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**IL-6 down-regulates HLA class II expression and IL-12 production of human dendritic cells to impair activation of antigen-specific CD4<sup>+</sup> T cells**

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**Key words:** Dendritic cells, Antigen presentation, HLA class II, IL-12, Helper T cells

**Abbreviations:**

7AAD: 7-amino-actinomycin D

ARG1: arginase-1

CFSE: carboxyfluorescein succinimidyl ester

COX2: cyclooxygenase-2

CTL: cytotoxic T lymphocyte

CTSL: cathepsin L

DC: dendritic cell

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-related kinase

FACS: fluorescence-activated cell sorting

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GM-CSF: granulocyte macrophage colony-stimulating factor

gp130: glycoprotein 130

HLA: human leukocyte antigen

IHC: immunohistochemistry

IL-6R: interleukin-6 receptor

IL: interleukin

JAK: Janus kinase

mAb: monoclonal antibody

MAPK: mitogen-activated protein kinase

MDSC: myeloid derived suppressor cell

Median FI: median fluorescence intensity

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

MoDC: monocyte derived dendritic cell

nor-NOHA: N<sup>w</sup>-hydroxy-L-arginine

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PD-1: programmed cell death protein 1

PD-L1: programmed cell death ligand 1

PI3K: phosphoinositide 3-kinase

SD: standard deviation

STAT3: signal transducer and activator of transcription 3

STEAP: 6-transmembrane epithelial antigen of prostate

TGF: transforming growth factor

Th: helper T

TIL: tumor-infiltrating lymphocyte

**Précis:** IL-6 attenuates activation of cancer antigen-specific CD4<sup>+</sup> T cells by down-regulation of DC functions.

Therefore, blocking IL-6-mediated signaling pathways may be a promising strategy to improve anti-tumor immunity.

**Foot note:**

This study was presented in part as an abstract in a poster at the 105th AACR Annual Meeting 2014, San Diego, CA, USA, April 5–9, 2014 (Abstract 3615) and the Annual Meeting IMMUNOLOGY 2014, Pittsburgh, PA, USA, May 2-6, 2014 (TUM4P.917).

**Abstract**

Immunosuppression in tumor microenvironments critically affects the success of cancer immunotherapy. Here, we focused on the role of interleukin (IL)-6/signal transducer and activator of transcription (STAT3) signaling cascade in immune regulation by human dendritic cells (DCs). IL-6-conditioned monocyte-derived DCs (MoDCs) impaired the presenting ability of cancer-related antigens. Interferon (IFN)- $\gamma$  production attenuated by CD4<sup>+</sup> T cells co-cultured with IL-6-conditioned MoDCs corresponded with decreased DC IL-12p70 production. Human leukocyte antigen (HLA)-DR and CD86 expression was significantly reduced in CD11b<sup>+</sup>CD11c<sup>+</sup> cells obtained from peripheral blood mononuclear cells (PBMCs) of healthy donors by IL-6 treatment, and was STAT3-dependent. Arginase-1 (ARG1), lysosomal protease, cathepsin L (CTSL), and cyclooxygenase-2 (COX2) were involved in the reduction of surface HLA-DR expression. Gene expressions of *ARG1*, *CTSL*, *COX2*, and *IL6* were higher in tumor-infiltrating CD11b<sup>+</sup>CD11c<sup>+</sup> cells compared with PBMCs isolated from colorectal cancer patients. Expression of surface HLA-DR and CD86 on CD11b<sup>+</sup>CD11c<sup>+</sup> cells was down-regulated and T cell-stimulating ability was attenuated compared with PBMCs, suggesting an immunosuppressive phenotype might be induced by IL-6, ARG1, CTSL, and COX2 in tumor sites of colorectal cancer patients. There was a relationship between HLA-DR expression levels in tumor tissues and the size of CD4<sup>+</sup> T and CD8<sup>+</sup> T cell compartments. Our findings indicate that IL-6 causes a dysfunction in human DCs that activates cancer antigen-specific Th cells, suggesting that blocking the IL-6/STAT3 signaling pathway might be a

promising strategy to improve cancer immunotherapy.

## Introduction

Dysfunctional immune responses in the tumor microenvironment are a critical issue affecting the development of cancer immunotherapies. Immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$  produced at high levels in tissues containing tumors block the functions of antitumor effector T cells. Myeloid-derived suppressor cells (MDSCs), induced from immature myeloid cells in tumor microenvironments, also block antitumor immunity [1-3]. To develop more effective cancer treatments, immune dysfunction in cancer patients needs to be overcome.

Recently, the effectiveness of immuno-checkpoint therapy using anti-programmed cell death protein 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1), and/or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies was reported in solid tumors [4-6]. These results indicate that the removal of blocking signals can restore lymphocyte antitumoral functions.

Dendritic cells (DCs) are antigen-presenting cells that induce antigen-specific immune responses through the activation of CD4<sup>+</sup> T and CD8<sup>+</sup> T cells. In cancer patients, DCs expressing HLA-class I, class II, and co-stimulatory molecules on the cell surface are crucial for inducing cancer-related antigen-specific helper T (Th) cells and cytotoxic T lymphocytes (CTLs) [7-10]. Correct regulation of DC function in tumors is important for the induction of anti-tumor immunity.



IL-6 is a pleiotropic cytokine that has a variety of effects on cells and tissues. It is produced by many different cells, including immune cells, fibroblasts, endothelial cells, and tumor cells [11, 12]. IL-6 binds to the IL-6 receptor (IL-6R) and this IL-6/IL-6R complex associates with the signal-transducing membrane protein, glycoprotein 130 (gp130), inducing its dimerization to initiate IL-6 signaling. Dimerization of gp130 is followed by the rapid activation of the Janus Kinase (Jak) family and several signaling pathways, including phosphoinositide 3-kinase (PI3K)/extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) and STAT3. STAT3 activation induces many effector genes that are involved in cell growth, differentiation, and survival.

We previously showed that IL-6 signaling suppressed major histocompatibility complex (MHC) class II expression on murine DCs via STAT3 activation, and was attenuated in CD4<sup>+</sup> T cell-mediated immune responses [13, 14]. Furthermore, we demonstrated that administration of a monoclonal antibody (mAb) against IL-6R enhanced T cell responses and inhibited tumor growth *in vivo* [15]. In mice with tumors, IL-6 suppressed CD4<sup>+</sup> T cell-mediated immunity through the down-regulation of MHC class II via enhanced arginase activity in DCs [16]. Administration of arginase-1 inhibitors, nor-NOHA or L-arginine, blocked the reduction of MHC class II levels in DCs during tumorigenesis. Furthermore, the injection of nor-NOHA at peri-tumor sites enhanced CD4<sup>+</sup> T cell responses, resulting in an inhibition of tumor growth.

IL-6-mediated STAT3 activation and the subsequent reduction in MHC class II expression in DCs appears to be a critical mechanism for inducing immune system dysfunction in cancer. Blocking IL-6/STAT3 signaling cascades might therefore be a promising approach to overcoming dysfunctional antitumor immunity.

In the current study, we focused on the IL-6/STAT3-signaling pathway in human DCs. We report the effects of IL-6 on the antigen-presenting ability of DCs in T cell-mediated anti-tumor immunity of colorectal cancer patients.

## Material and Methods

### Antibodies and reagents

APC-conjugated anti-human CD11c (3.9), APC-Cy7-conjugated CD4 (SK3), APC-Cy7-conjugated CD8 (RPA-T8), PE-conjugated HLA-DR (G46-6), FITC-conjugated anti-human HLA-A/B/C (G46-2.6), PE-conjugated CD80 (L307.4), and PE-conjugated CD86 (2331, FUN-1) mAbs, PE-conjugated mouse IgG1 $\kappa$  isotype control and FITC-conjugated mouse IgG1 $\kappa$  isotype control were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Control rat IgG was purchased from MP Biomedicals (Santa Ana CA, USA). 7-amino-actinomycin D (7AAD) was purchased from Beckman Coulter (Brea, CA, USA). Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) and human IL-4 were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human IL-6 was purchased from Wako Chemical Industries, Ltd. (Osaka, Japan). A specific inhibitor of STAT3, 6-Nitrobenzo[b]thiophene-1,1-dioxide, a COX2 inhibitor (sc-58125), and the arginase inhibitor (N<sup>w</sup>-hydroxy-L-arginine; nor-NOHA) were purchased from Calbiochem (San Diego, CA, USA). Dimethyl sulfoxide (DMSO), which was used as a solvent, was purchased from Wako Chemical Industries. Anti-human CD3 mAb (OKT3) was purchased from BioLegend (San Diego, CA, USA) and anti-human IL-12 mAb (C8.6) was purchased from BD Biosciences. Anti-human CD4, CD8 and CD14

microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). We used AIM-V (Invitrogen, Carlsbad, CA, USA) without serum for cell culture.

### **Informed consent**

Research protocols involving human subjects were approved by the Institutional Review Boards of Hokkaido University Graduate School of Medicine and the Institute for Genetic Medicine. Written informed consent was obtained from each patient or healthy donor.

### **Generation of MoDCs and IL-6 conditioned MoDCs**

We obtained PBMCs from healthy donors using Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) gradient centrifugation. CD14<sup>+</sup> cells, separated from PBMCs of healthy donors by magnetic cell sorting, were seeded in 12-well culture dishes ( $4 \times 10^5$  cells/well). MoDCs were induced from CD14<sup>+</sup> cells by culturing in the presence of recombinant human IL-4 (50 ng/mL) and GM-CSF (50 ng/mL) for 7 days. Recombinant human IL-6 (50 ng/mL) was added at day 6. MoDCs cultured in the presence of IL-6 for 24 h were designated IL-6-conditioned DCs.

### **Preparation of CD4<sup>+</sup> T cells from PBMCs of healthy donors**

CD4<sup>+</sup> T cells were sorted by magnetic cell sorting from the PBMCs of healthy donors. The isolated CD4<sup>+</sup> T cells ( $1 \times 10^5$  cells) were co-cultured with autologous MoDCs ( $1 \times 10^4$  cells) for cytokine production assays. Purities of the isolated CD4<sup>+</sup> T cells were > 95%.

### **Induction of cancer antigen-specific Th cells**

We cultured PBMCs ( $2 \times 10^6$  cells) in the presence of Survivin-derived peptide (EHKKHSSGCAFLSVKKQFEELTLGFEFLKLDREERAKNKIAK, 5  $\mu$ M) [17], or 6-transmembrane epithelial antigen of prostate (STEAP)-derived peptides (SLLLGTIHALIFAWN, 5  $\mu$ M and QFVWYTPPTFMIAVF, 5  $\mu$ M) [18] for 7 days. PBMCs were then treated with a streptococcus-derived anticancer immunotherapeutic agent, OK-432 (0.1 KE/ml), mitomycin-treated autologous MoDCs ( $1 \times 10^5$  cells), the same peptide that was previously used for stimulation, and IL-2 (10 IU/ml) for 7 days. Cells were re-stimulated and cultured for another 7 days. At day 21, the specificity of induced CD4<sup>+</sup> T cells for each antigen was evaluated by co-culture with MoDCs in the presence of the corresponding antigen (**Supplemental Fig. 1**). For cytokine production assays, antigen-specific CD4<sup>+</sup> T cells ( $5 \times 10^4$ ) were co-cultured with MoDCs or IL-6-conditioned MoDCs ( $5 \times 10^3$ ) in the presence of cancer antigen

peptides or control peptides for 24 h.

### **Preparation of DCs and co-culture with autologous T cells from colorectal cancer patients**

TILs and PBMCs were collected from patients with colorectal cancers. All colorectal cancer tissues were immediately minced using scissors after surgical resection and digested with 1 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. Single cell suspensions were obtained by passage through a 100- $\mu$ m nylon cell strainer (BD Biosciences). Surface HLA-DR and CD86 expression of 7AAD<sup>-</sup>CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells from PBMCs and TILs were evaluated by flow cytometry (**Supplemental Fig. 2**). For polymerase chain reaction (PCR) analysis and co-culture with T cells, 7AAD<sup>-</sup>CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells from PBMCs or TILs were isolated using a fluorescence-activated cell sorting (FACS) system. Autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells were sorted from PBMCs of colorectal cancer patients for T cell proliferation assays. Purities of the isolated 7AAD<sup>-</sup>CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells, CD4<sup>+</sup> T and CD8<sup>+</sup> T cells were > 95%. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were pre-incubated in carboxyfluorescein succinimidyl ester (CFSE) (5 nM) at room temperature for 15 min. After washing with phosphate-buffered saline (PBS), CFSE-labeled CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $1 \times 10^5$  cells) were co-cultured with CD11b<sup>+</sup>CD11c<sup>+</sup> cells ( $5 \times 10^4$  cells) from PBMCs or TILs in the presence of an anti-CD3 mAb

(OKT3, 2  $\mu\text{g}/\text{mL}$ ). 7AAD<sup>-</sup>CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells were added to T cells ( $1 \times 10^5$  cells) at different ratios (1:2–1:8) to measure IFN- $\gamma$  production. The level of IFN- $\gamma$  in culture supernatants was measured at 24 h by enzyme-linked immunosorbent assay (ELISA). Proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was evaluated at 72 h by flow cytometry.

### PCR analysis

Total RNA was extracted from human DCs or 7AAD<sup>-</sup>CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells derived from PBMCs and TILs with an RNeasy Mini kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized using 1  $\mu\text{g}$  of total RNA, oligo (dT) (Invitrogen), and Superscript III reverse transcriptase (Invitrogen). Genes encoding human *IL6*, *COX2*, *CTSL*, *ARG1*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were amplified in a LightCycler (Roche, Indianapolis, IN, USA). The primer sequences and numbers of universal probes used in this study were as follows: *IL6* (forward: 5'-caggagcccagctatgaact-3', reverse: 5'-gaaggcagcaggcaacac-3', universal probe: #7), *COX2* (forward: 5'-cttcacgcatcagttttcaag-3', reverse: 5'-tcaccgtaaatatgattaagtccac-3', universal probe: #23), *CTSL* (forward: 5'-gcaaggatgagtgtaggattca-3', reverse: 5'-gggagggcagttgaggac-3', universal probe: #75), *ARG1* (forward: 5'-tggcagaagtcaagaagaacg-3', reverse: 5'-atgctccaattgccaact-3', universal probe: #64), and *GAPDH*

(forward: 5'-agccacatcgctcagacac-3', reverse: 5'-gcccaatacgaccaaacc-3', universal probe: #60). Sample signals were normalized to the reference gene *GAPDH* using the  $\Delta\Delta Ct$  method ( $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{reference}}$ ).

### **Flow cytometry**

Surface expression of HLA-A/B/C, HLA-DR, CD80, and CD86 was evaluated with a FACSCantoII™ (BD Biosciences), and results were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). The mean fluorescence intensity (MFI) ratio (sample  $\Delta$  MFI (Specific marker MFI – isotype control MFI)/control sample  $\Delta$  MFI  $\times$  100) or the median FI ratio (sample  $\Delta$  median FI (Specific marker median FI – isotype control median FI)/control sample  $\Delta$  MFI  $\times$  100) was calculated for samples. A FACSriaII™ (BD Bioscience) was used for the isolation of CD11b<sup>+</sup>CD11c<sup>+</sup> DCs from PBMCs and TILs of colorectal cancer patients.

### **ELISA**

We determined IFN- $\gamma$ , IL-12p70, and IL-10 levels in culture supernatants using OptEIA™ human IFN- $\gamma$ , IL-12p70, and IL-10 ELISA kits (BD Bioscience), respectively, according to the manufacturer's



instructions. Total and active TGF- $\beta$  levels in culture supernatants were determined by using a Quantikine™ human TGF- $\beta$ 1 Immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

### **Immunohistochemistry (IHC)**

Normal and cancer tissues were obtained from colorectal surgical specimens (n = 27). Patient information is summarized in **Supplemental Table 1**. Colon specimens were fixed in formalin and embedded in paraffin. After deparaffinization, antigen retrieval was conducted by boiling for 10 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide for 15 min. After pre-incubation with Blocking I buffer (Nacalai Tesque, Kyoto, Japan) for 10 min, slides were incubated with mouse anti-human HLA-DR  $\alpha$ -chain mAb (clone TAL.1B5, Dako, Glostrup, Denmark), mouse anti-human CD4 mAb (clone 4B12, Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK), mouse anti-human CD8 mAb (clone 1A5, Novocastra Laboratories Ltd), or a polyclonal rabbit anti-human IL-6 (Rockland Immunochemicals, Inc, Gilbertsville, PA, USA) antibody overnight at 4°C. Sections were then incubated for 30 min with horseradish peroxidase (HRP)-labeled anti-mouse IgG polyclonal antibody (Nichirei Bioscience, Inc., Tokyo, Japan) or an HRP-labeled goat anti-rabbit antibody

(Dako). Positive signals were visualized using 3-3'-diaminobezidine-4HCL (DAB). Sections were counterstained with Mayer's hematoxylin.

### **Statistical analysis**

Statistical significance was evaluated using the Student's *t*-test for two groups of data. In some experiments, the results were evaluated by Dunnett's post-test (vs. control group). A P-value less than 0.05 was considered statistically significant.

## Results

### **IL-6 attenuates DC-mediated activation of cancer-related antigen-specific CD4<sup>+</sup> T cells**

We first investigated whether IL-6 influenced the antigen-presenting ability of human MoDCs. Cancer-related antigen, Survivin- or STEAP-specific CD4<sup>+</sup> T cells were co-cultured with MoDCs or IL-6-conditioned MoDCs in the presence of each antigen peptide. IFN- $\gamma$  production by Survivin- and STEAP-specific CD4<sup>+</sup> T cells was significantly decreased after co-culturing with IL-6-conditioned MoDCs compared with control MoDCs (**Fig. 1A, 1B**). We confirmed that IFN- $\gamma$  production by antigen-specific CD4<sup>+</sup> T cells was inhibited in the presence of anti-HLA-DP/DQ/DR mAbs (**Supplemental Fig. 3**). These findings suggest that IL-6 attenuates the DC-mediated activation of cancer-related antigen specific CD4<sup>+</sup> T cells.

### **IL-6 reduces IFN- $\gamma$ production by CD4<sup>+</sup> T cells**

Next, CD4<sup>+</sup> T cells, isolated from healthy donors, were cultured with MoDCs or IL-6-conditioned MoDCs in the presence of an agonistic anti-CD3 mAb. IFN- $\gamma$  production by CD4<sup>+</sup> T cells co-cultured with IL-6 conditioned MoDCs was significantly decreased compared with cells co-cultured with MoDCs (**Fig. 1C, 1D**). We further confirmed that IFN- $\gamma$  production by anti-CD3 mAb-stimulated CD4<sup>+</sup> T cells

was reduced when they were co-cultured with IL-6 conditioned BDCA1<sup>+</sup> DCs from PBMCs of healthy donors (**Supplemental Fig. 4**). However, the effects of IL-6 on mature type BDCA1<sup>+</sup> DCs were lower than for MoDCs. In addition, we confirmed that the HLA-DR expression of BDCA<sup>+</sup> DCs from blood was not down regulated by IL-6 treatment (**data not shown**). Therefore, these data suggest that IL-6 suppresses the maturation of MoDCs as well as T cell activation and cytokine production.

Additionally, we found that IL-12p70 production in culture supernatants from IL-6-conditioned DCs was significantly reduced. We confirmed that IFN- $\gamma$  production by CD4<sup>+</sup> T cells co-cultured with MoDCs was decreased to levels similar to those for IL-6 conditioned DCs in the presence of neutralizing anti-IL-12 mAb (**Fig. 1C, 1D**). These data suggest that IL-6 attenuates the induction of Th1 cells by decreasing IL-12p70 production by DCs.

#### **IL-6-mediated STAT3 activation reduces the surface expression of HLA class II and CD86**

We investigated whether IL-6 contributed to the surface expression levels of HLA class I, HLA class II, and costimulatory molecules on human DCs. HLA-DR and CD86 expression on CD11b<sup>+</sup>CD11c<sup>+</sup> cells was significantly reduced following treatment with IL-6. The expression of HLA-A/B/C and CD80 was not altered. The reduction of HLA-DR and CD86 expression levels was blocked by the addition of a

STAT3 inhibitor (**Fig. 2A, 2B**). These findings suggest that IL-6-STAT3 signaling in human DCs may suppress the surface expression levels of HLA class II and CD86 molecules.

### **COX2, lysosomal protease, and arginase activities are involved in the IL-6-mediated down-regulation of surface HLA-DR expression levels**

We further examined the ability of IL-6-induced effector molecules to decrease HLA class II and CD86 expression in human DCs. IL-6 stimulation induced the expression of *COX2*, *CTSL*, and *ARG1* genes in CD11b<sup>+</sup>CD11c<sup>+</sup> cells. (**Fig. 3A**) We confirmed that IL-6 induced a down-regulation of HLA-DR expression that was restored by the addition of COX2, lysosomal protease, or arginase inhibitors. The COX2 inhibitor partially restored the surface expression levels of CD86 (**Fig. 3B**). These data indicate that the IL-6-STAT3 signaling might negatively regulate HLA-DR expression levels on human DCs through the activation of COX2, lysosomal protease, and arginase.

### **Down-regulation of surface expression levels of HLA-DR and CD86 on CD11b<sup>+</sup>CD11c<sup>+</sup> cells in tumor tissues from colorectal cancer patients**

We next investigated whether tumor microenvironments suppressed the function of CD11b<sup>+</sup>CD11c<sup>+</sup>

cells in cancer patients. We examined the expression levels of various genes in CD11b<sup>+</sup>CD11c<sup>+</sup> cells isolated from the tumor tissues and PBMCs of colorectal cancer patients. Expression of the *IL6*, *COX2*, *CTSL*, and *ARG1* genes in CD11b<sup>+</sup>CD11c<sup>+</sup> cells from TILs was greater than that in PBMCs from colorectal cancer patients (**Fig. 4A**). We confirmed that tumor-infiltrating CD11b<sup>+</sup>CD11c<sup>+</sup> cells were an IL-6-producing population in the tumor microenvironment. HLA-DR and CD86 expression levels on CD11b<sup>+</sup>CD11c<sup>+</sup> cells from TILs were lower compared with those from PBMCs of colorectal cancer patients (**Fig. 4B**).

#### **Tumor-infiltrating CD11b<sup>+</sup>CD11c<sup>+</sup> cells have impaired T cell-stimulating functions**

We further examined the T cell-stimulating ability of tumor-infiltrating DCs. The proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was reduced in the presence of CD11b<sup>+</sup>CD11c<sup>+</sup> cells from TILs compared with those from PBMCs (**Fig. 4C**). IFN- $\gamma$  production by T cells was attenuated in the presence of CD11b<sup>+</sup>CD11c<sup>+</sup> cells from TILs and increased production was dependent on the number of cells added (**Fig. 4D**). These data suggested that the tumor microenvironment impaired the T cell-stimulating ability of tumor-infiltrating antigen-presenting cells such as DCs in colorectal cancer patients.

**HLA-DR expression levels are related to the infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells at tumor sites**

Finally, HLA-DR expression levels and the frequency of infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells were investigated in the tumor microenvironment of colorectal cancer patients. In this study, we observed infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in colorectal tumor tissues with high HLA-DR expression levels (**Fig. 5A**). We evaluated the HLA-DR expression levels and frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in tumor sites (**Fig. 5B, 5C**). HLA-DR expression levels in tumor tissues were closely related to the size of the CD4<sup>+</sup> T and CD8<sup>+</sup> T cell compartments in colorectal cancer patients.

## Discussion

Patients with advanced cancer are considered immunocompromised. Therefore, improving the immune status of cancer patients is required for effective cancer immunotherapies. Prospective and retrospective studies have shown that serum IL-6 levels are related to tumor stage and size, metastasis, survival of colon cancer patients [19], chemotherapy efficacy for advanced pancreatic cancer [20], advanced stage and metastasis-related morbidity of breast cancer [21], and the efficacy of personalized peptide vaccination for advanced biliary tract cancer [22]. Results from these studies suggest that IL-6 levels in cancer are not simply useful as promising prognostic biomarkers, but are also related to tumorigenesis and anti-tumor immune responses. The precise mechanism by which IL-6 modulates anti-tumor responses in systemic and tumor microenvironments remains to be elucidated.

In the current study, IL-6-conditioned MoDCs attenuated IFN- $\gamma$  production by cancer-related antigen-specific effector CD4<sup>+</sup> T cells. We confirmed that IFN- $\gamma$  production by CD4<sup>+</sup> T cells was significantly reduced when they were co-cultured with IL-6-conditioned DCs (**Fig. 1A, 1B**). This finding suggests that IL-6 suppresses the DC-mediated induction of Th1 cells. We also observed that IL-12p70 production by DCs was also reduced in parallel with decreased IFN- $\gamma$  production by CD4<sup>+</sup> T cells (**Fig. 1C, 1D**).



It was previously reported that STAT3 regulated NF- $\kappa$ B recruitment to the IL-12p40 promoter in murine DCs [23], and that STAT3 inhibited IL-12p35 gene expression in mice [24]. We found that pre-treatment of IL-6-conditioned MoDCs with a STAT3 inhibitor increased IL-12p70 production along with IFN- $\gamma$  production by CD4<sup>+</sup> T cells after T cell receptor stimulation (**data not shown**). These findings suggest that IL-6-dependent STAT3 activation in MoDCs might be involved in reducing IL-12p70 production in humans.

In general, it is well known that IL-10 and TGF- $\beta$  cytokines suppress DC maturation. In this study, we confirmed that the production of IL-10 but not the active form of TGF- $\beta$  was enhanced in the culture supernatants of IL-6-conditioned MoDCs (**Supplemental Fig. 5**). Therefore, IL-10 production by IL-6-conditioned MoDCs might be involved in the reduction of IL-12p70 and the subsequent production of IFN- $\gamma$  by CD4<sup>+</sup> T cells.

IL-12 activates STAT4 in CD4<sup>+</sup> T cells, subsequently inducing IFN- $\gamma$  secretion [25]. IL-12 is an important cytokine for Th1 immunity [26, 27], as it is essential for inducing fully activated CTLs in tumor-bearing hosts [28, 29]. IL-12p35<sup>-/-</sup> mice are more susceptible to tumor development following exposure to carcinogens compared with wild-type mice [30]. We reported that cancer antigen-derived peptides containing helper epitopes efficiently induced CTLs *in vitro* according to the helper function of

antigen-specific CD4<sup>+</sup> T cells [17]. In a clinical trial, a cancer peptide vaccine containing helper epitopes induced CTLs in an advanced cancer patient [31]. Blocking the IL-6/STAT3 signaling cascade might promote IL-12p70 production by DCs to induce cancer-specific Th1 cells in immunosuppressed cancer patients.

We observed that IL-6 stimulation reduced HLA-DR and CD86 expression levels on immature DCs derived from the PBMCs of healthy donors in a STAT3-dependent manner (**Fig. 2**). Arg1, lysosomal proteases, and COX2 were responsible for the down-regulation of HLA-DR on human DCs (**Fig. 3**). We previously demonstrated that arginine was required for the gene expression of MHC class II and arginase activation induced by IL-6 caused dysfunction of DCs in a tumor-bearing mouse model [16]. The IL-6-induced activation of lysosomal proteases reduced MHC class II  $\alpha\beta$ -dimer levels in murine DCs [13]. Previous reports indicated that activation of the COX2/PGE2 cascade inhibited the maturation of DCs [32, 33]. We speculated that these functional molecules might be involved in the mechanisms of IL-6-induced down-regulation of HLA class II in human DCs.

We observed that tumor-infiltrating CD11b<sup>+</sup>CD11c<sup>+</sup> cells from colorectal cancer specimens had reduced surface expression levels of HLA-DR and CD86 and attenuated CD4<sup>+</sup> and CD8<sup>+</sup> T cell-stimulating abilities compared with CD11b<sup>+</sup>CD11c<sup>+</sup> cells from the PBMCs of autologous patients

(Fig. 4). We speculated that intratumoral DCs had a reduced ability to activate effector Th1 cells and CTLs in the tumor microenvironment.

In this study, we observed that IFN- $\gamma$  production by T cells in the presence of CD11b<sup>+</sup>CD11c<sup>+</sup> cells derived from TILs was higher at a 2:1 ratio (T cells-APC) than at an 8:1 ratio, although the number of CD11b<sup>+</sup>CD11c<sup>+</sup> cells was greater at the 2:1 ratio (Fig. 4D). In these experiments, IFN- $\gamma$  was produced by T cells co-cultured with CD11b<sup>+</sup>CD11c<sup>+</sup> cells from TILs. Previous studies demonstrated that DCs induced from bone marrow in the presence of IFN- $\gamma$  effectively activated T cells by enhancing their maturation. Therefore, we speculated that IFN- $\gamma$ -conditioned CD11b<sup>+</sup>CD11c<sup>+</sup> cells from TILs altered the maturation status during culture resulting in the gain of T cell-stimulating ability *in vitro*.

IL-6 can be produced by various cells, including cancer cells, cancer-associated fibroblasts, and immune cells to cause chronic inflammation at tumor sites in cancer patients. We confirmed that the gene expression level of IL-6 was elevated in tumor-infiltrating CD11b<sup>+</sup>CD11c<sup>+</sup> cells (Fig. 4A). This was an indicator that CD11b<sup>+</sup>CD11c<sup>+</sup> cells might be a major IL-6-producing population in the tumor microenvironment of colorectal cancer patients. Gene expression levels of *ARG1*, *CTSL*, and *COX2* were increased in tumor-infiltrating CD11b<sup>+</sup>CD11c<sup>+</sup> cells. These results suggest that the inhibition of IL-6-induced activation of arginase, lysosomal proteases, and COX2 in tumor-infiltrating DCs might

restore the down-regulation of surface HLA class II expression, and effectively activate cancer-specific Th1 cells in the tumor microenvironment.

HLA class II expression levels were closely related to the invasion of tumor sites by CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The presence of intratumoral T cells is considered a good prognostic factor in colorectal cancer [34-38]. Recent studies indicated that an elevated number of intratumoral T cells, elevated HLA class I expression levels, and STAT1 activation are beneficial prognostic biomarkers for colorectal cancer [39]. Additionally, PD-L1 expression by cancer cells was related to invasion by immune cells and the efficiency of anti-PD-L1 therapy [40]. These studies show that the presence of tumor-infiltrating T cells and adequate activation of effector T cells are required for cancer immunotherapy. We postulated that maintaining the antigen-presenting ability of DCs would be critical for the induction and activation of cancer antigen-specific T cells in tumor microenvironments. Therefore, a therapeutic strategy for improving the function of DCs will hopefully be effective for increasing the number of good responders to a cancer immunotherapy.

Recent clinical trials using a chimeric anti-IL-6 mAb were performed on patients with advanced solid tumors [41], B-cell non-Hodgkin's lymphoma, multiple myeloma, Castleman disease [42], metastatic castration-resistant prostate cancer [43], and ovarian cancer [44]. Results from these trials showed that

these treatments were tolerated by patients. In addition, serum C-reactive protein levels were decreased, and anemia caused by chronic inflammation was improved following the administration of anti-IL-6 mAb. These results suggest that anti-IL-6 mAb might control the status of chronic inflammation in patients with advanced cancer. It also suggested that anti-IL-6 mAb assist with recovering the function of tumor-infiltrating DCs through the inhibition of IL-6/STAT3 signaling in the tumor microenvironment. We expect that the administration of anti-IL-6 mAb combined with cancer immunotherapy, such as a peptide vaccine, might demonstrate greater clinical efficacy.

In summary, we found that IL-6 induced STAT3 activation and reduced the surface expression levels of HLA class II and CD86 molecules. Concurrently, IL-12p70 production by human DCs was reduced. This resulted in the subsequent suppression of T-cell-mediated anti-tumor immunity. Therefore, IL-6 may be related to immunosuppression in cancer patients by causing DC dysfunction, suggesting that inhibition of the IL-6/STAT3 signaling pathway might be a promising strategy for improving cancer immunotherapies.

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**Conflict of interest**

All authors declare no conflict of interest.

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**Figure legends**

**Fig. 1** IL-6 impairs the DC-mediated activation of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells. **A.** Survivin- or STEAP-specific CD4<sup>+</sup> T cells ( $5 \times 10^4$ ) were co-cultured with MoDCs or IL-6-conditioned DCs ( $5 \times 10^3$ ) in the presence of cancer antigen peptides or control peptides for 24 h. IFN- $\gamma$  productions were determined by ELISA. Four independent experiments were conducted, and similar results were confirmed for each experiment. The mean and standard deviation (SD) of representative data are presented. **B.** Percentages of IFN- $\gamma$  production levels are indicated. Data are mean  $\pm$  SD (n = 4). \*P < 0.05 vs. Control. **C.** CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were co-cultured with control MoDCs or IL-6-conditioned MoDCs ( $1 \times 10^4$ ) in the presence of anti-CD3 mAb (2  $\mu$ g/mL) for 24 h. A neutralizing anti-IL-12 mAb or control Ab (2  $\mu$ g/mL) was added to cultures. IFN- $\gamma$  and IL-12p70 productions were evaluated by ELISA. Five independent experiments were conducted, and similar results were confirmed for each experiment. The mean and SD of representative data are presented. **D.** Percentages of IFN- $\gamma$  and IL-12p70 production levels are indicated. Data are mean  $\pm$  SD (n = 5). \*P < 0.05 vs. Control. n.s., not significant.

**Fig. 2** IL-6 down-regulates HLA class II and CD86 expression levels in CD11b<sup>+</sup>CD11c<sup>+</sup> cells via STAT3-dependent mechanisms. Adherent cells from PBMCs of healthy donors were stimulated with IL-6 in the presence of a STAT3 inhibitor or DMSO and surface expression levels of HLA-A/B/C, HLA-DR,

CD80, and CD86 on immature CD11b<sup>+</sup>CD11c<sup>+</sup> cells were evaluated by flow cytometry. **A.** Representative profiles. **B.**  $\Delta$  median FI ratios are presented as mean and SD (n = 5). \*P < 0.05 vs. Control.

**Fig. 3** *COX2*, *CTPL* and *ARG1* gene expression is related to the down-regulation of surface HLA-DR expression levels. Adherent cells collected from PBMCs of healthy donors were stimulated with IL-6. **A.** Expression of *COX2*, *CTPL*, and *ARG1* genes were evaluated by quantitative PCR. The mean and SD for expression levels of each gene are presented (n = 3). \*P < 0.05 vs. Control. **B.** Surface expression levels of HLA-DR and CD86 on CD11b<sup>+</sup>CD11c<sup>+</sup> cells following IL-6 stimulation in the presence of a COX-2 inhibitor (sc-58125; 10  $\mu$ g/mL), a lysosomal protease inhibitor (E64d; 20 nM), or an arginase inhibitor (nor-NOHA; 3  $\mu$ g/mL) were evaluated by flow cytometry. The  $\Delta$  median FI ratio is presented as the mean and SD (n = 5). \*P < 0.05 vs. IL-6.

**Fig. 4** Maturation and T cell-stimulating ability of CD11b<sup>+</sup>CD11c<sup>+</sup> cells are reduced in the tumor microenvironments of colorectal cancer patients. 7AAD<sup>-</sup>CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> cells derived from PBMCs and TILs were collected. **A.** Expression levels of *IL6*, *COX2*, *CTSL*, and *ARG1* genes in

CD11b<sup>+</sup>CD11c<sup>+</sup> cells from PBMCs and TILs were evaluated by quantitative PCR. Representative data are shown as the mean and SE of relative gene expression levels. **B.** HLA-DR and CD86 expression levels on the surface of CD11b<sup>+</sup>CD11c<sup>+</sup> cells from PBMCs and TILs were evaluated by flow cytometry. Representative profiles are shown with  $\Delta$  MFI ratios presented as mean and SD (n = 8). \*P < 0.05 vs. CD11b<sup>+</sup>CD11c<sup>+</sup> cells from PBMCs. **C.** CFSE-labeled CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $1 \times 10^5$ ) were stimulated with an anti-CD3 mAb in the presence of CD11b<sup>+</sup>CD11c<sup>+</sup> cells ( $5 \times 10^4$ ) from PBMCs or TILs for 72 h. Proliferation of T cells in the presence of CD11b<sup>+</sup>CD11c<sup>+</sup> cells from PBMCs (solid line) or TILs (dotted line) was evaluated by flow cytometry. Three independent experiments were conducted on samples from colorectal cancer patients. Representative profiles are shown. **D.** IFN- $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $1 \times 10^5$ ) co-cultured with CD11b<sup>+</sup>CD11c<sup>+</sup> cells (0,  $1.25 \times 10^4$ ,  $2.5 \times 10^4$ , or  $5 \times 10^4$ ) from PBMCs or TILs was measured by ELISA. Three independent experiments were conducted on samples from colorectal cancer patients, and similar results were confirmed for each experiment. Representative data is shown.

**Fig. 5** HLA-DR expression levels correlated with the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells at tumor sites in colorectal cancer patients. Tumors were collected from colorectal surgery specimens (n = 27). **A.**

Immunohistochemistry was used to detect the expressions of HLA-DR, CD4, and CD8. Subjects were classified as Grade 0–2 based on HLA-DR expression levels. Representative images ( $\times 200$  magnification) of samples from six patients (Pt#1-6) with Grade 0–2 HLA-DR expressions are shown. Scale bars indicate 200  $\mu\text{m}$ . **B.** The numbers of cases with HLA-DR Grade 0–2 are shown. **C.** Levels of CD4 and CD8 expression in patient tissues with HLA-DR Grade 0–2 expression were classified as absent, faint (-), moderate (+), and high (++). The numbers of cases for CD4 and CD8 scores are shown.

Figure 1. Ohno, Y. et al.

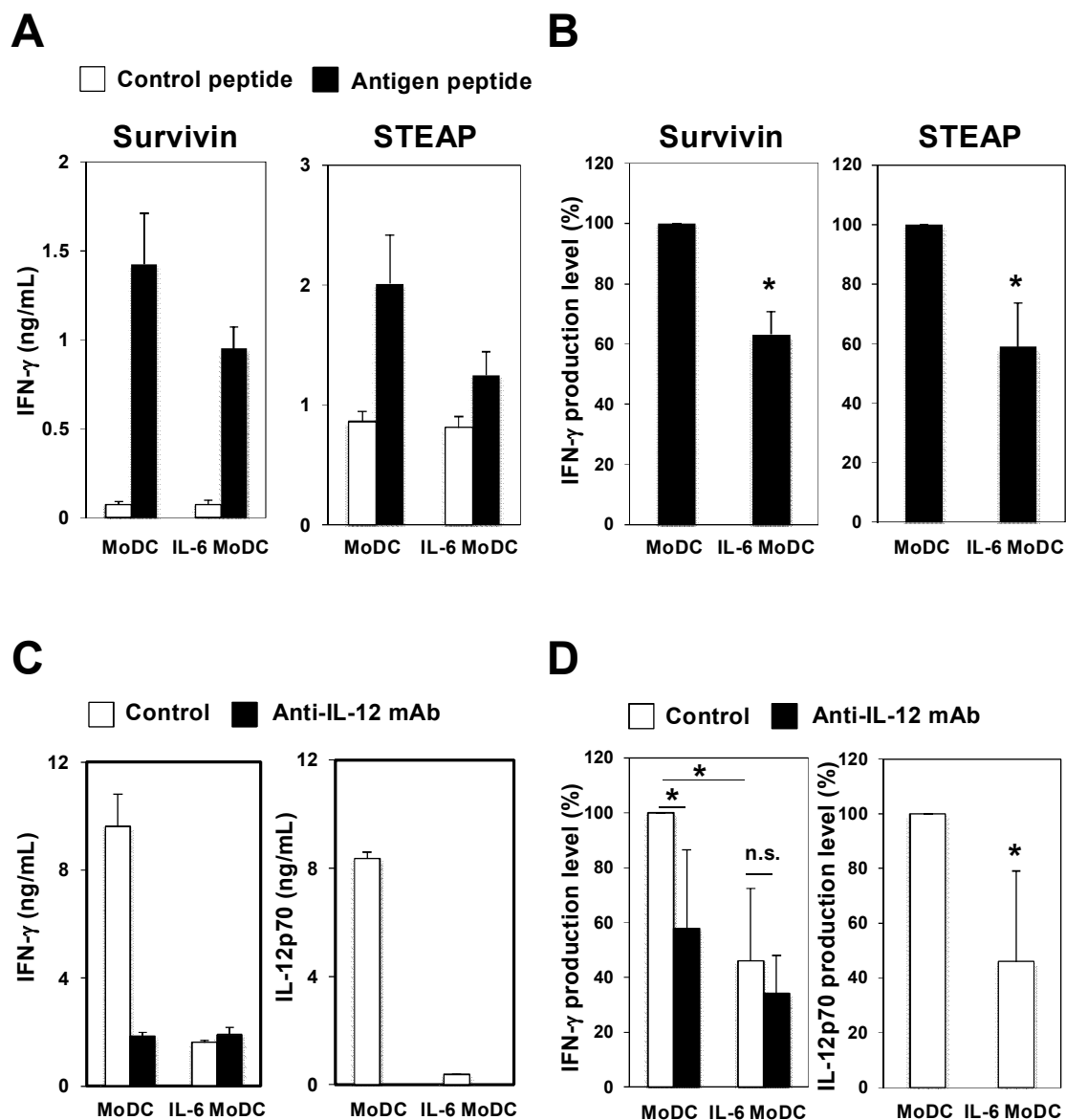


Figure 2. Ohno, Y. et al.

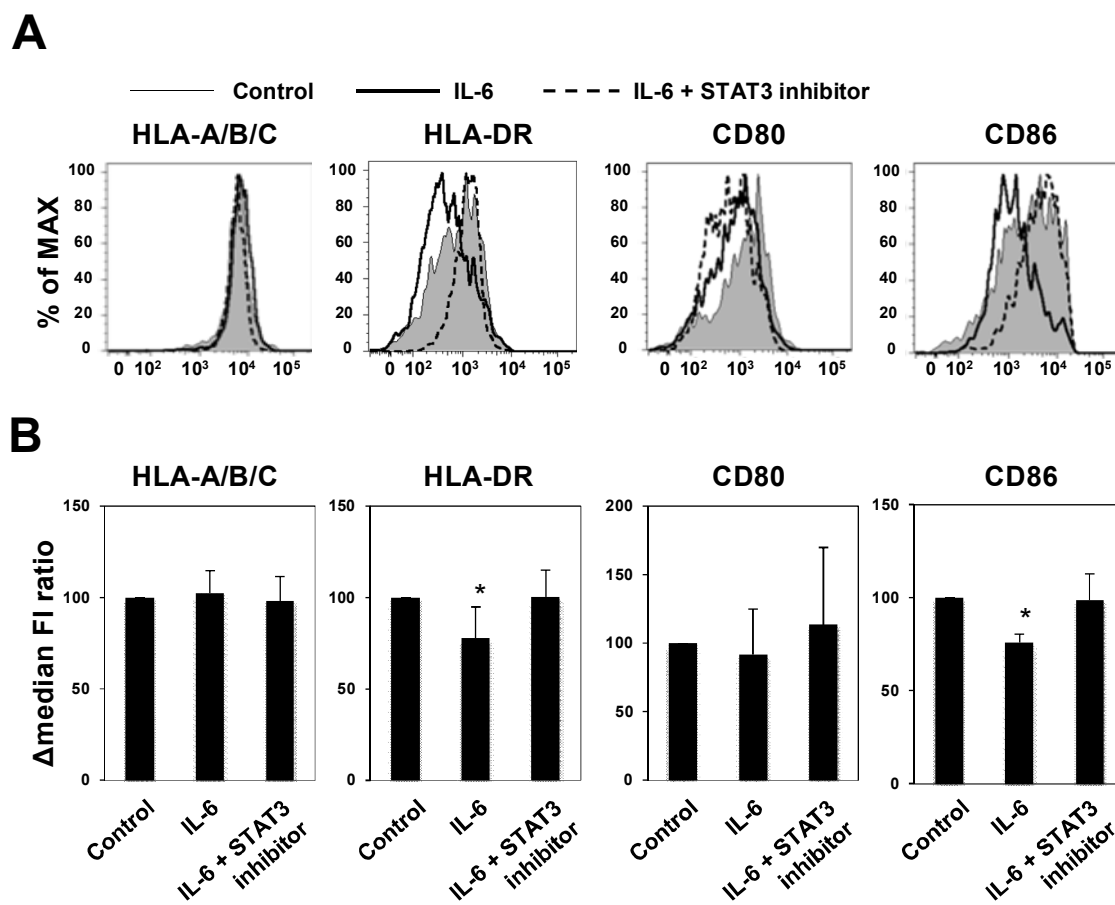


Figure 3. Ohno, Y. et al.

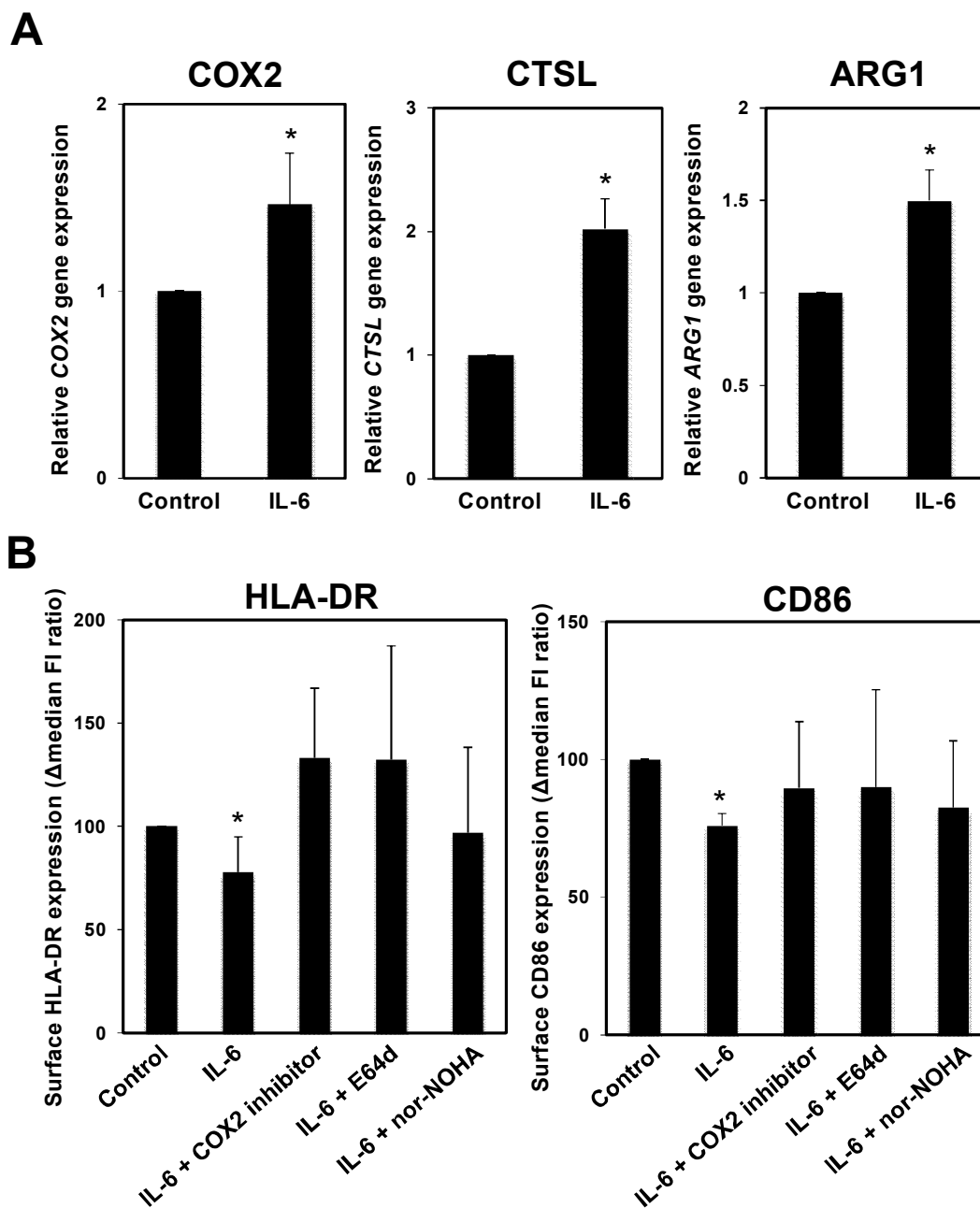




Figure 4. Ohno, Y. et al.

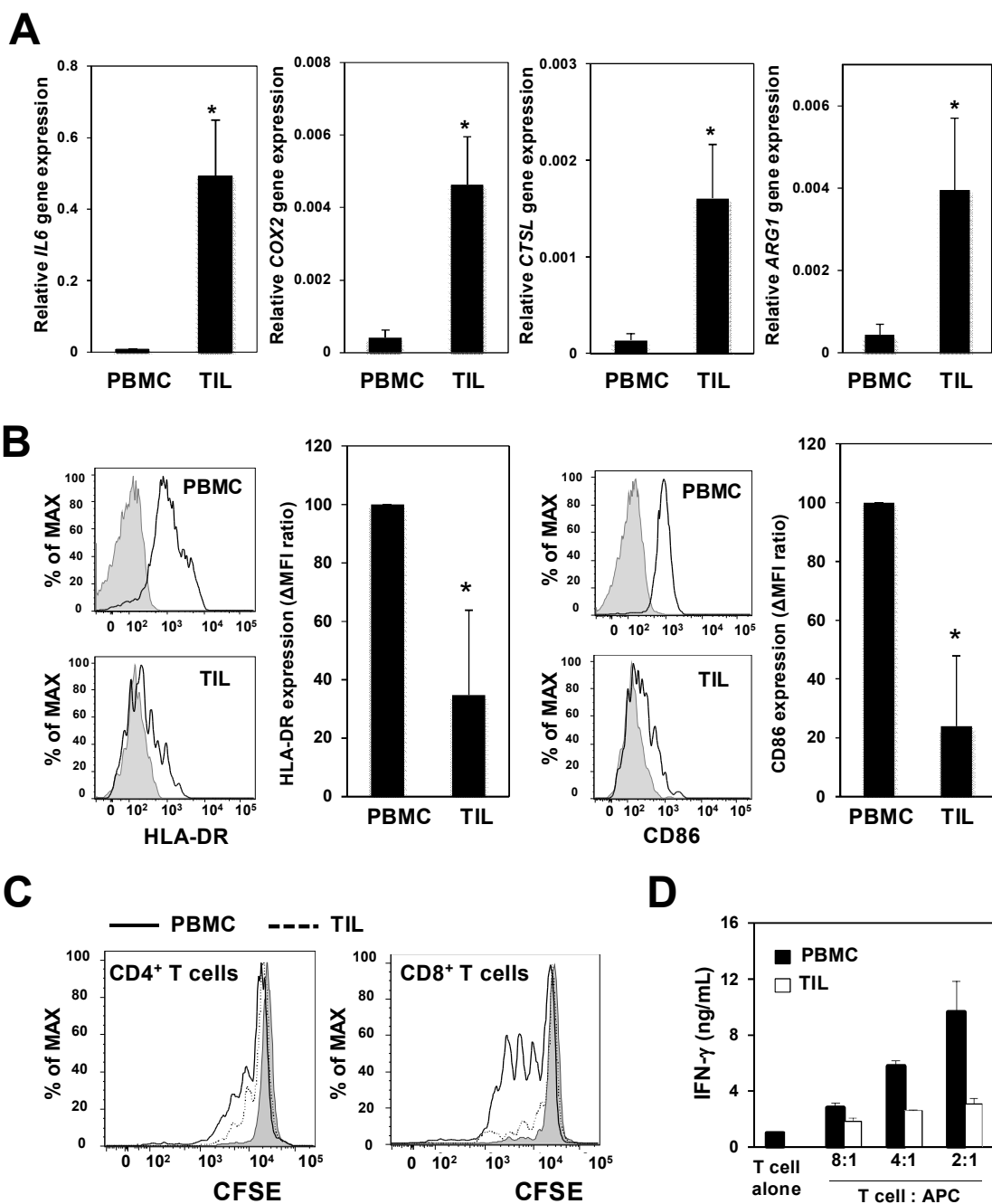
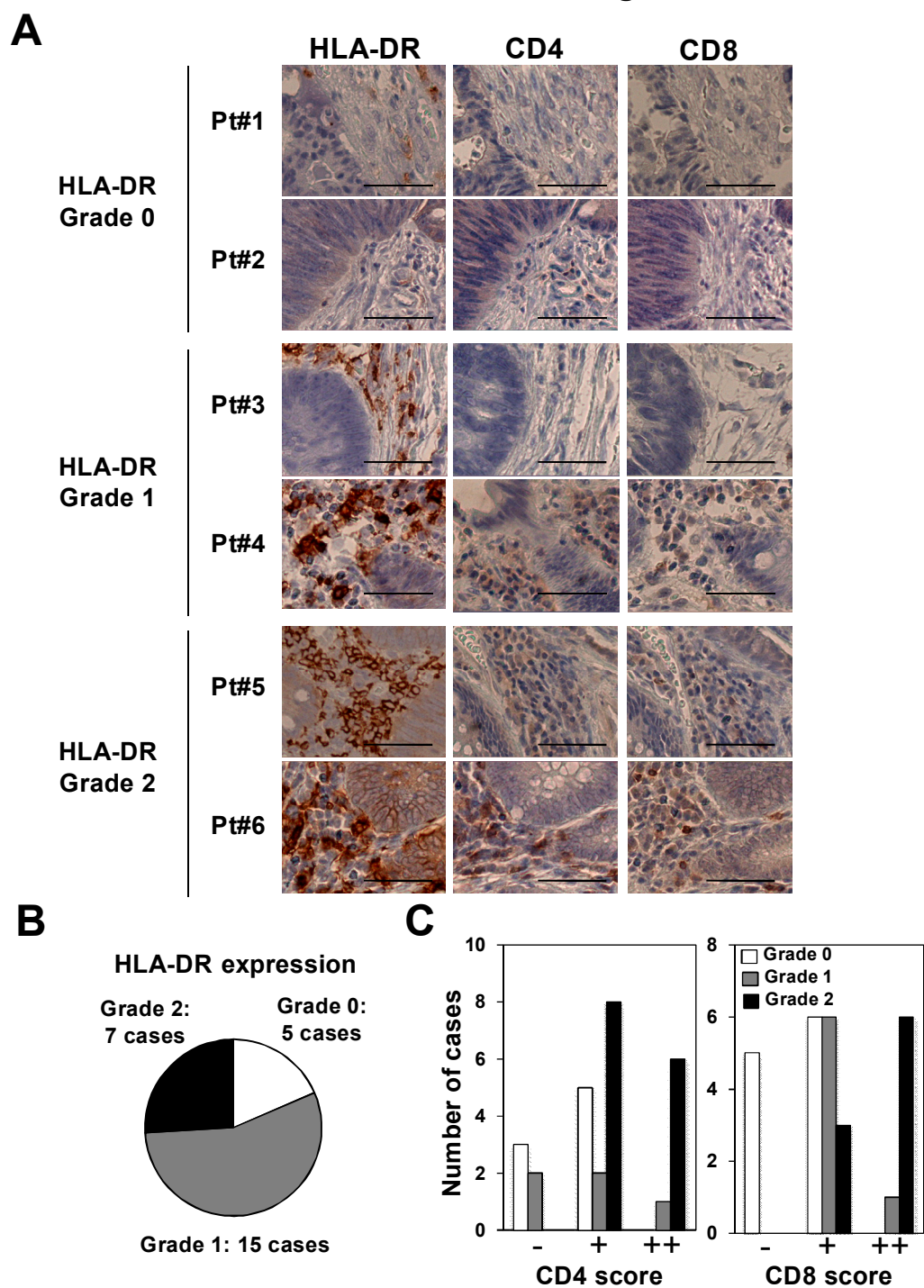
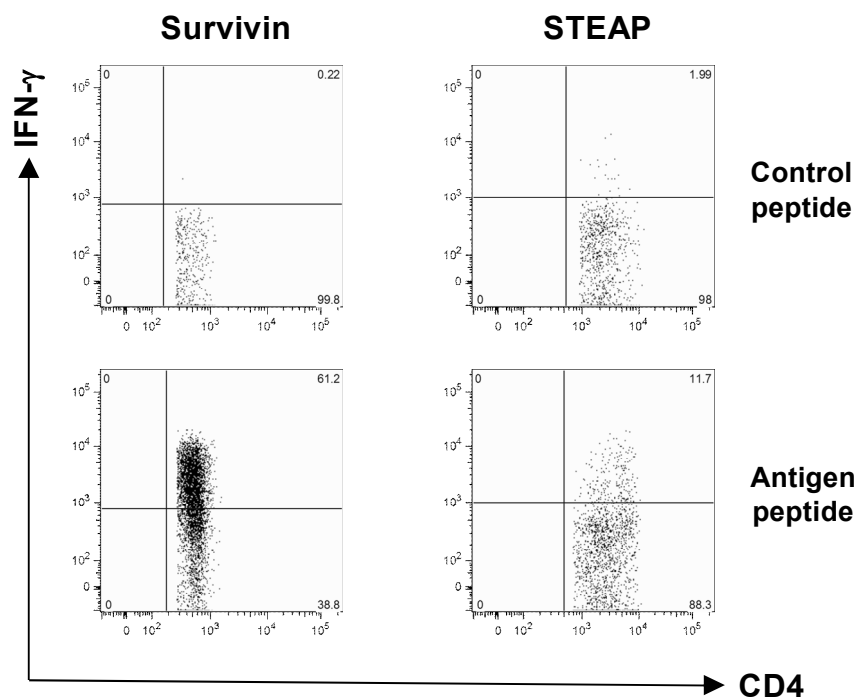


Figure 5. Ohno, Y. et al.



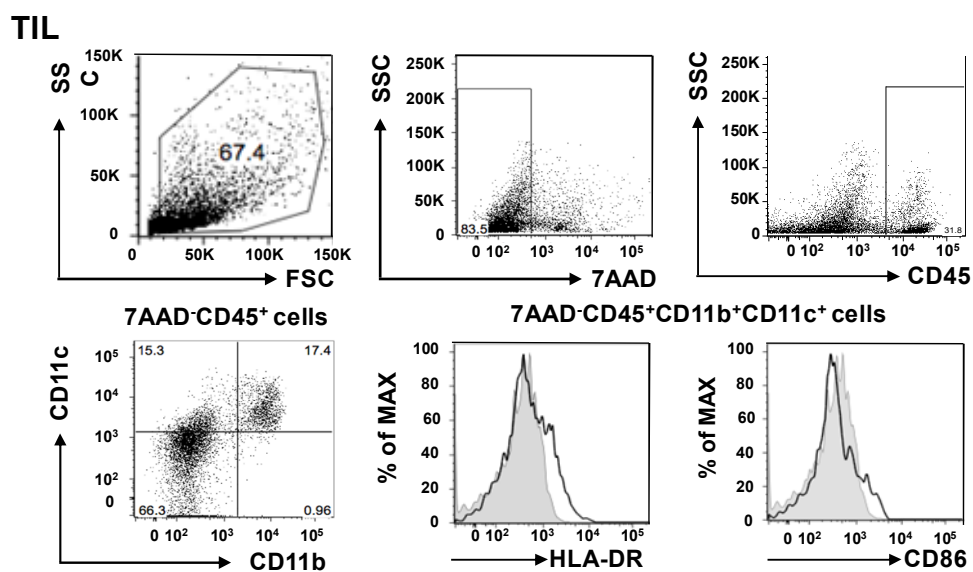
**Supplemental Figure 1. Ohno, Y. et al.****Supplemental Figure 1. Induction of cancer-related antigen-specific CD4 $^+$  T cells.**

Survivin-, or STEAP-specific CD4 $^+$  T cells were induced from the PBMCs of healthy donors in the presence of antigen-derived peptides. Induced cells ( $5 \times 10^4$ ) were stimulated with MoDCs ( $5 \times 10^3$ ) in the presence of antigen peptides or control peptides for 24 h. IFN- $\gamma$  levels were evaluated by flow cytometry. Representative scattergrams are shown.

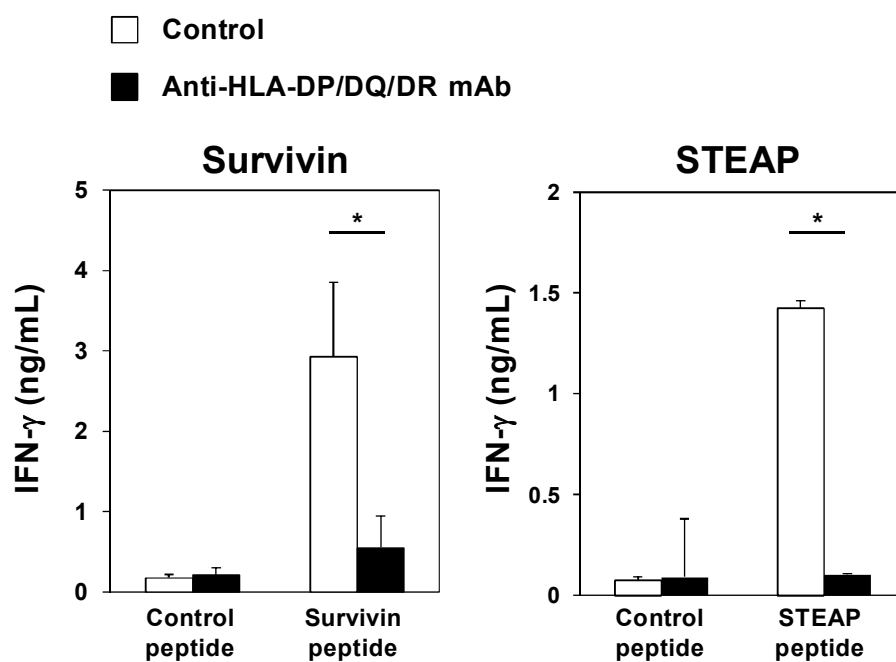
## Supplemental Figure 2. Ohno, Y. et al.

A  
PBMC

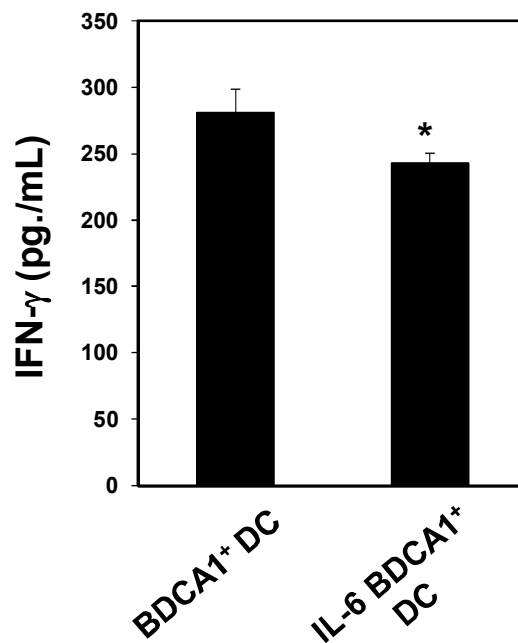
## B

**Supplemental Figure 2. Characteristic features of CD11b<sup>+</sup>CD11c<sup>+</sup> cells in PBMCs and TILs.**

PBMCs and TILs were obtained from colorectal cancer specimens. Collected cells were identified as live singlets based on side scatter, and 7-AAD and CD45 staining. 7AAD-CD45<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> singlets in PBMCs (A) and TILs (B) were indicated. Expression levels of HLA-DR and CD86 were evaluated for the cells from PBMCs and TILs. Representative profiles are presented.

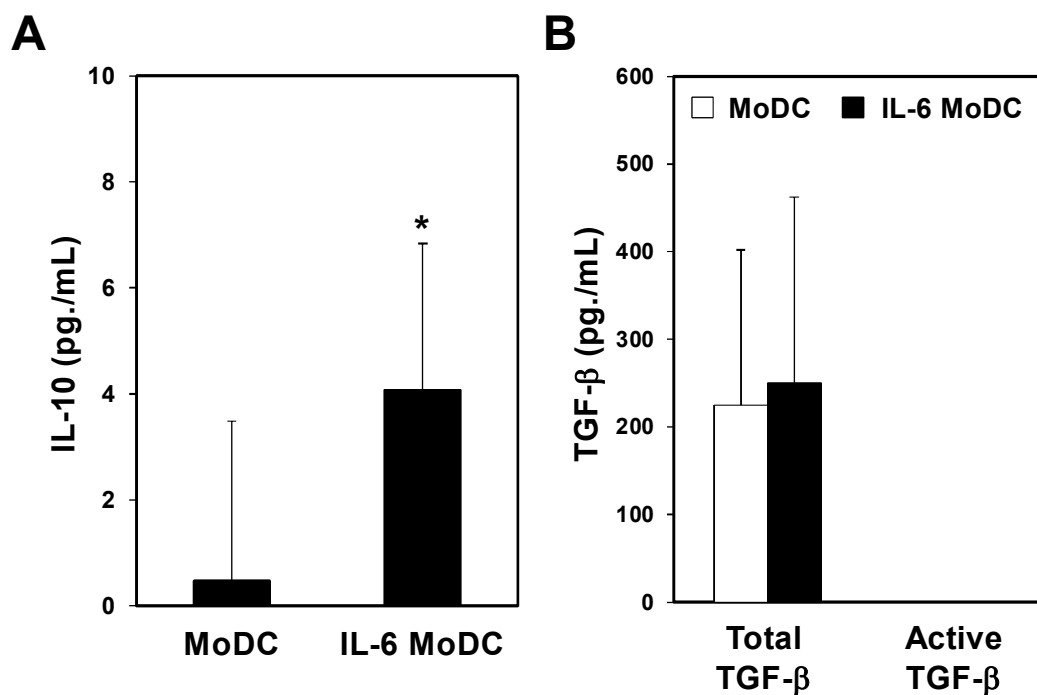
**Supplemental Figure 3. Ohno, Y. et al.****Supplemental Figure 3. HLA class II-dependent IFN- $\gamma$  production by antigen-specific CD4<sup>+</sup> T cells.**

Survivin- or STEAP-specific CD4<sup>+</sup> T cells ( $5 \times 10^4$ ) induced from PBMCs of healthy donors were stimulated with MoDCs ( $5 \times 10^3$ ), and then treated with the corresponding antigen peptides or control peptides in the presence of a control antibody or anti-HLA-DP/DQ/DR mAbs for 24 h. IFN- $\gamma$  levels were evaluated by ELISA. The mean and SD of representative data are presented.

**Supplemental Figure 4. Ohno, Y. et al.****Supplemental Figure 4. IFN- $\gamma$  production by CD4<sup>+</sup> T cells cocultured with BDCA1<sup>+</sup> DCs.**

BDCA1<sup>+</sup> DCs were isolated from PBMCs of healthy donors. Purities of the isolated BDCA1<sup>+</sup> DCs were more than 95 %. BDCA1<sup>+</sup> DCs were treated with IL-6 (50 ng/mL) for 24 h. CD4<sup>+</sup> T cells were isolated from autologous PBMCs of healthy donors. Purities of the isolated CD4<sup>+</sup> T cells were more than 95 %. CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were stimulated with BDCA1<sup>+</sup> DCs ( $1 \times 10^4$ ) in the presence of anti-CD3 mAb (2  $\mu$ g/mL) for 24 h. IFN- $\gamma$  levels were evaluated by ELISA. The mean and SD of representative data are presented (n=4).

## Supplemental Figure 5. Ohno, Y. et al.

**Supplemental Figure 5. IL-10 and TGF- $\beta$  productions by IL-6-conditioned MoDCs.**

MoDCs ( $4 \times 10^5$ ) induced from PBMCs of healthy donors were stimulated with or without IL-6 (50 ng/mL) for 24 h. IL-10 (**A**) and the total or active forms of TGF- $\beta$  (**B**) levels in the culture supernatants were evaluated by ELISA. The mean and SD of representative data are presented.

### Supplemental Table 1. Ohno, Y. et al.

Supplemental Table 1. Patient information for those enrolled in this study.

Total #	Age y.o. (median)	Sex	Stage	TMN (UICC ver.7)		
27	39-83 (64)	Male :14 Female:13	Stage I : 1	T1 : 1	N0 : 15	M0 : 21
			Stage II : 12	T2 : 2	N1 : 3	M1 : 6
			Stage III : 4	T3 : 12	N2 : 3	
			Stage IV : 3	T4 : 6		