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Blockade of PD-1/PD-L1 Pathway Enhances the Antigen-Presenting Capacity of Fibrocytes

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Fibrocytes, a distinct population of collagen-producing, monocyte-derived cells, are involved in wound healing as well as fibrotic diseases. Recently, fibrocytes have been revealed to play a role in the tumor microenvironment, particularly under antiangiogenic therapy. In addition, combination cancer immunotherapy with immune checkpoint inhibitor and antiangiogenic agents have been developed for various cancers in the clinical setting, although the immunological background is not clear. In the current study, we aimed to determine the function of fibrocytes in tumor immunity induced by immune checkpoint inhibitor therapy. Human and murine fibrocytes were generated from PBMCs and lungs, respectively. The expression of costimulatory and inhibitory molecules on fibrocytes was examined by flow cytometry. The stimulation of $CD8^+$ T cells by fibrocytes was examined in MLRs with a ^{3}H-thymidine incorporation assay. Fibrocytes expressed $CD80^{low}$ and $CD86^{high}$ as a costimulatory molecule, and expressed $PD\text{-}L1^{high}$, but not PD-L2, as a coinhibitory molecule. Without any stimulation, fibrocytes strongly enhanced the proliferation of $CD8^+$ T cells in mice and humans. Treatment with anti-CD86 and -CD54 Abs inhibited the growth of $CD8^+$ T cells induced by fibrocytes. Anti–PD-L1 Ab further enhanced the proliferation of $CD8^+$ T cells, even in the OVA-specific MLR with OT-$1Rag^{-/-}$ mice. Importantly, fibrocytes derived from PBMCs of patients with lung adenocarcinoma or murine MC38 tumors augmented the proliferation of $CD8^+$ T cells with PD-L1 blockade. These results suggest that fibrocytes infiltrating tumor sites may play a role in the antitumor immunity mediated by $CD8^+$ T cells when the activity is further enhanced by PD-L1/PD-1 blockade. ***The Journal of Immunology*****, 2021, 206: 000–000.**

Fibrocytes, a distinct population of collagen-producing, monocyte-derived cells, exhibit diverse morphological and molecular characteristics of both macrophages and fibroblasts (1–3). In the peripheral circulation, fibrocytes account for ∼0.5% of the leukocyte pool. Morphologically, fibrocytes appear as adherent, large, spindle-shaped cells on in vitro culture. At the time of writing, there is no single specific marker to identify fibrocytes, the combination of intracellular collagen staining along with the expression of hematopoietic marker CD45 and chemokine receptor CXCR4 is thought to be appropriate to isolate fibrocytes (2, 4–8).

In health, fibrocytes home to the sites of injury and promote wound healing by producing proinflammatory cytokines (IL-6 and IL-8), chemokines (CXCL3 and CXCL4), and collagen (2, 9, 10). However, fibrocytes have also been reported to involve in the pathogenesis of various fibrotic diseases, such as interstitial lung disease, bronchial asthma, cardiovascular disease, and rheumatoid arthritis (6, 11–14). In addition, fibrocytes have been demonstrated to function as a potent APC (15). The surface expression of immune molecules (CD80 and CD86) and MHC class II are thought to potentiate the immune response by fibrocytes (16–18).

However, there is an increasing body of evidence suggesting that fibrocytes from cancer patients suppress the T cell function and, as a result, support tumor progression. Fibrocytes instigate lung metastasis by inducing the influx of Ly-$6C^+$ monocytes via CCL2 (19). The production of indolamine oxidase by fibrocytes is another of their immunosuppressive properties (20). Based on these data, it is unclear whether fibrocytes stimulate or suppress the T cell function.

We and others have reported the proangiogenic property of fibrocytes via the production of angiogenic factors, including fibroblast growth factor 2 (FGF-2) (21, 22). In the tumor microenvironment,

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Abbreviations used in this article: DC, dendritic cell; hPD-L1, human PD-L1; ^{3}H-TdR, ^{3}H-thymidine; ICI, immune checkpoint inhibitor; mIgG, mouse IgG; PD-1, programmed cell death 1; rh, recombinant human; rm, recombinant murine.

under treatment with the anti–VEGF-A Ab bevacizumab, we found a remarkable increase in tumor-infiltrating fibrocytes that mediated acquired resistance in mice and humans (21).

Recent advances in cancer immunotherapy have resulted in the development of immune checkpoint inhibitors (ICIs) of several Abs against CTLA4 and programmed cell death 1 (PD-1) and its ligand PD-L1, which have been approved for various malignancies (23, 24). In addition, clinical trials involving combination therapy with anti–PD-1/PD-L1 Abs and other modalities have demonstrated promising efficacy against some cancers (25, 26). Among these, the combination of ICIs with antiangiogenic agents, which eventually increase the infiltration of fibrocytes into tumors, has been expected, whereas an immunological background showing additive or synergistic effects remains unclear.

It is of importance to understand the role of immune checkpoint molecules expressed in various types of immune cells, including fibrocytes in the tumor microenvironment. In the current study, we analyzed the expression and role of PD-L1 in the Ag-presenting capacity of both human and murine fibrocytes in vitro. We also compared the Ag-presenting and T cell–stimulatory ability of fibrocytes with those of dendritic cells (DCs) and several types of macrophages. Finally, we examined the immune regulatory function of PD-L1–expressing cancer-associated fibrocytes derived from PBMCs of patients with human lung adenocarcinoma and MC38 tumor in mice.

Materials and Methods

Mice

C57BL/6 female and BALB/C male mice were purchased from The Jackson Laboratory and Charles River Laboratory, respectively. OT-I Rag1$^{-/-}$ transgenic mice expressing a TCR specific for OVA$_{257\text{-}264/Kb}$ were kindly provided by Prof. K. Yasutomo (Tokushima University, Tokushima, Japan). All mice were maintained under specific pathogen-free conditions in the animal research center of Tokushima University, and all animal experiments were approved by the animal research committee of Tokushima University and performed in accordance with our institution's guidelines for animal care and use.

Isolation of human fibrocytes

Human fibrocytes were isolated according to previously published methods (7). Informed consent was obtained from all volunteers, and the protocol was approved by the institutional review board of Tokushima University Hospital (no. 2838). Briefly, PBMCs were isolated from the peripheral blood of healthy volunteers using Ficoll density centrifugation. The isolated cells were cultured in DMEM supplemented with 20% FBS, penicillin, and streptomycin on fibronectin (no. 1030-FN; R&D Systems, Minneapolis, MN)–coated, 150-mm cell culture dishes (BD Pharmingen, Franklin Lakes, NJ). After 7–8 d of incubation at 37˚C with 5% CO_2, only strongly adherent cells (washed three times with sterile PBS) were determined to be circulating fibrocytes by a flow-cytometric analysis. A flow-cytometric analysis revealed that harvested fibrocytes expressed CD45, collagen I, and CXCR4 and showed >90% purity.

Fibrocytes were also isolated from patients with lung cancer (adenocarcinoma) without any chemotherapy/radiotherapy/intervention.

Purification of fibrocytes from the mouse lung

The isolation of murine fibrocytes follows a previously published protocol (4). In brief, lungs removed from female C57BL/6 mice were minced with scissors and incubated with DMEM containing 1 mg/ml BSA (no. A9418; Sigma-Aldrich, St. Louis, MO), 1 mg/ml collagenase type 1 (no. LS004196; Worthington, Lakewood, NJ) and 500 μg/ml DNase 1 (no. LS002139; Worthington) for 1 h at 37˚C. The single-cell suspensions from whole lungs were incubated in DMEM supplemented with 20% FBS in 150-mm, fibronectin-coated dishes. After 5 d, the trypsinized cells were incubated with anti-CD45 Ab coupled to magnetic beads (130-052-301; Miltenyi Biotec, Auburn, CA) for 15 min at 4–8˚C. CD45$^+$ populations were purified using an autoMACS instrument (Miltenyi Biotec) according to the manufacturer's instructions. In flow cytometry, intracellular staining revealed that more than 95% of these cells were positive for collagen 1; these were therefore used as fibrocytes.

Flow cytometry

Detection of human fibrocytes by flow cytometry has been described previously (7, 27). Briefly, FITC-conjugated anti-CD45 (1:25 dilution, HI30; BioLegend, San Diego, CA) and PE-conjugated anti-CXCR4 Abs (1:25 dilution, 12G5; eBioscience, San Diego, CA) were used to stain the surface molecules before proceeding to intracellular staining. Cells were then fixed and permeabilized (BD Pharmingen) for staining with biotin-conjugated anti–collagen I Ab (1:100 dilution, no. 600-406-103; Rockland, Limerick, PA) and PE-Cy7 streptavidin (1:500 dilution, no. 405206; BioLegend).

The expression of immune checkpoint molecules on fibrocytes has been documented with regard to anti-human PE CD274 (PD-L1, MIH-1), CD273 (PD-L2, MIH18), CD152 (CTLA-4, L3D10), CD366 (TIM-3, F38-2E2), CD40 (5C3), CD80 (2D10.4), CD86 (IT2.2), HLA-ABC (W6/32), and HLA-DR (LN3) Abs and mouse IgG (mIgG) 1 k (P3.6.2.8.1) (eBioscience). Anti-human PE Galectin-9 (9M1-3), CD54 (HA58) Abs (BioLegend), and anti-human PE-Cy7 CD279 (PD-1, EH12.1) Ab and mIgG1 (MOPC-21) (BD Pharmingen), anti-human PE CD3 (UCHT1), CD19 (HIB19), and CD14 (61D3) Abs (eBioscience) were also used.

To identify murine fibrocytes, cells were fixed and permeabilized for intracellular staining. Before permeabilization, PE-conjugated Ab to CD45 (1:25, 30-F11; eBioscience) and FITC-conjugated Ab to CXCR4 (1:25 dilution, no. 557967; BD Biosciences) were applied. Biotin-conjugated anti–collagen I Ab (1:100 dilution, 600-406-103; Rockland) and PE-Cy7 Streptavidin (1:500 dilution, 405206; BioLegend) were applied to detect intracellular collagen simultaneously with CD45 and CXCR4.

For murine immune checkpoint expression, anti-murine PE CD274 (MIH5), CD273 (122), CD40 (1C10), CD86 (GL1), and CD54 (YN1/1.7.4) Abs and rat IgG2a (eBR2a) (eBioscience), and anti-murine PE CD80 (1G10; life Technology, Carlsbad, CA), PE Galectin-9 (RG9-35; BD Pharmingen), PE MHC-H-2K^b (28-8-6), PE-Cy7 anti-mouse I-A/I-E (M5/114.15.2), PE CD152 (UC10-4B9), PE CD366 (TIM-3) and PE CD279 (RMPI-14) Abs, and rat IgG2b (RTK 4530) (BioLegend) were used.

For the detection of DCs, anti-human PE CD83 (HB15e; eBioscience) and anti-murine PE CD11c (N418; BioLegend) Abs were used, respectively, for humans and mice. Monocyte-derived macrophage polarization to M1 and M2 was documented with anti-human PE CD14 (61D3), CD80 (2D10.4), CD86 (IT2.2), CD163 (GH1/61), and CD206 (19.2) Abs (eBioscience) in humans and anti-mouse FITC F4/80 (BM8), PE CD80 (1G10), CD86 (GL1), CD206 (MR6F3; eBioscience), CD200R (OX-110; BioLegend), and CD11b Abs (M1/70; BD Pharmingen) in mice. The stained cells were analyzed by flow cytometry using a BD LSRFortessa (BD Biosciences) for acquisition and were analyzed with the FlowJo software program (Tree Star, Ashland, OR).

Proliferation assay of CD8$^+$ T cells

In human MLR, CD8$^+$ T cells were isolated from the PBMCs of healthy donors with a CD8$^+$ T cell isolation kit (no. 130-045-201; Miltenyi Biotec). Human CD8$^+$ T cells were plated in a 96-well plate (flat bottom; Falcon) coated with anti-CD3 Ab (OKT3, 5 μg/ml, 37˚C for 2–3 h; eBioscience) and/or soluble anti-CD28 Ab (CD28.2, 2 μg/ml; eBioscience) at a density of 1×10^5 cells per well. Fibrocytes or DCs were added to CD8$^+$ T cells at 1×10^4 cells per well after irradiation (25 Gy) with a Hitachi Medico MBR-1520A-TW (Hitachi, Chiba, Japan) or treatment with mitomycin C (25 μg/ml) at 37˚C for 30 min (no. M4287; Sigma-Aldrich). Control cultures contained CD8$^+$ T cells, fibrocytes, or DCs alone.

In murine MLR, splenocytes were used after depleting RBCs with RBC lysis buffer (no. R7757; Sigma-Aldrich). CD8$^+$ splenic T cells were harvested with a CD8a$^+$ T cell isolation kit, as described (no. 130-104-075; Miltenyi Biotec). CD8$^+$ T cells (2×10^5 per well) were stimulated with fibrocytes or DCs (2×10^4 per well) in a 96-well, flat-bottom plate (Falcon) coated with anti-CD3e Ab (145-2C11, 10 μg/ml) and/or soluble anti-CD28 Ab (37.51, 2 μg/ml) (eBioscience).

After 72 and 48 h of coculture, cells were pulsed with 1 μCi/well of ^{3}H-thymidine (^{3}H-TdR) (PerkinElmer, Boston, MA) for 15 and 12 h before termination in human and murine MLRs, respectively, and DNA synthesis was measured by a ^{3}H-TdR incorporation with a β-scintillation counter (Aloka instrument).

Ab blocking studies

In blocking experiments for MLRs, the antagonistic Abs, for CD80 (2D10.4), CD86 (IT2.2), or CD54 (HA58) (10 μg/ml) in humans and

CD80 (16-10A1), CD86 (GL1), or CD54 (eBioKAT-1) in mice were used with the respective control Abs, mIgG1 (P3.6.2.8.1; eBioscience), rat IgG2a (eBR2a), or Armenian Hamster IgG (eBio299Arm). In all experiments, fibrocytes were preincubated with the corresponding Abs at 37°C for 1 h before being added to $CD8^+$ T cells.

The effect of ICIs on the proliferation of $CD8^+$ T cells stimulated with fibrocytes

To determine the effect of PD-L1/PD-1 interaction on $CD8^+$ T cell proliferation, recombinant human PD-L1 (hPD-L1)–Fc chimera protein (no. 156-B7; R&D Systems) was added to $CD8^+$ T cells (1×10^5 cells per well) stimulated with precoated anti-CD3 Ab and anti-CD28 (1 μg/ml). Likewise, BALB/C splenic $CD8^+$ T cells were plated at 2×10^5 cells per well with anti-CD28 (2 μg/ml), which had previously been coated with anti-CD3e Ab (10 μg/ml), and the recombinant murine (rm) PD-L1–Fc chimera protein (no.1019-B7; R&D Systems) was added at the designated concentrations.

To assess the effect of ICIs on fibrocyte-induced $CD8^+$ T cell proliferation, we introduced autologous and allogeneic MLR. Autologous studies designated fibrocytes (1×10^4 per well) and $CD8^+$ T cells (1×10^5 per well) from the same donor (human) and the same mouse species (C57BL/6). Anti-CD3 Ab was used to mimic antigenic stimulation (OKT3, 5 μg/ml), and (145-2C11, 10 μg/ml) was added to human and murine autologous MLRs. Atezolizumab (a humanized anti–hPD-L1 Ab; Chugai Pharmaceutical, Tokyo, Japan) and anti-DNP human IgG1 (N297A) (Acro Biosystem, Newark, DE) or Ultra-LEAF Purified Human IgG1 isotype control recombinant (QA16A12; BioLegend) were applied at a concentration of 20 μg/ml for preincubation with fibrocytes at 37°C for 1 h in both autologous and allogeneic human MLRs. In allogeneic studies, fibrocytes were exposed to the allogeneic $CD8^+$ T cells of different donors (human) and different mouse species. Purified $CD8^+$ T cells (2×10^5 per well) isolated from the spleen of male BALB/c mice were cocultured at a 10:1 ratio with female C57BL/6 fibrocytes (2×10^4 per well). Anti–PD-L1 Ab (10F.9G2; Bio X Cell, Lebanon, NH) and LEAF Purified Rat IgG2b isotype control (RTK4530; BioLegend) were used at a concentration of 20 μg/ml for preincubation with fibrocytes in murine MLRs. To explain the PD-1 blocking effect, anti–human PD-1 Ab (EH12.2H7; BioLegend) and mIgG1 k isotype control (P3.6.2.8.1; eBioscience), at concentrations of 10 μg/ml, were preincubated with $CD8^+$ T cells for 1 h at 37°C before mixing with fibrocytes. Similarly, anti-mouse PD-1 Ab (BE0146; Bio X Cell) with isotype control rat IgG2a at a concentration of 10 μg/ml (BE0089; Bio X Cell) were used. All experiments described the coculture stands for 72 and 48 h for human and murine MLR, respectively, before the cell proliferation assessment.

Murine Ag–dependent autologous T cell proliferation assay

Fibrocytes derived from the lungs of OT-I $Rag^{-/-}$ mice were pulsed with 2 μg/ml Ova peptide (SIINFEKL; Sigma-Aldrich) at 37°C for 4 h in 10% RPMI 1640 and washed extensively five times with sterile PBS before further application. Pulsed fibrocytes were further preincubated with anti–PD-L1 Ab and control rat IgG2b at 37°C for 1 h after recounting. In this study, H-$2K^b$ TRP-2 peptide (SVYFFVWL; MBL Life Science) was used as control (28).

Generation of human monocyte-derived DCs and M1 and M2 macrophages

Human peripheral blood-derived monocytes were isolated from healthy donors by magnetic sorting using a human monocyte isolation kit (no. 130-050-201; Miltenyi Biotec) according to the manufacturer's protocol). CD14-positive cells were stimulated with recombinant human (rh) GM-CSF (no. 074-05603; Wako Pure Chemicals, Osaka, Japan) and IL-4 (no. 098-03964; Wako Pure Chemicals) for 7 d; in the last 48 h, they were additionally stimulated with TNF-α (no. 203-15263; Wako Pure Chemicals) at a concentration of 50 ng/ml to harvest mature DCs.

M1 and M2 macrophages were obtained after 6 d of culturing in medium (RPMI 1640) with 10% FBS supplemented with 50 ng/ml rhGM-CSF or rhM-CSF (no.138-16101; Wako Pure Chemicals), respectively. For coculturing with $CD8^+$ T cells, DCs or each type of macrophage were pretreated with mitomycin C at 15 or 25 μg/ml, respectively, for 30 min at 37°C.

To assess the fibronectin effect, these cells were plated in fibronectin-coated (no. 1030-FN; R&D Systems) six-well plates following the above protocol.

Generation of murine bone marrow–derived DCs and M1 and M2 macrophages

Murine monocyte precursor cells were obtained by flushing bone marrow out from the femur and tibia of 6–10-wk age of C57BL/6 mice. To generate murine DCs, bone marrow cells were cultured with murine rGM-CSF (no. 415-ML/CF; R&D Systems) and IL-4 (no. 214-14; PeproTech, Rocky Hill, NJ) for 5 d at a 20 ng/ml, then 2 additional d with GM-CSF, IL-4, and TNF-α (no. 315-01A, as 20 ng/ml; PeproTech). Bone marrow cells were also polarized into M1 and M2 macrophages by culturing with rmGM-CSF and rmM-CSF (no. 416-ML; R&D Systems), respectively, at 20 ng/ml for 6 d (29, 30).

Cross-presentation study

OT-I $Rag^{-/-}$ murine splenic $CD8^+$ T cells were purified using a $CD8a^+$ T cell isolation kit. Fibrocytes and DCs were also collected from the same species and pulsed with Ova protein (albumin of chicken egg white, no. 5503; Sigma-Aldrich) in different concentration (1 and 4 mg/ml) for 4 h at 37°C. After washing extensively, fibrocytes or DCs (1×10^4 per well) were added to $CD8^+$ T cells (1×10^5 per well) at a 1:5 ratio. In some experiments, pulsed fibrocytes were preincubated with anti–MHC class I (H-$2K^b$, clone: AF6.88.5.5.3, no. BE0121; Bio X Cell) (10 μg/ml) or anti–PD-L1 Ab (20 μg/ml) with control mIgG2a (clone: C1.18.4, no. BE0085; Bio X Cell) at a 1:10 ratio for 1 h before starting MLR. Fibrocytes were pulsed with Ova protein for 2 h to determine the PD-L1 blocking effect on $CD8^+$ T cells by cross-presenting fibrocytes. Cellular proliferation was evaluated by a ^{3}H-TdR incorporation assay.

Purification of tumor-infiltrating fibrocytes

MC38 tumor cells (1×10^6) were inoculated s.c. into syngeneic C57BL/6 mice. One group ($n = 6$) was treated with i.p. injection of semaxanib (SU5416, VEGFR-2 inhibitor; Abcam, Cambridge, U.K.), 10 mg/kg/d from day 7 to 21. The tumor tissues collected were minced and suspended to ensure single cells on day 21. The cells were counted by using a hemocytometer, and 6×10^7 cells were subjected to magnetic sorting of $CD45^+$ cells with an autoMACS instrument. Tumor-derived $CD45^+$ cells were plated in fibronectin-coated, 100-mm dishes supplemented with 20% FBS containing DMEM. After 7 d, adherent cells were labeled with anti-mouse $CD45^+$ microbeads again to purify tumor-infiltrating fibrocytes. About 50% of the adherent population expressed collagen I and CXCR4 in addition to CD45 by a flow-cytometric evaluation. The number of fibrocytes counted, treated with anti–PD-L1 Ab as 20 μg/ml (Bio X Cell), and mixed with $CD8^+$ T cells originated from healthy BALB/c mice in a 96-well plate to proceed allogeneic MLRs.

Statistical analyses

Data are presented as the mean ± SEM. The statistical analyses were performed using a one-way ANOVA, followed by Tukey multiple-comparison post hoc test, using the Prism software program (version 8.0; GraphPad Software, La Jolla, CA). All p values <0.05 were considered to indicate statistical significance. All the experiments were triplicated unless otherwise specified.

Results

The cell surface expression of immune checkpoint molecules on fibrocytes

First, we examined the cell surface expression of coinhibitory checkpoint molecules like PD-L1, PD-L2, PD-1, CTLA-4, Galectin-9, and TIM-3 at the same time as costimulatory molecules such as CD40, CD80, CD86, CD54, and both HLA molecules on murine and human fibrocytes. Flow cytometry revealed that human fibrocytes derived from PBMCs expressed high levels of PD-L1, CD86, and CD54 in addition to both HLA class I (HLA-ABC) and class II (HLA-DR) (Fig. 1A). Regarding other coinhibitory molecules, human fibrocytes expressed the lower level of galectin-9 and TIM-3, which depended on the donor, whereas no PD-L2, PD-1, or CTLA-4 expression was found. Notably, the CD80 expression on fibrocytes was very low. When we analyzed murine fibrocytes, the expression pattern of coinhibitory and stimulatory molecules was similar to that in human fibrocytes, although the expression of galectin-9, TIM-3, CD40, and MHC class II was not detected (Supplemental Fig. 1A). In addition,

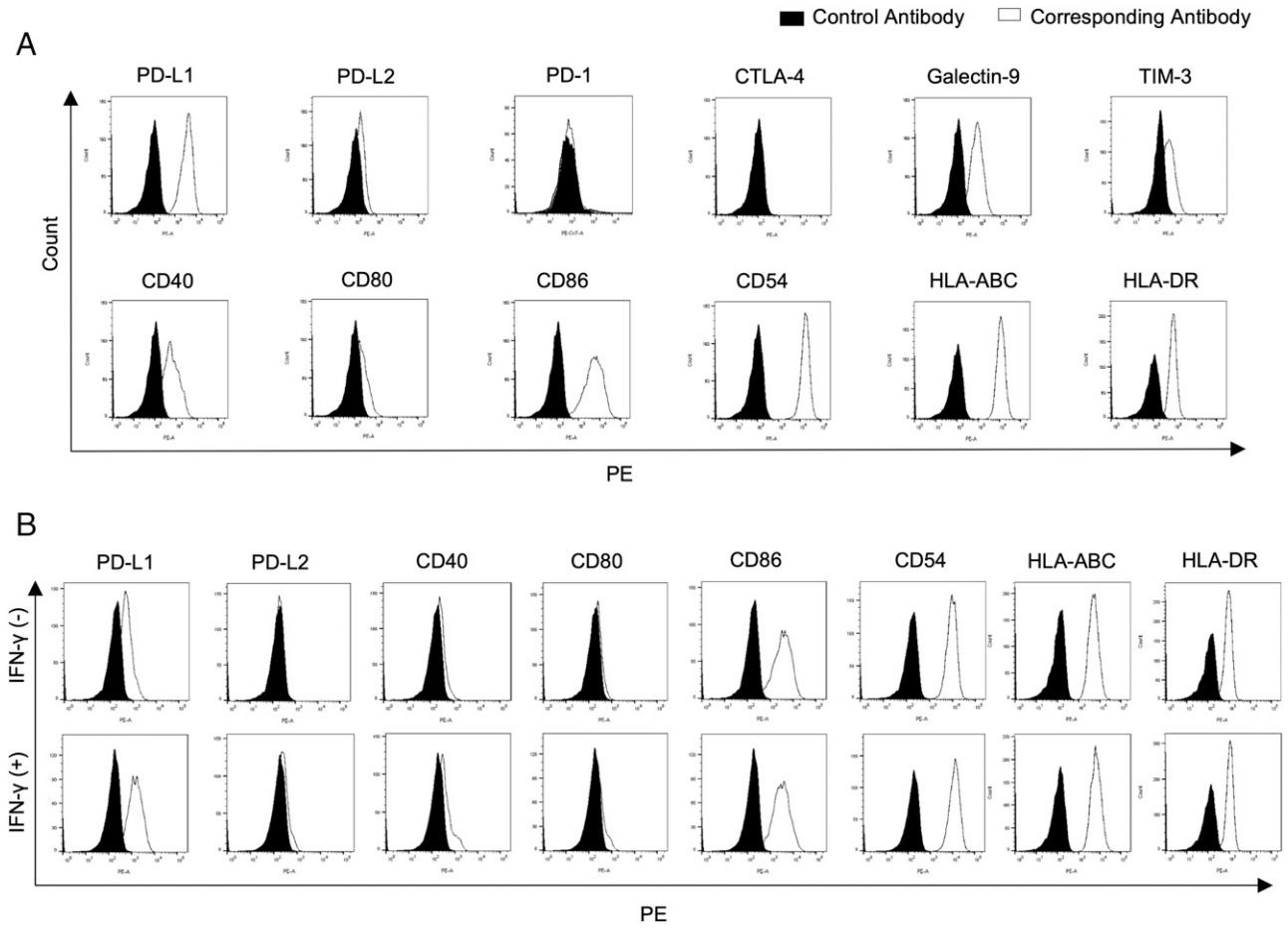

FIGURE 1. The cell surface expression of immune checkpoint molecules in fibrocytes and the effects of IFN-γ. A representative flow cytometric analysis of human PBMC-derived fibrocytes (**A**). The effects of IFN-γ on the expression of immune checkpoint molecules in human (**B**) fibrocytes. Data are representative of three independent experiments.

the level of CD86 on fibrocytes in mice was relatively low in comparison with that in humans. The high expression of PD-L1 is a characteristic finding of fibrocytes.

IFN-γ is a known inducer of PD-L1 via the activation of the JAK/STAT/IRF1 downstream signaling pathway (31). Thus, harvested fibrocytes were cultured with IFN-γ (10 ng/ml) for 48 h in vitro and analyzed by flow cytometry. As a result, treatment with IFN-γ was found to significantly upregulate the expression of PD-L1 (Fig. 1B, Supplemental Fig. 1B). The expression of CD86, CD54, HLA-ABC, and HLA-DR was also enhanced by IFN-γ. However, CD80 failed to upregulate the expression, even with IFN-γ stimulation, in both murine and human fibrocytes (Fig. 1B, Supplemental Fig. 1B).

Fibrocytes stimulate the proliferation of CD8$^+$ T cells

To examine the stimulatory activity induced by fibrocytes in T cells, fibrocytes were cocultured with CD8$^+$ T cells in several experimental conditions, and the activity was measured with ^{3}H-TdR incorporation of T cells. First, we used an allogeneic human system of MLR with fibrocytes and CD8$^+$ T cells harvested from PBMCs (Fig. 2A). As shown in Fig. 2A, fibrocytes showed stimulatory activity of the proliferation of CD8$^+$ T cells. However, DCs had higher capacity to stimulate CD8$^+$ T cells than fibrocytes. Similarly, murine fibrocytes augmented the growth of CD8$^+$ T cells, although the activity was lower than that of DCs (Supplemental Fig. 2A).

Next, we determined which costimulatory molecules play an important role in CD8$^+$ T cell–stimulatory activity by fibrocytes with the same model used in Fig. 2A. As shown in Fig. 2B, the enhancement of the growth of CD8$^+$ T cells by human fibrocytes was blocked with anti-CD86 or -CD54, but not -CD80. These results were confirmed in the murine system, whereas anti-CD80 Ab inhibited the growth of CD8$^+$ T cells stimulated by murine fibrocytes (Supplemental Fig. 2B).

The ability of fibrocytes to activate CD8$^+$ T cells was also examined using autologous CD8$^+$ T cells activated with anti-CD3 and -CD28 Abs. Both human and murine fibrocytes enhanced the growth of CD8$^+$ T cells, and the stimulatory activity was blocked by anti-CD86 and -CD54 Abs (Fig. 2C, Supplemental Fig. 2C). However, CD80 was not involved in the activity in human or mice (Fig. 2C, Supplemental Fig. 2C), suggesting that the stimulatory activity of CD8$^+$ T cells by fibrocytes was mainly mediated through CD86 and not CD80.

Blockade of the PD-1/PD-L1 pathway enhances the activation of CD8$^+$ T cells in the MLR models

To investigate the involvement of the PD-1/PD-L1 pathway on fibrocyte-mediated activation of CD8$^+$ T cells, we first investigated the effect of addition of an exogenous soluble form of hPD-L1 (hPD-L1–Fc). As shown in Fig. 3A, the addition of recombinant hPD-L1–Fc inhibited the activation of human CD8$^+$ T cells induced by anti-CD3 Ab in a dose-dependent manner, indicating that the PD-1/PD-L1 pathway was involved in the activation of CD8$^+$ T cells. Subsequently, to clarify the effect of PD-L1 blockade in the human system, we examined the effect of a humanized anti–PD-L1 Ab, atezolizumab, which has been

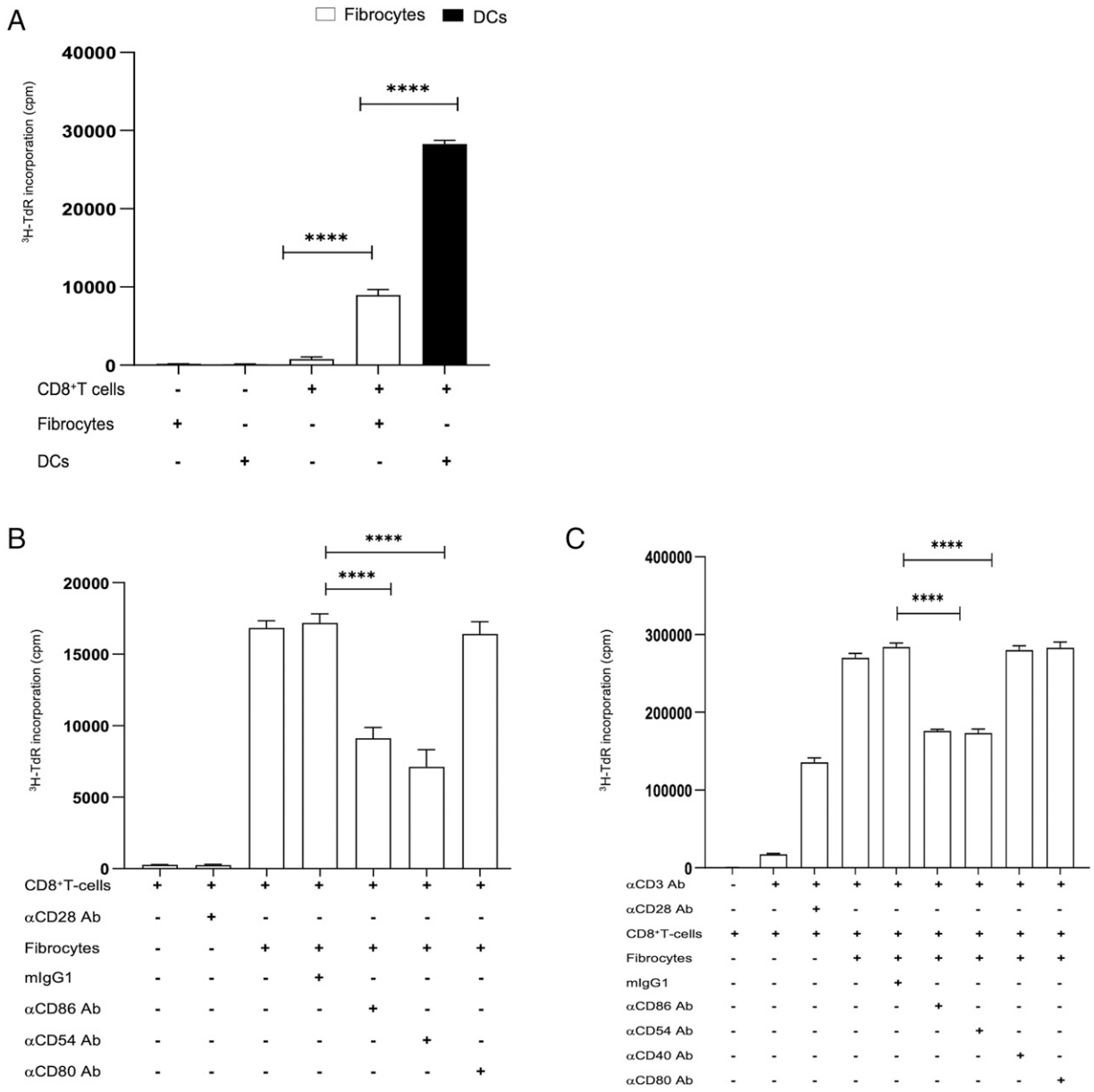

FIGURE 2. Fibrocytes stimulate the proliferation of CD8$^+$ T cells through CD86 and CD54. (**A**) Human allogenic MLR with fibrocytes and DCs ($n = 4$). (**B**) The effects of blocking Abs against CD54, CD80, and CD86 in human allogeneic MLR stimulated with fibrocytes. (**C**) The effects of blocking Abs against CD54, CD80, and CD86 in human autologous MLR stimulated with fibrocytes and anti-CD3 Ab. ****$p < 0.0001$ by a one-way ANOVA, and data are shown as the mean ± SEM.

approved for cancer immunotherapy in the clinical setting. In autologous MLR, the addition of atezolizumab significantly enhanced the fibrocyte-induced CD8$^+$ T cell proliferation (Fig. 3B). In allogeneic MLR, atezolizumab also enhanced the growth of CD8$^+$ T cells stimulated by human fibrocytes (Fig. 3C). When we examined the effect of anti–PD-1 Ab, the addition of anti–human PD-1 Ab, like anti–PD-L1 Ab, upregulated the proliferation of CD8$^+$ T cells induced by human allogeneic fibrocytes (Fig. 3D). In brief, the interruption of PD-1/PD-L1 interaction augments the growth of CD8$^+$ T cells stimulated by fibrocytes.

To further define the PD-L1 function, we cross-checked PD-L1 blocking in murine MLRs. The addition of rmPD-L1–Fc prevented the proliferation of CD8$^+$ T cells mediated by anti-CD3 and -CD28 Abs in a dose-dependent manner (Supplemental Fig. 3A). The addition of anti–PD-L1 Ab further enhanced the proliferation of CD8$^+$ T cells stimulated by murine fibrocytes and anti-CD3 Ab (Supplemental Fig. 3B). Next, in allogeneic MLR, CD8$^+$ T cells derived from BALB/c mice were stimulated by fibrocytes derived from C57BL/6 mice. As shown in Supplemental Fig. 3C, anti–PD-L1 Ab significantly enhanced the fibrocyte-induced proliferation of CD8$^+$ T cells. Similarly, anti–PD-1 Ab enhanced the growth of CD8$^+$ T cells induced by allogenic fibrocytes (Supplemental Fig. 3D).

Comparison between fibrocytes and other APCs

We next compared the expression levels and functions of coinhibitory molecules (particularly PD-L1) of fibrocytes with DCs or macrophages. We therefore generated DCs and M1 and M2 macrophages by culturing with GM-CSF and IL-4, GM-CSF alone, or M-CSF alone, respectively, from CD14$^+$ monocytes in humans and from bone marrow cells in mice. Like fibrocytes, M2 macrophages showed a spindle shape in culture (data not shown). Flow cytometry also showed that the expression pattern of coinhibitory molecules, including PD-L1, in fibrocytes resembles that in DCs

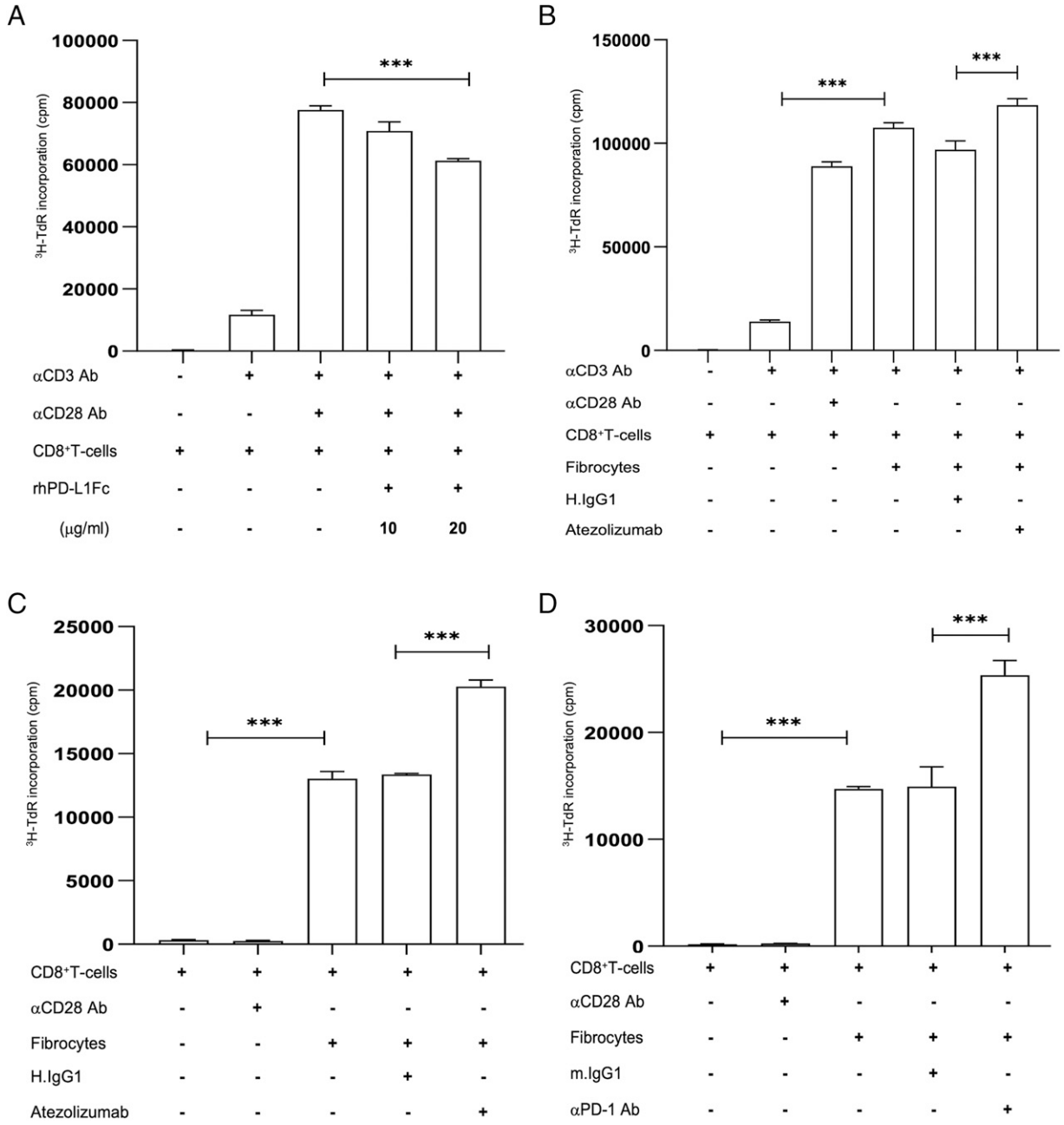

FIGURE 3. Blockade of the PD-1/PD-L1 pathway enhances the activation of $CD8^+$ T cells by fibrocytes in human MLRs. (**A**) The effect of recombinant hPD-L1–Fc on the proliferation of $CD8^+$ T cells activated by anti-CD3 and -CD28 Abs. (**B**) The effect of a humanized anti–hPD-L1 Ab, atezolizumab, on the proliferation of $CD8^+$ T cells in autologous MLR stimulated with fibrocytes and anti-CD3 Ab. (**C**) The effect of a humanized anti–hPD-L1 Ab, atezolizumab, on the proliferation of $CD8^+$ T cells in allogeneic MLR with fibrocytes. (**D**) The effect of anti–human PD-1 Ab on the proliferation of $CD8^+$ T cells in allogeneic MLR with fibrocytes. Data are representative of three independent experiments. $^{***}p < 0.001$, by a one-way ANOVA, and data are shown as the mean ± SEM.

and M1 macrophages (Supplemental Fig. 4). In comparison with DCs, a distinct difference existed in the expression of CD80. DCs expressed high levels of CD80 without any stimulation, whereas fibrocytes did not. These results indicated that the phenotype of fibrocytes was quite unique among macrophage-lineage cells. Next, we examined the functional differences of the PD-L1 expression on fibrocytes, DCs, and M1 and M2 macrophages with allogeneic MLR. Among these cells, DCs showed the highest stimulatory capacity of $CD8^+$ T cells, whereas anti–PD-L1 Ab, unlike fibrocytes, did not mediate further enhancement of the growth of $CD8^+$ T cells in human or mice (Fig. 4A, 4B, Supplemental Fig. 5A, 5B). Interestingly, anti–PD-L1 Ab tended to augment the growth of $CD8^+$ T cells stimulated by M1 macrophages in humans (Fig. 4C), but not in mice (Supplemental Fig. 5C), although the stimulatory activity by both macrophages was apparently lower than that by fibrocytes in humans and mice (Fig. 4C, 4D, Supplemental Fig. 5C, 5D).

To address the question as to whether the fibronectin used in the culture of fibrocytes affects the PD-L1 blocking effects on DCs and M1 and M2 macrophages, we investigated the expression of immune checkpoint molecules on DCs and M1 and M2 macrophages with fibronectin stimulation (Supplemental Fig. 6A). These results indicated that no change was found in their expressions, including PD-L1. Moreover, in an allogeneic MLR, DCs and M1 and M2

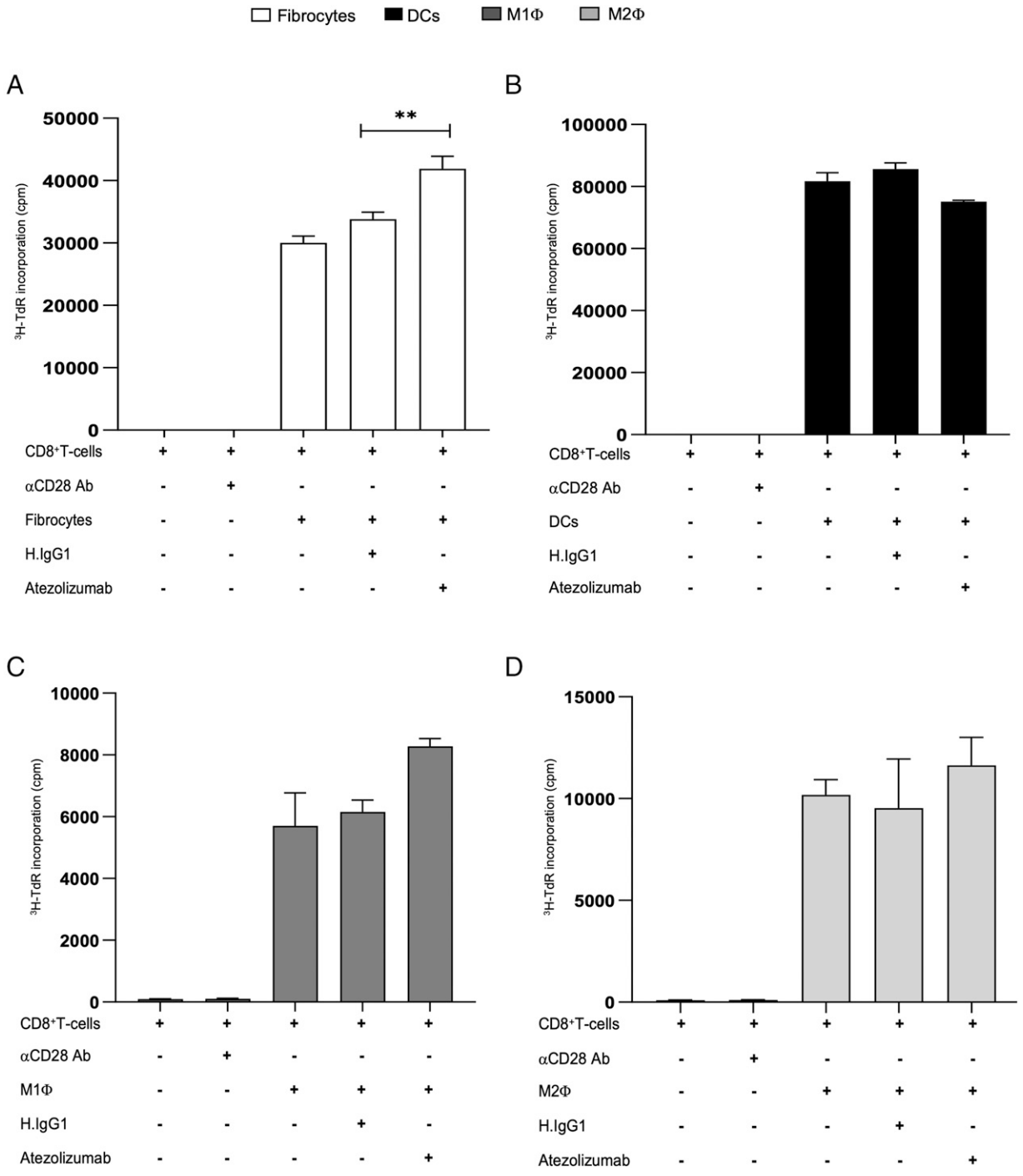

FIGURE 4. Comparison between fibrocytes and other APCs in allogeneic MLRs in human. Allogeneic MLRs with fibrocytes (**A**), DCs (**B**), and M1 (**C**) and M2 macrophages (**D**) from the same human donor. These data represent five or more repeated experiments and are shown as the mean ± SEM. $^{**}p < 0.01$, by a one-way ANOVA.

macrophages failed to enhance the proliferation of CD8$^+$ T cells with atezolizumab (Supplemental Fig. 6B), like the data in Fig. 4.

The role of PD-L1 in Ag-specific mouse model and cross-presentation by fibrocytes

Moreover, to confirm the role of the PD-1/PD-L1 pathway in Ag-specific CD8$^+$ T cell stimulation by fibrocytes, we used OVA-specific OT-1Rag$^{-/-}$ transgenic mice. In this system, fibrocytes pulsed with OVA peptide (SIINFEKL), but not the relevant TRP-2 peptide, and stimulated the proliferation of CD8$^+$ T cells from OT-1Rag$^{-/-}$ transgenic mice (Fig. 5A). Anti–PD-L1 Ab significantly enhanced the proliferation of CD8$^+$ T cells from OT-1Rag$^{-/-}$ transgenic mice (Fig. 5A). These data suggest that the PD-1/PD-L1 pathway was involved in an Ag-specific activation of CD8$^+$ T cells by fibrocytes.

Cross-priming of naive CD8$^+$ T cells, in which APCs can phagocytose an exogenous protein and present it via MHC class I, termed cross-presentation, is crucial for adaptive cell-mediated immunity. The cross-presentation of fibrocytes was examined using OT-1Rag$^{-/-}$ mice. Fibrocytes and bone marrow–derived DCs were pulsed with OVA protein in different concentrations. Importantly, OVA protein–pulsed fibrocytes have shown the ability of CD8$^+$

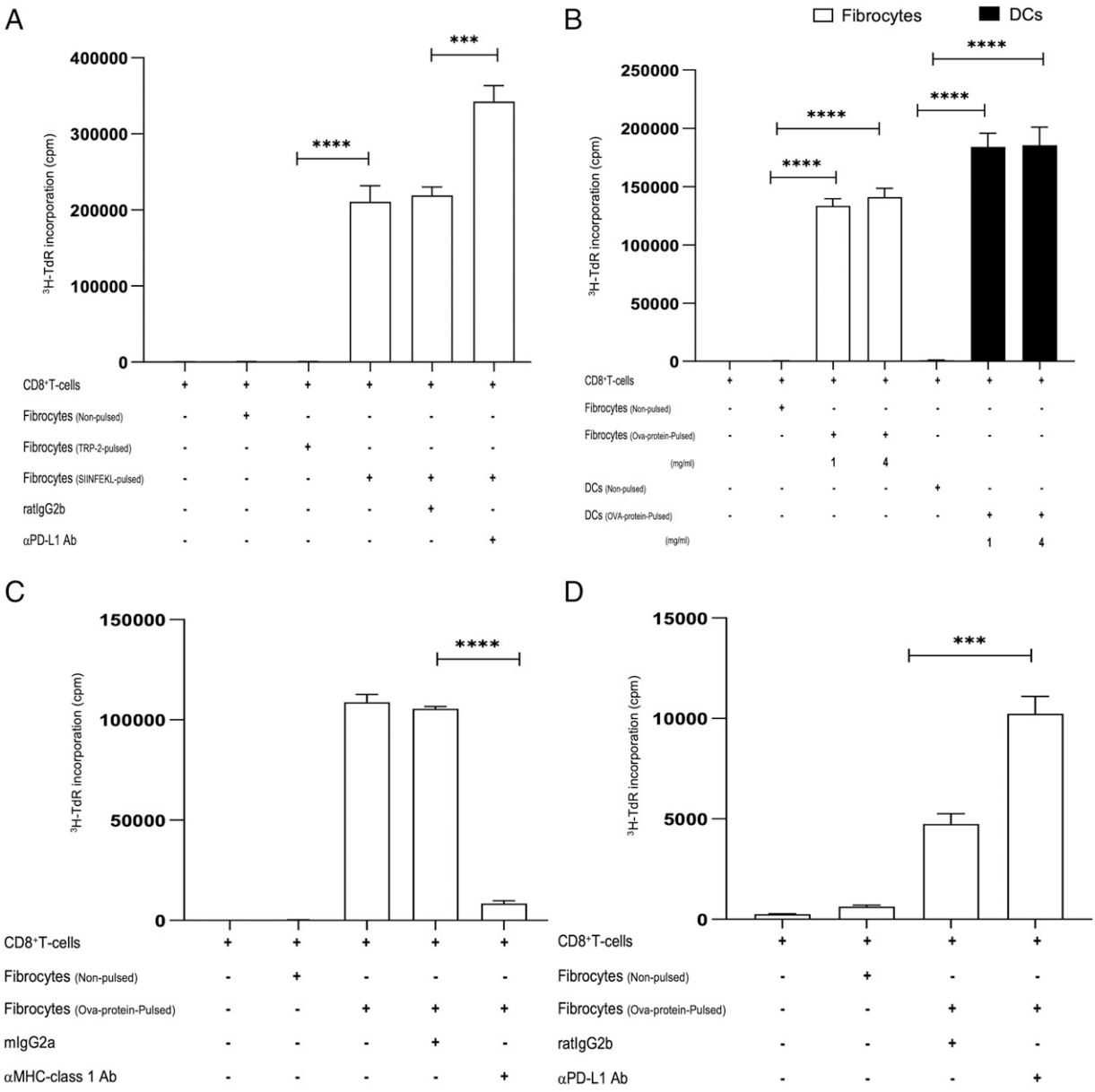

FIGURE 5. The role of PD-L1 in Ag-specific MLR and cross-presentation by fibrocytes. (**A**) The effect of anti–murine PD-L1 Ab on the proliferation of CD8$^+$ T cells from OT-1Rag$^{-/-}$ transgenic mice stimulated with fibrocytes pulsed with OVA peptide (SIINFEKL). (**B**) Murine MLR of CD8$^+$ T cells from OT-1Rag$^{-/-}$ transgenic mice with fibrocytes or DCs pulsed with Ova protein. (**C**) Blocking of cross-presentation of fibrocytes with anti–MHC class I Ab. (**D**) The effect of anti–murine PD-L1 Ab on the proliferation of CD8$^+$ T cells from OT-1Rag$^{-/-}$ transgenic mice with fibrocytes pulsed with OVA protein. Data are representative of three or more independent experiments. ****$p < 0.0001$, ***$p < 0.001$, by a one-way ANOVA, and data are shown as the mean ± SEM.

T cell proliferation, like DCs (Fig. 5B), and this activity was strongly inhibited by anti–MHC class I Ab (Fig. 5C). These results clearly indicate that fibrocytes have the ability of cross-presentation, similarly to DCs. Furthermore, anti–PD-L1 Ab significantly enhanced the growth of CD8$^+$ T cells mediated by OVA protein–pulsed fibrocytes (Fig. 5D). Again, these data suggest that PD-L1 plays an important role in cross-presentation by fibrocytes.

PD-L1 blockade enhanced the APC function of fibrocytes derived from patients with lung adenocarcinoma and murine tumors

Finally, we confirmed the APC function of fibrocytes derived from PBMCs of patients with lung adenocarcinoma or MC38 murine tumors. Therefore, we ascertained the blocking effect of atezolizumab on fibrocyte-induced proliferation of CD8$^+$ T cells. As shown in Fig. 6A, treatment with atezolizumab reproducibly enhanced the proliferation of CD8$^+$ T cells stimulated with fibrocytes derived from three patients with lung adenocarcinoma. To provide further evidence, we purified tumor-infiltrating fibrocytes from MC38 tumor syngeneic with C56BL/6 mice. The flow-cytometric analysis showed that ~50% of attached cells were CD45 positive, and the phenotype of these cells were compatible to those of fibrocytes (Fig. 6B). Addition of anti–PD-L1 Ab clearly enhanced the proliferation of CD8$^+$ T cells stimulated with fibrocytes derived from MC38 tumors with or without treatment of VEGFR-2 inhibitor (semaxanib) (Fig. 6C).

Discussion

In the current study, we demonstrated that fibrocytes express immune checkpoint molecules, including PD-L1, and the blockade of

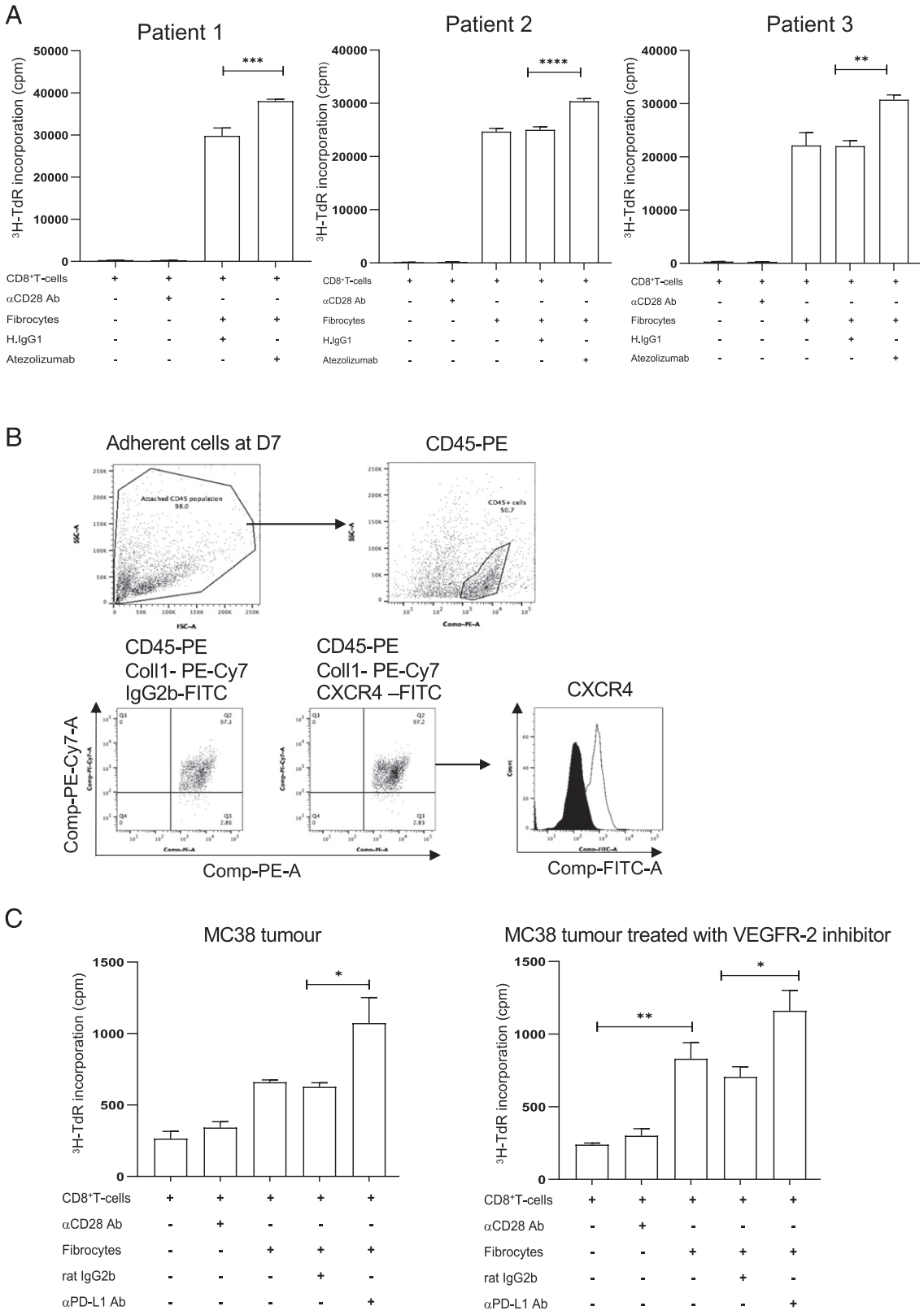

FIGURE 6. Blocking PD-L1 enhanced APC function of fibrocytes derived from patients with lung adenocarcinoma and murine tumors. (**A**) Allogeneic MLRs with CD8$^+$ T cells derived from healthy donor and fibrocytes derived from patients with lung adenocarcinoma with atezolizumab (anti–PD-L1 Ab). (**B**) Flow-cytometric assessment of tumor-infiltrating fibrocytes derived from murine MC38 tumor. (**C**) Effect of anti–PD-L1 Ab on the proliferation of CD8$^+$ T cells stimulated with fibrocytes in an allogeneic MLR. CD8$^+$ T cells were harvested from spleen of BALB/c mice. Fibrocytes were harvested from MC38 tumors with or without treatment with VEGFR-2 inhibitor (semaxanib). ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, by a one-way ANOVA, and data are shown as the mean ± SEM.

PD-L1 showed the significant enhancement of the APC function to stimulate $CD8^+$ T cells in several models in mice and humans. In addition, we found that fibrocytes isolated from peripheral blood of patients with lung cancer and murine tumor also had APC function that was significantly enhanced by anti–PD-L1 Ab.

Fibrocytes are known as a bone marrow–derived, collagen-producing cells (1–3). Thus, functional analyses have been performed to evaluate their involvement in wound healing as well as fibrogenesis in various organs (4, 5, 7, 10–14). However, fibrocytes have also been demonstrated to show Ag-presenting capacity to activate $CD8^+$ T cells (15–18) because they expressed costimulatory molecules such as CD86. In the current study, we further confirmed the expression levels of costimulatory molecules and then examined the expression of coinhibitory molecules, including PD-L1 in fibrocytes. As a result, fibrocytes were found to express a higher level of PD-L1. Notably, the PD-L1 expression in fibrocytes was functional because blocking with anti–PD-1/PD-L1 Abs clearly induced the upregulation of the proliferation of $CD8^+$ T cells. However, the blockade of the PD-L1 expression in DCs did not enhance the growth of $CD8^+$ T cells, although the PD-L1 expression level in DCs was higher than that in fibrocytes. Recently, Sugiura et al. (32) reported that *cis*–PD-L1/CD80 interactions on DCs block the binding of PD-L1 to PD-1. Therefore, PD-L1 did not work to inhibit T cell activation on DCs, and anti–PD-L1 Abs do not enhance the growth of $CD8^+$ T cells, although the stimulatory activity of $CD8^+$ T cells induced by DCs was highest among the APCs tested. We also found that M1, but not M2, macrophages stimulated the proliferation of $CD8^+$ T cells in humans. These results suggest that the immune cells that are directly involved in the enhancement of antitumor immunity mediated by anti–PD-1/PD-L1 Ab in tumor sites may be fibrocytes or M1 macrophages, rather than DCs.

Recent advances in cancer immunotherapy have resulted in the development of several Abs against CTLA4 and PD-1 and its ligand PD-L1, which have been approved for use as ICIs in the treatment of various malignancies (23, 24). In addition, clinical trials involving combination immunotherapy of anti–PD-1/PD-L1 Abs with other modalities have demonstrated the promising efficacy of this regimen against some cancers (25, 26). Among these the combination of ICIs with antiangiogenic agents, including an anti-VEGF Ab, bevacizumab, has been expected and has shown durable efficacy in several cancers, including advanced hepatocellular carcinoma (33–37). We previously reported that therapy with anti-VEGF agents, including bevacizumab or inhibitors, increased the accumulation of fibrocytes in tumors in mice and humans (21). Therefore, we asked whether fibrocytes infiltrating into tumors play a role in additive/synergistic antitumor effects in combination with anti–PD-1/PD-L1 Abs and anti-VEGF agents.

In the current study, we recharacterized fibrocytes in comparison with DCs and macrophages. Fibrocytes have shown similarity to M2 macrophages because of their profibrotic properties (38, 39). However, from the immunological viewpoint, the expression pattern of PD-L1 and the effect of anti–PD-L1 Ab (in human MLR) on fibrocytes resembled those of M1 macrophages. These data suggest that fibrocytes are a unique population among macrophage-lineage cells. We also investigated the cross-presentation ability of fibrocytes pulsed with chicken OVA albumin (exogenous protein) in the context of OT-1$Rag^{-/-}$ $CD8^+$ T cells. Pulsed fibrocytes take up and process the exogenous OVA protein and present it to cytotoxic T cells via MHC class I, as evidenced by the blocking effect of anti–MHC class I Ab. The data clearly indicated the function of fibrocytes as APCs.

However, little is known about the APC function of fibrocytes isolated from tumor tissues as well as PBMC of cancer patients. Tumor-derived fibrocytes stimulated the proliferation of $CD8^+$ T cells, and the activity was significantly enhanced by anti–PD-L1 Ab. Furthermore, we showed the stimulatory effects on $CD8^+$ T cells by fibrocytes isolated from PBMC of patients with lung cancer. Treatment with anti–PD-L1 Ab reproducibly enhanced the T cell–stimulatory activity of fibrocytes from cancer patients. These data strongly suggest that fibrocytes function in tumor microenvironment as APCs to enhance antitumor immunity, especially when cancer patients were treated with anti–PD-1/PD-L1 Ab. Further study is required to investigate the other functions of fibrocytes as APCs, including the ability to migrate into lymph nodes, as well as the production of immune-related cytokines.

In the current study, we showed that fibrocytes had the ability to enhance tumor immunity when anti–PD-1/PD-L1 Ab was used, suggesting that fibrocytes play a role as APCs at least within tumor sites. Although novel mechanisms involved in therapy with PD-1/PD-L1 blockade have just emerged (40, 41), to identify and analyze fibrocytes in tumor tissues may provide a much deeper understanding of tumor immunity and be useful for the further development of cancer immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

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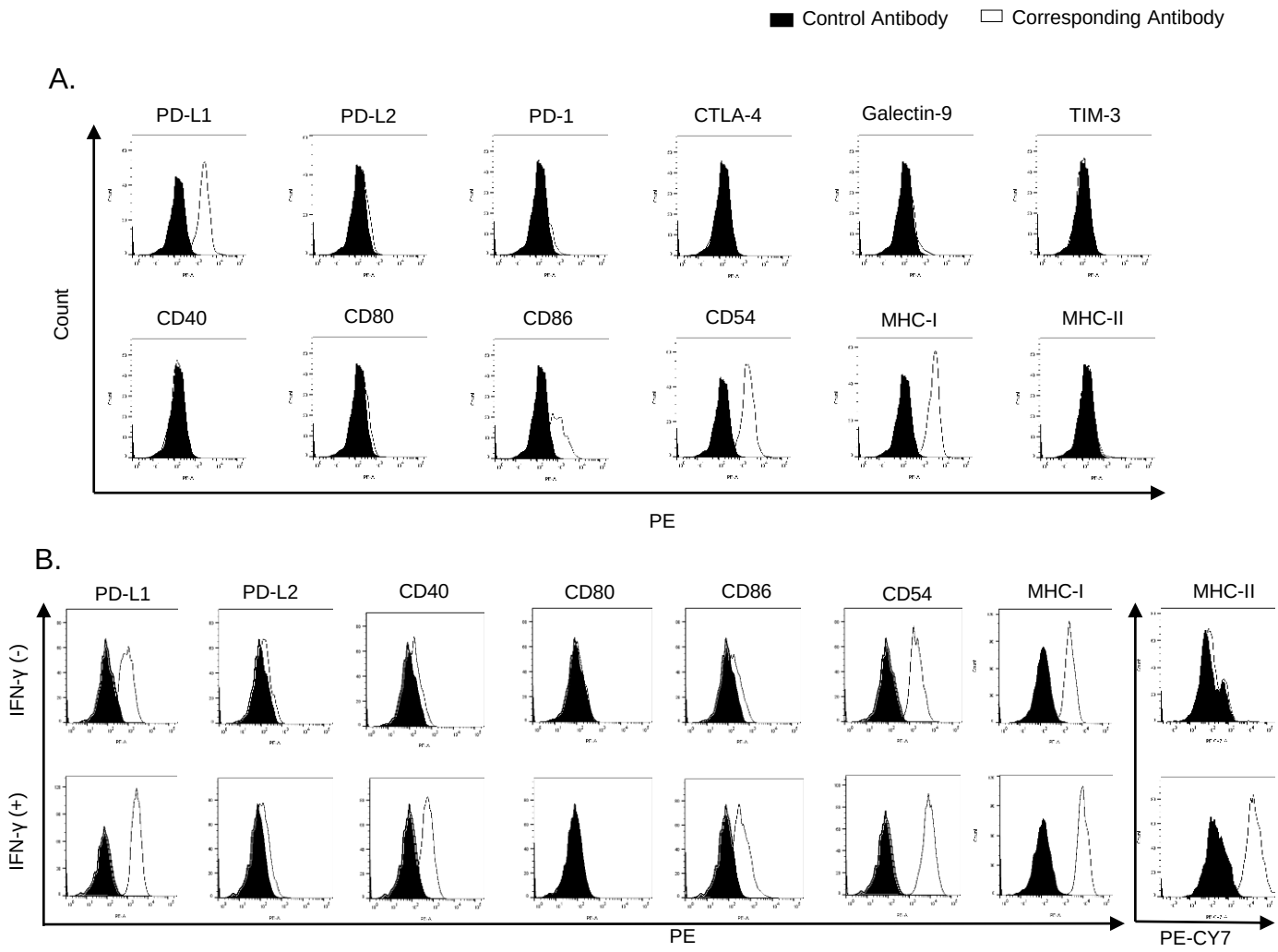
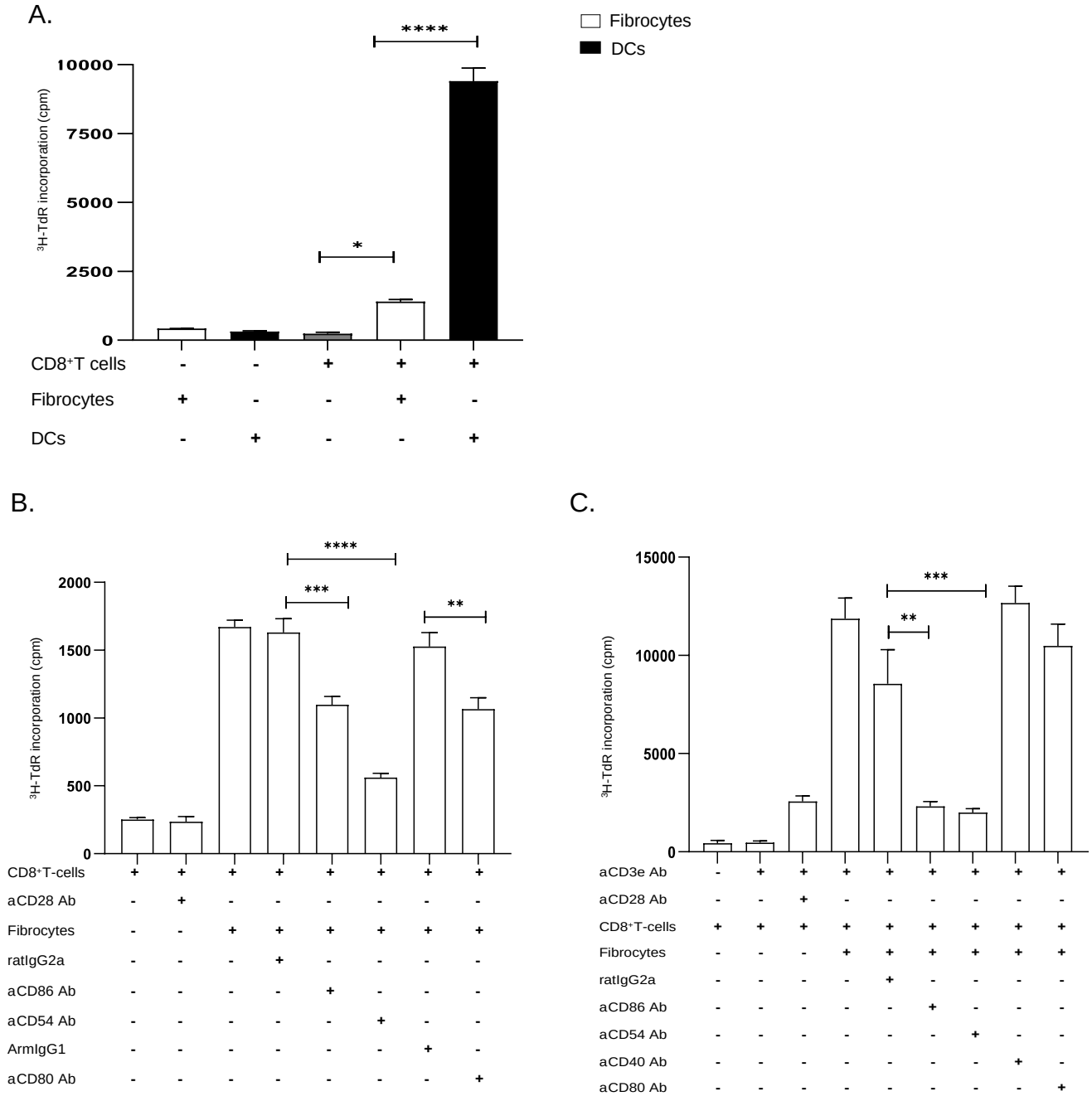
Supplementary Figure 1



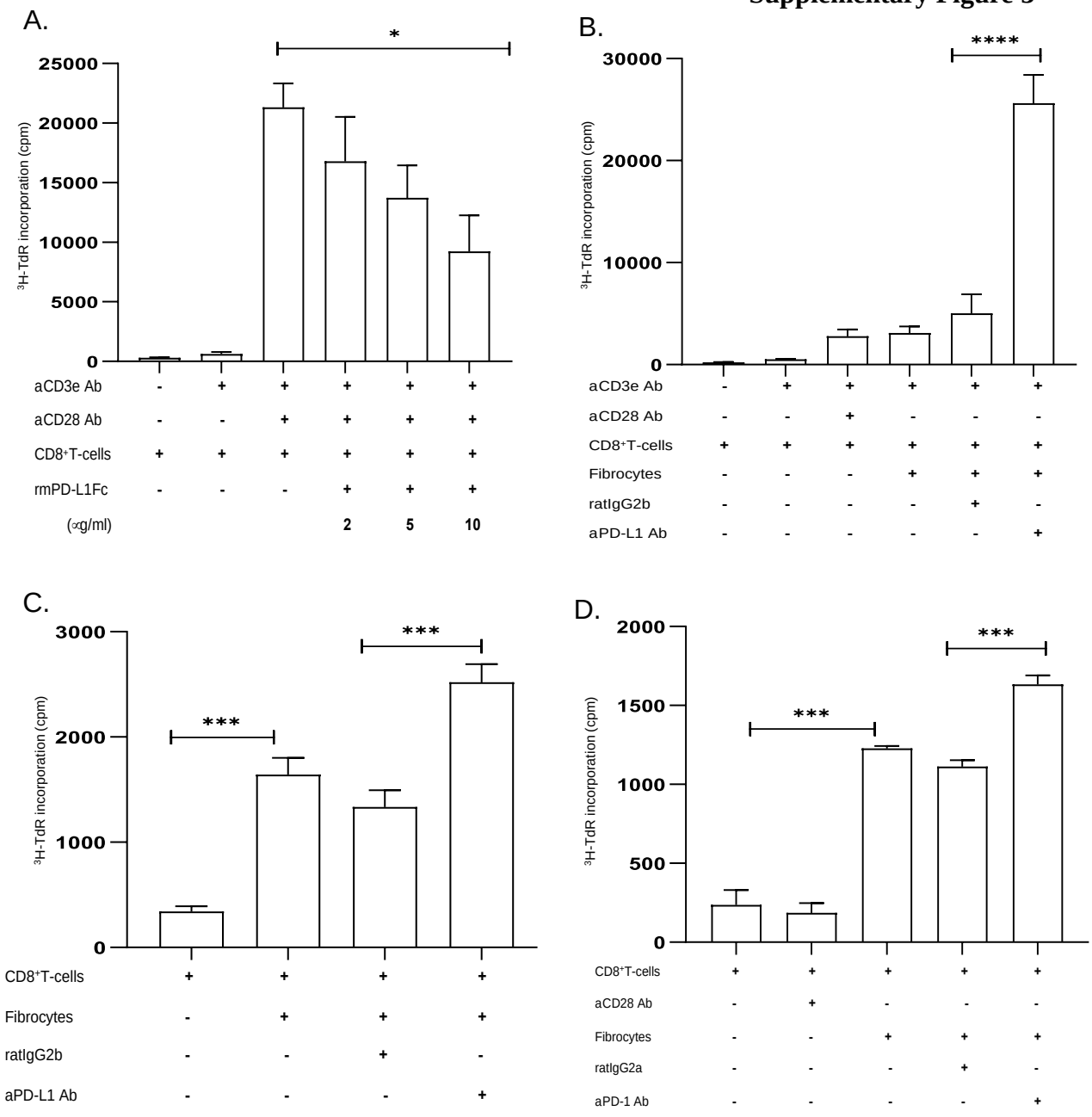
Supplementary Figure 1. The cell surface expression of immune checkpoint molecules in murine fibrocytes and the effects of IFN-γ. A representative flow cytometric analysis of murine fibrocytes derived from the lungs (A). The effects of IFN-γ on the expression of immune checkpoint molecules in murine (B) fibrocytes. Data are representative of three independent experiments.

Supplementary Figure 2



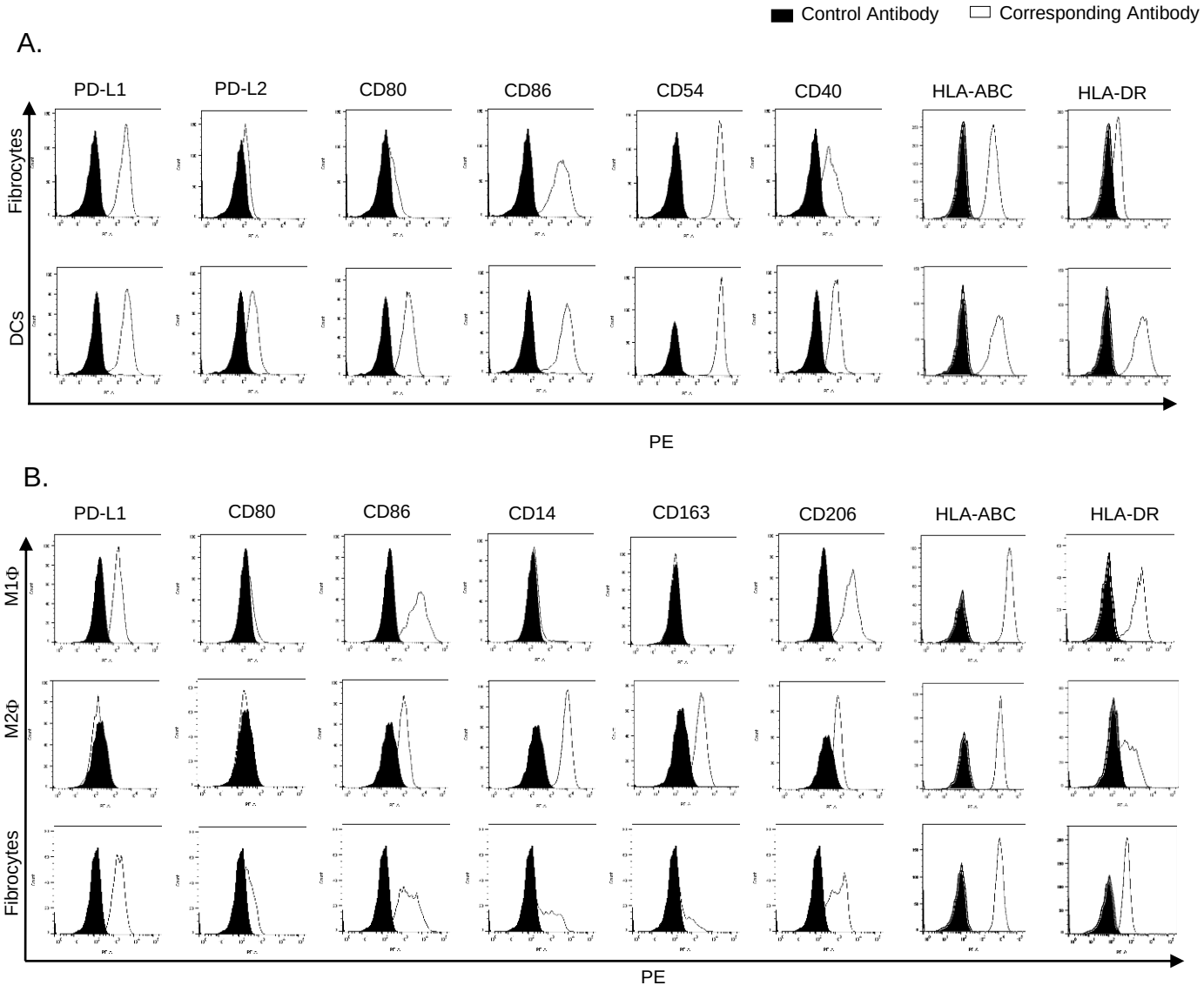
Supplementary Figure 2. Fibrocytes stimulate the proliferation of $CD8^+$ T cells through CD86 and CD54 in mice. **(A)** Murine allogeneic MLR with fibrocytes and DCs. **(B)** The effects of blocking Abs against CD54, CD80 and CD86 in murine allogeneic MLR stimulated with fibrocytes. **(F)** The effects of blocking anti-bodies against CD54, CD80 and CD86 in murine autologous MLR stimulated with fibrocytes and anti-CD3 Ab. Data are representative of three independent experiments. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ by a one-way ANOVA and data are shown as the mean ± SEM.

Supplementary Figure 3



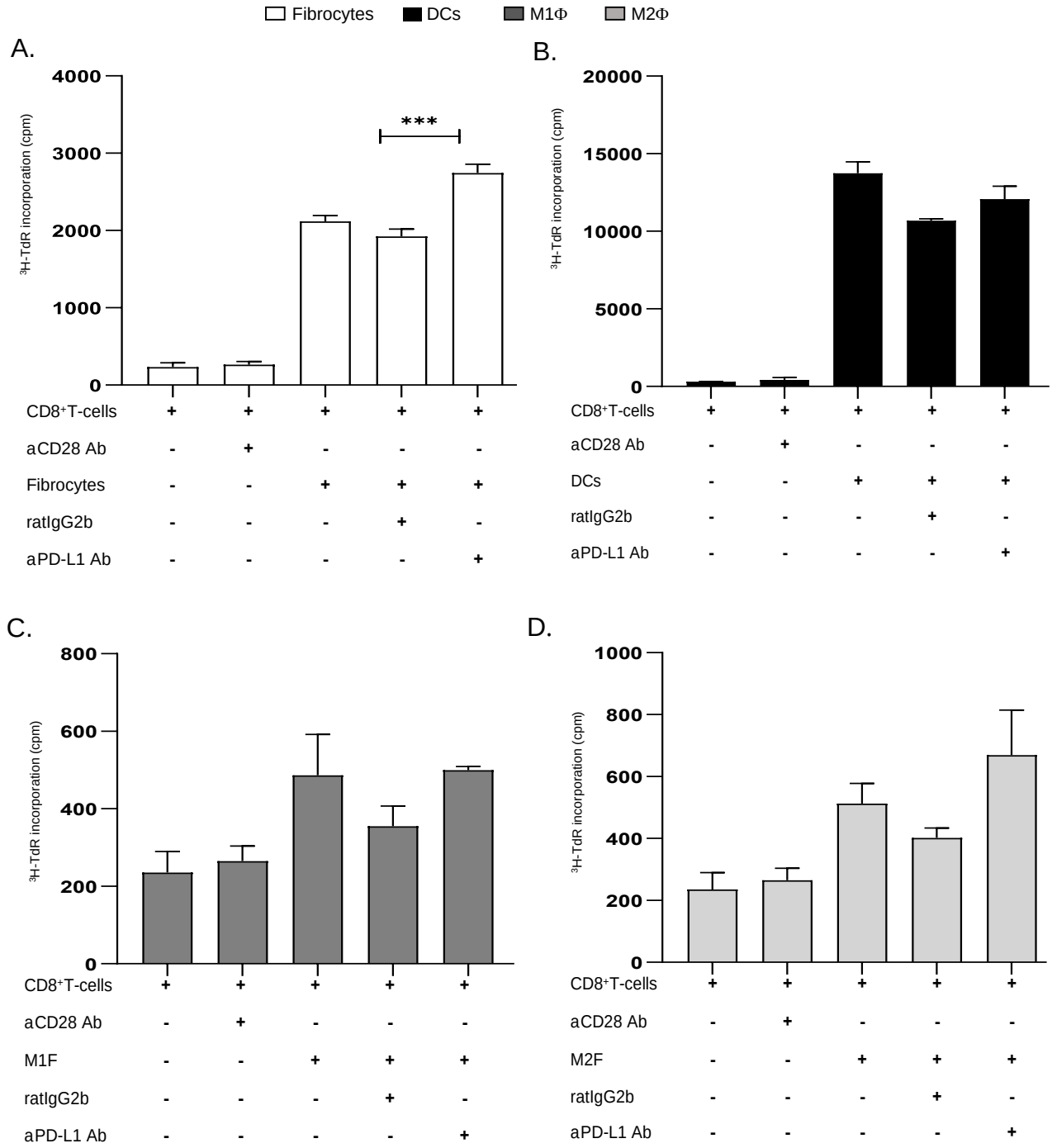
Supplementary Figure 3. Blockade of the PD-1/PD-L1 pathway enhances the activation of $CD8^+$ T cells in murine MLRs. **(A)** The effect of rmPD-L1Fc on the proliferation of $CD8^+$ T cells activated by anti-CD3 and CD28 Abs. **(B)** The effect of anti-murine PD-L1 Ab on the proliferation of $CD8^+$ T cells in autologous MLR stimulated with fibrocytes and anti-CD3 Ab. **(C)** The effect of anti-murine PD-L1 Ab on the proliferation of $CD8^+$ T cells in allogeneic MLR with fibrocytes. **(D)** The effect of anti-murine PD-1 Ab on the proliferation of $CD8^+$ T cells in allogeneic MLR with fibrocytes. **** $P < 0.0001$, ***$P < 0.001$, * $P < 0.05$ by a one-way ANOVA and data are shown as the mean ± SEM.

Supplementary Figure 4



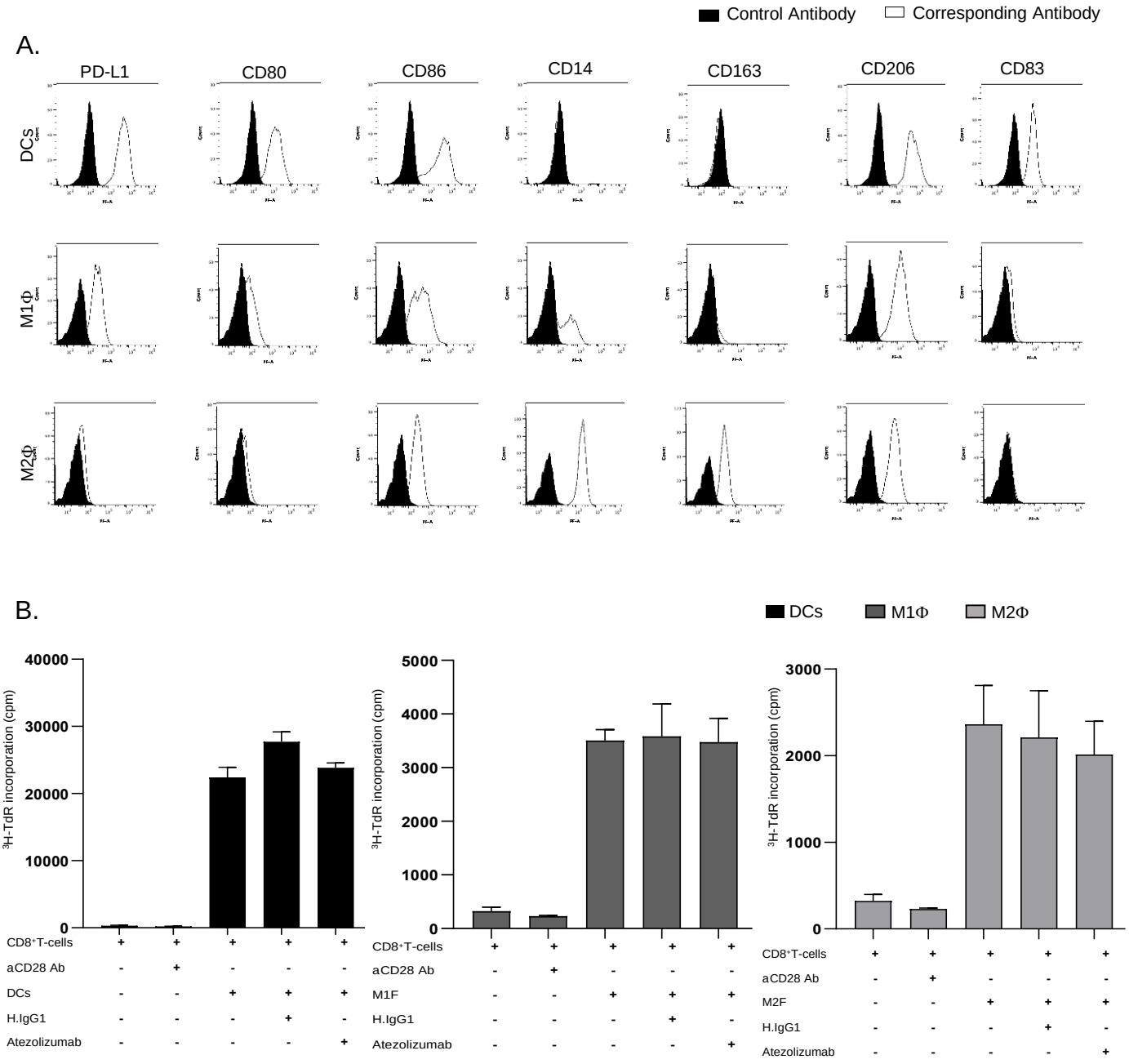
Supplementary Figure 4: The comparison of human fibrocytes to other APCs, including dendritic cells **(A)** and M1 and M2 macrophages **(B)**. Representative flow-cytometry data showing the differences in the expression of immune checkpoint molecules between fibrocytes and DCs **(A)**, and M1 or M2 macrophages **(B).**

Supplementary Figure 5



Supplementary Figure 5. Comparison between fibrocytes and other antigen-presenting cells in allogeneic MLRs in mice. Allogeneic MLRs with fibrocytes **(A)**, dendritic cells **(B)**, M1 **(C)** and M2 macrophages **(D)** from the same mouse donor. These data represent 5 or more repeated experiments and are shown as the mean ± SEM. ** $P<0.01$ by a one-way ANOVA.

Supplementary Figure 6



Supplementary Figure 6. The effect of fibronectin stimulation of human DCs, M1 and M2 (macrophages). **(A)** Representative flow-cytometric evaluation of immune check-points molecules on DCs, M1Φ and M2Φ upon fibronectin (coated) stimulation. **(B)** Blocking effect of anti-PD-L1 Ab (Atezolizumab) on $CD8^+$ T-cell proliferation, in allogeneic MLRs with DCs, M1Φ and M2Φ. Data are representative of three independent experiments. **** $P < 0.0001$, ***$P < 0.001$ by a one-way ANOVA and data are shown as the mean ± SEM.