, TNF-α levels were significantly increased by CD40 agonists (86.8±51.8 pg/mL) compared to isotype antibody controls (67.1±39.4 pg/mL) (p=0.036). In addition, TGF-β1 and IL-10 levels showed a tendency to decrease in response to the CD40 agonist in 1:1 and 1:10 (PANC-1: THP-1) cell cocultures. These results suggest that CD40 agonists induce the production of M1 cytokines (TNF-α) rather than M2 cytokines (TGF-β1 and IL-10) in cell cocultures. In organotypic slice cultures, TNF-α levels also increased after treatment with 2 μg/mL CD40

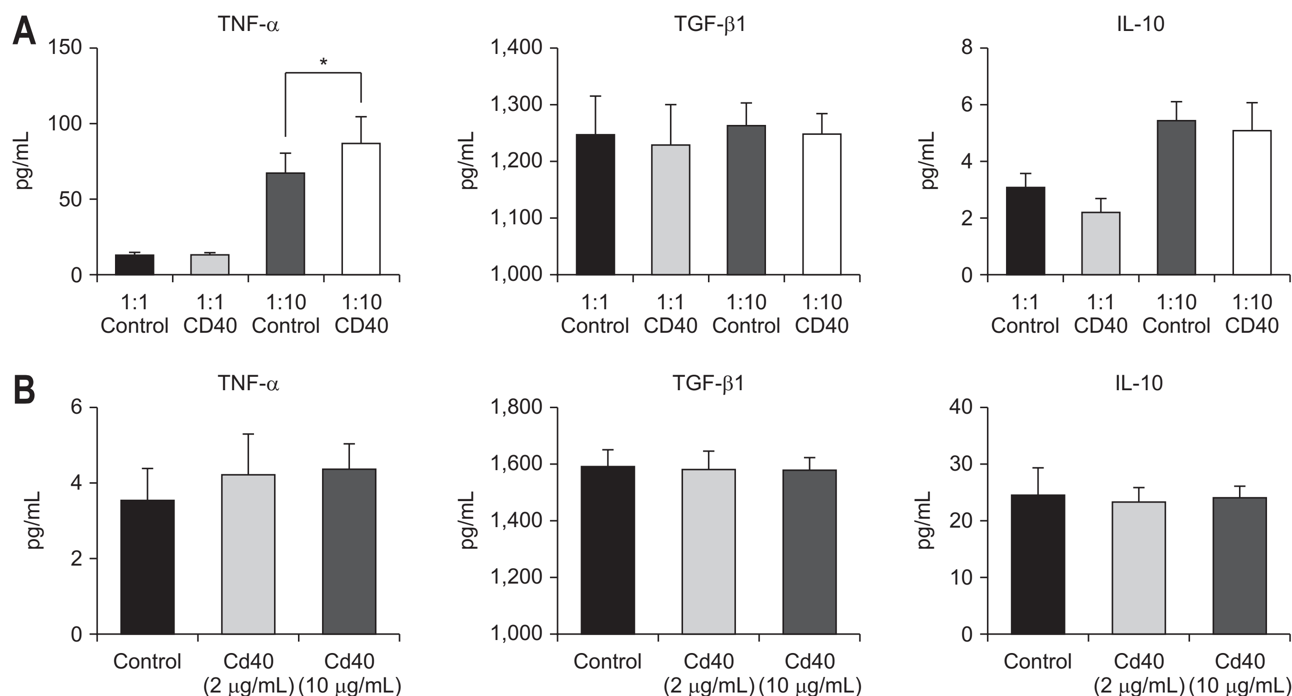

Fig. 6. Cytokine levels in the supernatants of cultures. (A) Cell cocultures and (B) organotypic slice cultures. Conditioned medium containing 10% fetal bovine serum has a TGF-β1 concentration of 1,153 pg/mL.
TNF, tumor necrosis factor; TGF, tumor growth factor; IL, interleukin. *$p<0.05$

agonists (4.2±3.9 pg/mL) and 10 μg/mL CD40 agonists (4.3±2.7 pg/mL) compared to isotype antibody controls (3.5±3.5 pg/mL); however, the increase was not statistically significant. TGF-β1 and IL-10 levels in the organotypic slice cultures showed no significant changes in response to CD40 agonists. Overall TGF-β1 and IL-10 levels in organotypic slice cultures were more than four times higher than those in cell cocultures, which indicated that the human pancreatic cancer microenvironment is more immunosuppressive, with higher levels of M2 cytokines.

DISCUSSION

The tumor microenvironment of pancreatic cancer contains a staggering milieu of cells such as immature monocytes, regulatory T cells, mast cells, dendritic cells, natural killer cells, neutrophils, pancreatic stellate cells, cancer-associated fibroblasts, and TAMs.[16] This heterogeneous population and their interactions with tumors and stroma contribute to tumorigenesis. During tumorigenesis, dysregulated signals lead to biased expression of various immune mediators, resulting in tumor growth, tumor progression and immunosuppression including differentiation of TAMs. TAMs represent the major inflammatory component of the tumor stroma, and the stroma of pancreatic cancer has a large number of macrophages.[17] TAMs have functional plasticity by adapting their phenotype to respond to stimuli and signals specific to the tumor microenvironment. According to their functions, macrophages can be broadly categorized into two subgroups; classically activated M1 or alternatively activated M2. M1 macrophages are involved in Th1 responses, which include the detection and clearance of tumor cells and the presentation of antigens to T cells by secreting IL-1β, IL-6, and TNF-α.[2] In contrast, M2 macrophages inhibit antitumor activity and induce Th2 responses by IL-10, IL-13, and TGF-β.[18] TAMs are traditionally characterized by M2 polarization, which is involved in the whole process of cancer progression from initiation to distant metastasis, including tumorigenesis, immune evasion, invasion, angiogenesis, and metastasis.[16,19] Although TAMs exhibit M2 characteristics, previous studies reported that TAMs can exhibits a mix of the M2 and M1 phenotypes,[1] where more than 20% of TAMs in pancreatic cancer had dual positive expression of CD163 (an M2 marker) and HLA-DR (an M1 marker), and TAMs secrete IL-1β as well as typical M2 cytokines.[1] The present study also revealed that diffuse expression of M1 and M2 markers in human pancreatic cancer specimens.

The role of TAMs in pancreatic cancer has been actively studied. M2-polarized TAMs promoted epithelial-mesenchymal transition in pancreatic cancer cells partially through the TLR4/IL-10 signaling pathway.[6] TAMs induced by conditioned media from pancreatic cancer cells

promote tumor metastasis by secreting IL-8.[20] In particular, CD163 expression was suggested as a prognostic factor in human pancreatic cancer. In 212 patients with pancreatic cancer, M2 macrophages (CD163$^+$ or CD204$^+$) were significantly associated with shorter survival, and %M1^{high}/M2^{low} was an independent prognostic factor.[5] Kurahara *et al.*[21] showed in 76 patients with pancreatic cancer that CD163$^+$ macrophages were related to lymph node metastasis and poor prognosis, although CD68$^+$ macrophages had no relation to either characteristic. Another study reported in 99 patients with pancreatic cancer that dense CD163$^+$ M2 macrophage infiltration into the stroma was an independent prognostic factor.[22] In addition, CD163 has been found to be a significant factor associated with prognosis in other malignancies, such as intrahepatic cholangiocarcinoma,[3] hepatocellular carcinoma,[4] and extrahepatic bile duct cancer.[23] Our present study demonstrated that CD163 is significantly related to overall survival in patients with pancreatic cancer, although CD68, HLA-DR, and FOXP3 are not. The fact that CD163 is a significant factor for survival suggests that M2 macrophages are closely related to the prognosis of pancreatic cancer.

Since TAMs are associated with tumorigenesis and tumor progression, immunotherapy targeting macrophages is a promising modality to overcome insensitivity to chemoradiotherapy and improve the prognosis of pancreatic cancer patients. Several molecules that target macrophages have been studied. The chemokine ligand 2/chemokine receptor 2 (CCR2) axis involves inhibition of macrophage recruitment from the bone marrow and migration to tumor microenvironment. In a murine pancreatic cancer model, blockade of CCR2 depletes monocytes and macrophages from the tumor, which results in enhanced antitumor immunity, decreased tumor growth, and reduced metastasis.[24] Colony-stimulating factor 1 receptor blockade in pancreatic cancer models reprograms TAMs to support antitumor interferon responses and T cell activities,[25] or to induce reduction in M2 TAM levels and increase in M1 TAM levels.[26] Pancreatic cancer cells secrete Bcl-2-associated athanogene 3 (BAG3), which binds to and activates macrophages. Anti-BAG3 antibody results in reduced tumor growth and prevents metastasis formation in a pancreatic cancer mouse model.[27] In addition to these targets, CD40, a member of the TNF receptor family, is a potential target that can alter macrophage activation. CD40 is a cell surface molecule on immune cells including macrophages. CD40 agonistic antibodies that bind to CD40 promote the maturation of antigen-presenting cells and enhance the tumoricidal activity of macrophages. A previous study showed that CD40 agonists alter the pancreatic tumor microenvironment to increase sensitivity to immune checkpoint blockade.[28] In a genetically engineered mouse model of pancreatic cancer, CD40-activated macrophages rapidly infiltrated tumors and became tumoricidal independent of T cells.[10] Our study demonstrated that CD40 agonists triggered macrophage polarization toward the M1 phenotype and increased TNF-α levels (an M1 cytokine) in cell cocultures. Additionally, CD40 agonist-activated M1 macrophages suppressed the proliferation of human pancreatic cancer cells. Our organotypic slice culture also showed the effect of CD40 agonists on macrophages in human pancreatic cancer tissues with increasing HLA-DR$^+$ (M1 phenotype) and decreasing CD163$^+$ (M2 phenotype) expression. Moreover, CD40 agonists led to a decrease in the infiltration of FOXP3$^+$ suppressive regulatory T cells and an increase in caspase-3$^+$ levels (indicative of apoptosis). These findings imply that CD40 agonists can trigger M1 macrophages polarization, reduce the number of infiltrating regulatory T cells, and induce apoptosis in human pancreatic cancer. Based on our results that CD163 expression is negatively associated with overall survival in patients with pancreatic cancer, CD40 agonists may positively affect the survival of patients.

CD40 agonists can mediate T cell-dependent and T cell-independent immune mechanisms of tumor regression.[8] T cell-independent mechanisms modulate the immune status of the tumor microenvironment by activating macrophages and destroying the tumor stroma.[10] T cell-dependent mechanisms involve upregulating antigen presentation and activating T cells. Previous preclinical models revealed synergistic enhancement from combining CD40 agonists with cytotoxic agents or T cell-inducing vaccines.[11,29] Chemotherapy may function as a vaccine by presumably releasing tumor antigens in an immunogenic fashion.[8] In our experiment, gemcitabine was combined with CD40 agonists in organotypic slice cultures to induce T cell-dependent effects in addition to macrophage effects. Although detailed T cell changes or effects were not evaluated in the present study, a decrease in regulatory T cells by CD40 agonists was demonstrated. Therefore, the apoptosis of pancreatic cancer observed in our study is considered to have been caused by T cell dependent and independent mechanisms by CD40 agonists.

We utilized an organotypic slice culture model in the present study. Organotypic slice cultures have been recently developed and utilized for studying several tumors including head, neck, breast, gastric, lung, and pancreatic cancers.[30-33] Existing preclinical models of pancreatic cancer, such as primary cell lines, tumor xenografts, genetically engineered mouse models, and organoids, have some limitations: lack of stroma, immunocompromised environment, nonhuman species, time requirement, and

costs. In contrast, organotypic slice culture preserves tumor cells and their unique tumor microenvironment and is an open system permitting direct and rapid access to drug treatment.[15] It has been demonstrated that organotypic slice cultures of pancreatic cancer maintains viability, proliferative activity, and microenvironment during cultivation.[15,33,34] Previous studies reported that this technique enables the rapid assessment of drug responses to viable pancreatic cancer tissue ex vivo and subsequent downstream analyses.[15,35] Thus, organotypic slice culture would prospectively allow for the prediction of drug efficacy and identification of effective drugs in an individual cancer patient for personalized treatment.

Regarding IHC and interpretation of macrophages, there are a few controversial points. First is the lack of robust markers to quantify TAMs. We used CD68 as a pan-macrophage marker, HLA-DR as an M1 marker, and CD163 as an M2 marker, all of which are commonly used markers. However, some studies used other markers, such as CD11c and iNOS for M1 and CD204 and CD206 for M2.[36] Although CD68 is a pan-macrophage marker, it is not specific for the monocyte/macrophage system and can be immunohistochemically detectable in a variety of other cell types. In addition, CD163 is an M2 macrophage marker but is also expressed in some dendritic and endothelial cells.[37] This could result in inaccurate M1 and M2 TAM counts. Second, it is difficult to quantify M1 and M2 TAMs in a tissue section, which is prone to bias and interobservational discrepancies. Digital image analysis can help reduce subjective human errors, ensuring precise and accurate results each time.[36] However, this application could incorrectly misidentify cells such as dendritic and endothelial cells as TAMs. In the present study, we used digital image analysis to reduce such bias. Since macrophage markers were stained diffusely and macrophages cannot be distinguished clearly, we utilized stained pixels interpreted by ImageJ. Although the pixels could include a few components other than macrophages, they did not hinder the relative comparison of responses by CD40 agonists. To support our findings, ELISA analysis of cytokine profiling in the supernatants was added. Although CD163 was related to survival in the present study, it cannot be concluded that CD163 affects survival independently due to the various factors affecting survival and the relatively small number of patients analyzed. However, we evaluated as many as 6 to 8 HPFs of surgically resected specimens per patient to obtain as much data as possible.

In summary, human pancreatic cancer showed more M2 polarization of macrophages, and CD163 was associated with advanced cancer stages and shorter overall survival in patients with pancreatic cancer. CD40 agonists alter macrophage differentiation to favor the M1 phenotype and suppress the proliferation of human pancreatic cancer cells in cell coculture. Organotypic slice culture demonstrated that CD40 agonists induce M1 polarization of TAMs and increase the apoptosis of human pancreatic cancer.

CONFLICTS OF INTERSET

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: J.H.C., C.Y.L. Methodology: C.Y.L., J.H.C., W.S.L. Materials: I.Y.P. Experiments: C.Y.L., J.H.C., W.S.L. Funding acquisition: J.H.C. Pathologic review: J.H.C., J.K. Writing - original draft: J.H.C. Writing - review and editing: C.Y.L., W.S.L. Approval of final manuscript: all authors.

ORCID

Chae Yoon Lim https://orcid.org/0000-0001-9645-1030
Jae Hyuck Chang https://orcid.org/0000-0003-1180-2693
Won Sun Lee https://orcid.org/0000-0003-3043-0071
Jeana Kim https://orcid.org/0000-0003-1021-0290
Il Young Park https://orcid.org/0000-0003-4590-2297

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