学　位　論　文

Studies on functional regulation of human dendritic cells by IL-6/STAT3 signaling pathway and the effects of anti-tumor immunity in a tumor microenvironment

(IL-6/STAT3 シグナル経路によるヒト樹状細胞の機能制御と抗腫瘍免疫への影響に関する研究)

2015 年 3 月

北海道大学

大野　陽介

Table of contents

List of Publications

1. Yosuke Ohno, Hidemitsu Kitamura, Norihiko Takahashi, Junya Ohtake, Shun Kaneumi, Kentaro Sumida, Shigenori Homma, Hideki Kawamura, Nozomi Minagawa, Susumu Shibasaki, and Akinobu Taketomi.

   IL-6 down-regulates HLA class II expression and IL-12 production of human dendritic cells to impair activation of antigen-specific $CD4^+$ T cells. Cancer Immunology, Immunotherapy, Submitted, 2014.

2. Kentaro Sumida, Yosuke Ohno, Junya Ohtake, Shun Kaneumi, Takuto Kishikawa, Norihiko Takahashi, Akinobu Taketomi, and Hidemitsu Kitamura.

   IL-11 induces differentiation of myeloid-derived suppressor cells through activation of STAT3-signlaing pathway. Cancer Research, Submitted, 2014.

3. Junya Ohtake, Yosuke Ohno, Shun Kaneumi, Takuto Kishikawa, Kentaro Sumida, Norihiko Takahashi, Akinobu Taketomi, and Hidemitsu Kitamura

   miR-20b and miR-224 regulate cytokine production by T cell immune responses. Cellular Immunology, Submitted, 2014.

List of Presentations

1. 大野陽介, 大竹淳也, 高橋典彦, 北村秀光, 西村孝司, 武冨紹信

   革新的癌ワクチン H/K-HELP ロングペプチドによる癌特異的 T 細胞免疫応答の増強機構

   第 113 回日本外科学会定期学術集会　2013 年 4 月 10～12 日　福岡

2. 大野陽介, 角田健太郎, 本間重紀, 川村秀樹, 高橋典彦, 北村秀光, 武冨紹信

大腸癌微小環境下における Tumor associated macrophages の機能解析

第 114 回日本外科学会定期学術集会　2014 年 4 月 3～5 日　京都

3. Yosuke Ono, Junya Ohtake, Shun Kaneumi, Kazutaka Masuko, Kentaro Sumida, Takuto Kishikawa, Satoshi Terada, Toshiyuki Kita, Norihiko Takahashi, Akinobu Taketomi, Hidemitsu Kitamura.

IL-6/STAT3-dependent immunosuppressive function of tumor infiltrating dendritic cells in colorectal cancer.

105th AACR Annual Meeting 2014, April 5-9, 2014, San Diego, CA, USA

4. 大野陽介, 大竹淳矢, 金海俊, 岸川拓斗, 寺田聖, 角田健太郎, 喜多俊行, 高橋典彦, 武富紹信, 北村秀光

IL-6/STAT3 シグナル経路による樹状細胞の機能制御とがん免疫治療への応用

第 79 回日本インターフェロン・サイトカイン学会学術集会　2014 年 6 月 19～20 日　札幌

5. Yosuke Ono, Junya Ohtake, Shun Kaneumi, Takuto Kishikawa, Satoshi Terada, Kentaro Sumida, Toshiyuki Kita, Hidemitsu Kitamura, Norihiko Takahashi, Akinobu Taketomi

IL-6 impairs ability of dendritic cells for inducing Th1 cells to tumor microenvironment.

第 73 回日本癌学会学術総会　2014 年 9 月 25～27 日　横浜

6. 大野陽介, 北村秀光, 大竹淳也, 金海俊, 吉田雅, 柴崎晋, 本間重紀, 川村秀樹, 高橋典彦, 武冨紹信

IL-6 シグナル経路による樹状細胞の機能制御と癌細胞の免疫逃避

第 25 回日本消化器癌発生学会　2014 年 11 月 13〜14 日　福岡

7. Yosuke Ono, Hidemitsu Kitamura, Junya Ohtake, Shun Kaneumi, Kentaro Sumida, Shigenori Homma, Hideki Kawamura, Norihiko Takahashi, Akinobu Taketomi.

IL-6/STAT3 signaling impaired induction of cancer-antigen specific T cells via dendritic cells in tumor microenvironment.

ASCO Gastrointestinal Cancers Symposium 2015, January 15-17, 2015, San Francisco, CA, USA

Introduction

Since the discovery of cancer-related antigens in the 1990s[1], cancer immunotherapy has developed as no side-effect therapy based on cancer-specific activation of the host immune system[2]. To date, several clinical trials of cancer peptide vaccine therapies and cell therapies have been conducted for the treatment of cancer patients. Although some trials reported clinical efficacy[3,4], cancer immunotherapy is not a standard therapy for cancer patients. For the development of more effective cancer immunotherapies, it is important to overcome dysfunctional immune responses in the tumor microenvironment. Various immunosuppressive cytokines, such as IL-10 and TGF-β, are produced at high levels in tumor-bearing tissues, and block the function of antitumor effector T cells[5]. In addition, some immune suppressive cells such as $Foxp3^{+}CD4^{+}$ regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), induced from immature myeloid cells in tumor microenvironments, are well known to block antitumor immunity[5-7] **(Figure 1)**.

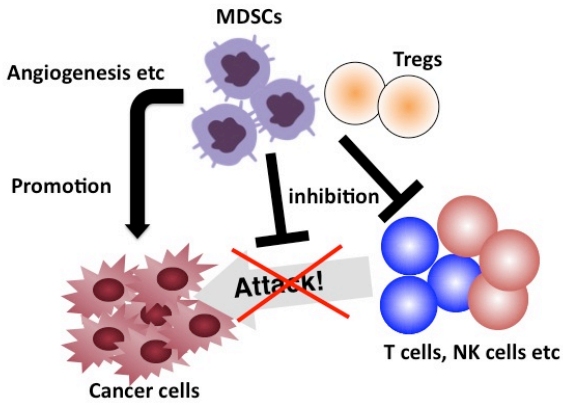

Figure 1. Schema of immunosuppression in the tumor microenvironment.

Recently, effective immuno-check point therapy using anti-PD-1, PD-L1, and/or CTLA-4 antibodies to activate effector T cells in cancer patients was reported in various solid tumors[8-10]. These results indicate that cancer antigen-specific T cells, which eliminate cancer cells, potentially exist in tumor microenvironments. As a result, blocking of negative signals to tumor-infiltrating T cells can restore the cytotoxic function against the target cancer cells. Thus, introduction of cancer antigen-specific T cells to the tumor microenvironment is required for the first step towards more effective cancer immunotherapy.

Dendritic cells (DCs), representative antigen-presenting cells, strongly induce antigen-specific immune responses through activation of $CD4^+$ T and $CD8^+$ 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 Th cells and cytotoxic T lymphocytes (CTLs) through T cell-DC interaction[11-14]. Therefore, proper regulation of DC function in tumor-bearing hosts is important for inducing anti-tumor immunity.

IL-6, a pleiotropic cytokine with a variety of effects on cells and tissues, is produced by many different cells including immune cells, fibroblasts, endothelial cells, and tumor cells[15,16]. IL-6 first binds to the IL-6 receptor (IL-6R); this IL-6/IL-6R complex then associates with the signal-transducing membrane protein, gp130, inducing its dimerization to initiate IL-6 signaling. Gp130 dimerization is followed by the rapid activation of the Janus Kinase (JAK) family and several signaling pathways, including PI3K/ERK/MAPK and STAT3. STAT3 activation induces numerous effector genes

involved in cell proliferation, differentiation and survival.

Previously, it was reported that IL-6 signaling suppresses MHC class II expression on murine DCs via STAT3 activation and attenuates $CD4^+$ T cell-mediated immune responses[17,18] **(Figure 2)**.

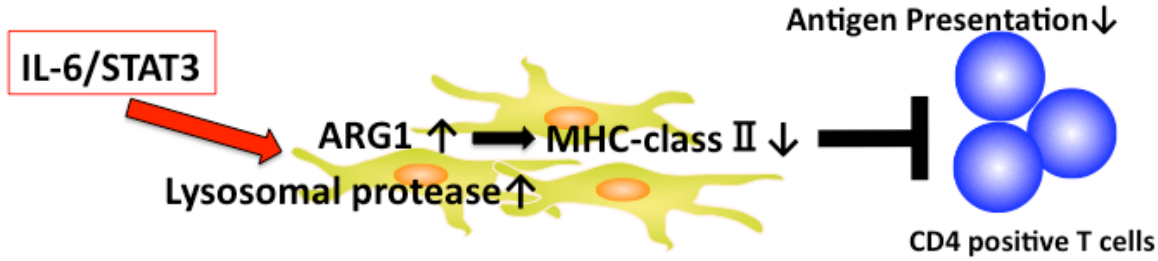

Figure 2. IL-6/STAT3 signaling attenuates the antigen-presenting function of murine DCs via arginase or lysosomal protease activities.

Furthermore, the previous study indicated that administration of monoclonal antibody antagonizing IL-6R (anti-IL-6R mAb) enhanced T cell responses and inhibited tumor growth *in vivo*[19]. In tumor-bearing mice, IL-6 suppressed $CD4^+$ T cell-mediated immunity through down-regulation of MHC class II by enhanced arginase activity of DCs[20]. Thus, IL-6-mediated STAT3 activation appears to be a critical mechanism for inducing dysfunctional immune system responses in the tumor microenvironment via regulation of antigen-presenting cells. Blockade of IL-6/STAT3 signaling cascades may therefore be a promising approach to overcome the dysfunction of antitumor immunity in cancer patients. However, the precise effects of IL-6 on human cancer immunology are not clear.

Therefore, work in this dissection focused on the IL-6/STAT3-signaling pathway in human DCs, aiming to translate the information obtained by murine studies for clinical benefit in human subjects, and demonstrating the possibility of improving cancer immunotherapy by regulation of the IL-6/STAT3 signaling pathway.

In this study, IL-6/STAT3 signaling attenuated the antigen-presenting ability of human monocyte-derived dendritic cells (MoDCs), and impaired Th1 immunity via reduced IL-12 production. Moreover, IL-6 inhibited maturation of $CD11b^{+}CD11c^{+}$ myeloid cells as antigen-presenting cells, and tumor-infiltrating DCs in human colorectal cancer might be affected by the IL-6/STAT3 signaling pathway, resulting in the impaired T cell stimulating ability. Therefore, proper regulation of the antigen-presenting ability of DCs is important for the activation of effector T cells infiltrated into the tumor microenvironment, thus, the IL-6/STAT3 signaling pathway is a promising target for improving cancer immunotherapy.

Abbreviations

ARG1: arginase 1

COX2: cytochrome c oxidase subunit II

CTL: cytotoxic T lymphocyte

CTSL: cathepsin L

DC: dendritic cell

mAb: monoclonal antibody

MFI: mean fluorescence intensity

MDSC: myeloid derived suppressor cell

MoDC: monocyte derived dendritic cell

PBMC: peripheral blood mononuclear cell

TIL: tumor-infiltrating leukocyte

Treg：regulatory T cell

PMA: paramethoxyamphetamine

Poly I:C : Polyinosinic-polycytidylic acid (poly I:C)

WT: wild-type

Materials and Methods

Antibodies and reagents

APC-conjugated anti-mouse CD4 (RM4-5), PE-Cy7-conjugated anti-mouse CD45 (3D-F11), PE-conjugated anti-mouse IFN-γ (XMG1.2), APC-conjugated anti-human CD11c (3.9), PE-conjugated HLA-DR (G46-6), FITC-conjugated anti-human HLA-A/B/C (G46-2.6), PE-conjugated anti-human CD80 (L307.4), and PE-conjugated anti-human CD86 (2331, FUN-1) mAbs, and PE-conjugated mouse IgG1κ isotype control and FITC-conjugated mouse IgG1κ isotype control were purchased from BD Bioscience (Franklin Lakes, NJ, USA). Control rat IgG was purchased from MP Biomedicals, LLC (Eschwege, Germany). 7-Amino-actinomycin D (7AAD) was purchased from Beckman Coulter (Brea, CA, USA). Recombinant human GM-CSF and human IL-4 was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Recombinant human IL-6 was purchased from Wako Chemical Industries, Ltd. (Osaka, Japan). Specific inhibitors of STAT3 (6-Nitrobenzo[b]thiophene-1,1-dioxide), COX2 (sc-58125), and arginase (N^{w}-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, Ltd. (Osaka, Japan).

Anti-human CD3 mAb (OKT3) was purchased from BioLegend (San Diego, CA, USA). Anti-human IL-12 mAb (C8.6) was purchased from BD Bioscience (Franklin Lakes). Anti-human CD4, CD8 and CD14 microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). AIM-V (Invitrogen, Carlsbad, CA, USA) without serum was used for human cell culture in this study. Phorbol 12- myristate 13-acetate (PMA) and A23187 calcium ionophore were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyinosinic-polycytidylic acid (poly I:C) was purchased from InvivoGen (San Diego, CA, USA).

Animals

Wild-type BALB/c mice were obtained from Charles River Japan (Kanagawa, Japan). BALB/c background IL-$6^{-/-}$ mice were kindly donated by Dr. Y. Iwakura (Tokyo University of Science). The mice were maintained in specific pathogen-free conditions and used at 5-10 weeks of age according to the guidelines of the animal department at Hokkaido University.

Cell line

A murine colorectal cancer cell line, CT26, was obtained from American Type

Culture Collection (Manassas, VA, USA). CT26 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum.

Tumor-bearing mouse model

CT26 cells (1 x 10^6 cells) were intradermally inoculated into wild-type and IL-$6^{-/-}$ mice. Tumor volume was calculated using the following formula: volume (mm^3) = 0.2 × (length [mm] × width [mm]) × (height [mm])^2. Tumors and regional lymph nodes were collected from tumor-bearing mice at day 12. The retrieved tissues were minced using scissors and digested in 1 mg/mL collagenase solution (Sigma, Tokyo, Japan). Cell suspensions were prepared from the tumors and regional lymph nodes and used for experiments.

Intracellular cytokine staining

For detection of cytoplasmic IFN-γ expression of T cells, single cell suspensions from tumors and regional lymph nodes (1 × 10^6 cells in 12-well culture dish) were stimulated with PMA (25 ng/mL) and A23187 calcium ionophore (1 μg/mL) for 4 h in the presence of Brefeldin A. Then, the cells were harvested and stained with anti-CD4

mAb, anti-CD45 mAb, and 7-AAD, and fixed with 4 % paraformaldehyde. After permeabilization, the fixed cells were stained with anti-IFN-γ mAb. Data were acquired by flow cytometry.

Informed consent of healthy volunteers and cancer patients

Research protocols for 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 subject.

Generation of monocyte-derived dendritic cells (MoDCs) and IL-6-conditioned MoDCs

PBMCs were obtained from healthy donors by Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) gradient centrifugation. $CD14^+$ cells, separated by magnetic cell sorting from PBMCs of healthy donors, were seeded in 12-well culture dishes (4×10^5 cells). MoDCs were induced from the $CD14^+$ cells cultured 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 used as IL-6-conditioned DCs..

Induction of cancer antigen-specific Th cells and evaluation of antigen-presenting function for MoDCs

PBMCs (2×10^6 cells) were cultured in the presence of MAGE-A4-derived peptide (ALAETSYVKVLEHVVRVNARVR, 5 μmol/L)[21], survivin-derived peptide (EHKKHSSGCAFLSVKKQFEELTLGEFLKLDRERAKNKIAK, 5 μmol/L)[22], or STEAP-derived peptide (SLLLGTIHALIFAWN, 5 μmol/L and QFVWYTPPTFMIAVF, 5 μmol/L)[23] for 7 days, and restimulated with OK-432 (0.1 KE/ml)-stimulated mitomycin-treated autologous MoDCs (1×10^5 cells) in the presence of each peptide and IL-2 (10 IU/ml) for 7 days. Then, the re-stimulation was carried out and the cells were cultured for another 7 days. At day 21, the induced antigen-specific $CD4^+$ T cells (5 x 10^5 cells) for each antigen were co-cultured with MoDCs or IL-6 MoDCs (5×10^3 cells) in the presence of corresponding antigen (5 μmol/L) for 24 h (96-well U bottom plates), and IFN-γ production in the culture supernatants was determined by ELISA.

Stimulation of MoDCs with Poly I:C

MoDCs and IL-6-conditioned MoDCs were stimulated with Poly I:C (5 μg/mL) for 4 h,

and the gene expression levels of *IL12A* and *IL12B* were determined by PCR. Culture supernatant was collected at 24 h after stimulation, and IL-12 production was measured by ELISA.

Co-culture of MoDCs with $CD4^+$ T cells

Autologous $CD4^+$ T cells were isolated from PBMCs by magnetic cell sorting. $CD4^+$ T cells (1×10^5 cells) were co-cultured with control MoDCs or IL-6-conditioned MoDCs (1×10^4 cells) in the presence of anti-CD3 mAb (2 μg/mL) for 24 h. In some experiments, MoDCs were pretreated with STAT3 inhibitor (5 μg/mL) for 24 h. Neutralizing anti-IL-12 mAb or control antibody (2 μg/mL) was added to the culture. IFN-γ, IL-12, and IL-4 production levels were evaluated by ELISA.

Preparation of adherent cells, evaluation of maturation marker, and co-culture with autologous T cells

PBMCs (2×10^6 cells) obtained from healthy donors were seeded in 12-well plates. After 2 h, non-adherent cells were removed and adherent cells were cultured in the presence of IL-6 (50 ng/mL) for 3 days. The adherent cells were harvested and the expression levels of HLA-A/B/C, HLA-DR, CD80, and CD86 were evaluated by flow

cytometry. In addition, the harvested cells (5×10^5 cells) were co-cultured with autologous T cells, which were sorted from non-adherent cells by magnetic cell isolation in the presence of anti-CD3 antibody for 24 h (96-well U bottom plates), and IFN-γ production in the culture supernatant was determined by ELISA.

Preparation of $CD11b^+CD11c^+$ cells and co-culture with autologous T cells

Tumor-infiltrating leukocytes (TILs) and PBMCs were collected from patients with colorectal cancer. All colorectal cancer tissues were minced using scissors and digested with 1 mg/mL collagenase (Sigma, Tokyo, Japan). Single cell suspensions were obtained by passage through 100 μm nylon cell strainers (BD Bioscience). Surface HLA-DR and CD86 of $7AAD^-CD45^+CD11b^+CD11c^+$ cells from PBMCs and TILs were evaluated by flow cytometry. For PCR analysis and co-culture with T cells, $CD11b^+CD11c^+$ cells were isolated from PBMCs and TILs by fluorescence-activated cell sorting (FACS). Autologous $CD4^+$ T and $CD8^+$ T cells were sorted from PBMCs by magnetic cell isolation before FACS. For T cell proliferation assays, $CD4^+$ T and $CD8^+$ T cells were pre-incubated in CFSE (5 nM) at room temperature for 15 min. After washing with PBS, CFSE-labeled $CD4^+$ T and $CD8^+$ T cells (1×10^5 cells) were co-cultured with $CD11b^+CD11c^+$ cells (5×10^4 cells) from PBMCs or TILs in the

presence of anti-CD3 mAb (OKT3: 2 μg/mL). IFN-γ production levels of the culture supernatants were measured at 24 h by ELISA. Proliferation of $CD4^+$ T and $CD8^+$ T cells was evaluated at 72 h by flow cytometry.

PCR

Total RNAs were extracted from human DCs with an RNeasy Mini kit (Qiagen, Hilden, Germany) and first strand cDNAs were synthesized using 1 μg total RNA template, oligo (dT) (Invitrogen), and Superscript III reverse transcriptase (Invitrogen). Human *IL6*, *COX2*, *CTSL*, *ARG1*, and *GAPDH* were amplified with a thermal cycler (LightCycler, Roche, Indianapolis, IN). The primer sequences used in this study are listed in **Table 1**. Sample signals were normalized to the *GAPDH* housekeeping gene according to the $^{\Delta\Delta}Ct$ method: $^{\Delta}Ct = {}^{\Delta}Ct_{sample} - {}^{\Delta}Ct_{reference}$. Percentages relative to control samples were calculated for each sample.

Table 1. PCR primer sequences.

Gene name	Forward	Reverse	Probe (#)
IL12A	5'-cactcccaaaacctgctgag-3'	5'-caatctcttcagaagtgcaagg-3'	#50
IL12B	5'-ccctgacattctgcgttca-3'	5'-aggtcttgtccgtgaagactcta-3'	#37
IL6	5'-caggagcccagctatgaact-3'	5'-gaaggcagcaggcaacac-3'	#7
COX2	5'-cttcacgcatcagtttttcaag-3'	5'-tcaccgtaaatatgatttaagtccac-3'	#23
CTSL	5'-gcaaggatgagtgtaggattca-3'	5'-gggagggcagttgaggac-3'	#75
ARG1	5'-tggcagaagtcaagaagaacg-3'	5'-atgcttccaattgccaaact-3'	#64
GAPDH	5'-agccacatcgctcagacac-3'	5'-gcccaatacgaccaaatcc-3'	#60

Flow cytometry

FACSCanto II (BD Bioscience) was used for flow cytometry and the results were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). MFI ratio (Sample MFI / control sample MFI × 100) was calculated in paired samples. FACSAriaTM II (BD Bioscience) was used for isolation of $CD11b^{+}CD11c^{+}$ cells from PBMCs and TILs of colorectal cancer patients.

ELISA

IL-2, IFN-γ, IL-4, and IL-12 levels in culture supernatants were measured by OptEIA human IL-2, IFN-γ, IL-4, and IL-12 ELISA kits (BD Bioscience), respectively, according to the manufacturer's instructions.

Immunohistochemistry (IHC)

Normal and cancer tissues were obtained from colorectal surgical specimens. Colon specimens were fixed in formalin and embedded in paraffin. After deparaffinization, antigen retrieval was performed by boiling for 10 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide for 15 min. After pre-incubation with Blocking I (Nacalai Tesque, Kyoto, Japan) for 10 min to block non-specific interaction of primary and secondary antibodies, slides were treated with mouse anti-human HLA-DR α-chain, (clone TAL.1B5, DAKO), mouse anti-human CD4 (clone 4B12, Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK), mouse anti-human CD8 (clone 1A5, Novocastra Laboratories Ltd), rabbit anti-human IL-6 (polyclonal, Rockland Immunochemicals, Inc, Gilbertsville, PA, USA), or phospho-STAT3 (clone D3A7, Cell Signaling Technology, Inc, Danvers, MA, USA) antibodies over night at 4 °C. All sections were incubated for 30 min with peroxidase labeled anti-mouse IgG polyclonal antibody (Nichirei Bioscience, Inc., Tokyo, Japan)

or peroxidase labeled goat anti-rabbit immunoglobulin antibody (Dako) as secondary antibody. Protein expression was visualized using 3-3'-diaminobezidine-4HCL (DAB). Finally, sections were counterstained with Mayer's hematoxylin.

Statistical analysis

Statistical significance was evaluated by the paired t-test for paired data, and *P* values of < 0.05 were considered statistically significant.

Results

IL-6 produced in tumor-bearing mice significantly attenuates Th1 immunity and promotes tumor growth

Previous reports demonstrated that IL-6 attenuated the antigen-presenting ability of murine DCs via reduction of MHC-class II[17,18,20]. In this study, the relationship of IL-6 deficiency with tumor growth *in vivo* was first investigated. Murine CT26 colorectal cancer cells were intradermally inoculated into wild-type (WT) or IL6$^{-/-}$mice. Tumor growth was significantly reduced in IL6$^{-/-}$ mice **(Figure 3)**.

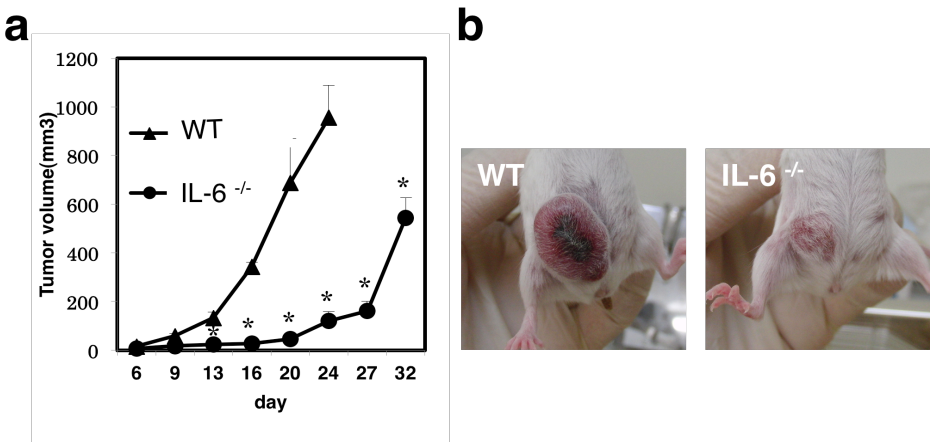

Figure 3. CT26-derived tumor growth was significantly inhibited by lack of IL-6 in the tumor microenvironment. CT26 murine colon cancer cells were intradermally inoculated into wild-type (WT) or IL6$^{-/-}$ BALB/c mice. Tumor size was monitored for 32 days after inoculation. (a) The means and SDs of tumor volume are indicated (n=3). (* P<0.05 *vs.* WT mice) (b) Representative images of tumors at day 15 are shown.

To investigate the immune status of tumor-bearing WT or IL-$6^{-/-}$ mice, immune cells were collected from tumor tissues at day 12 and the proportion of tumor-infiltrating T cells was evaluated. $CD45^{+}$ immune cells were more highly infiltrated into the tumor tissues of IL-$6^{-/-}$ mice compared with WT mice. In addition, $CD4^{+}$ T and $CD8^{+}$ T cells were confirmed to be more highly infiltrated into the tumor microenvironment of IL6-deficient mice **(Figure 4a and b)**.

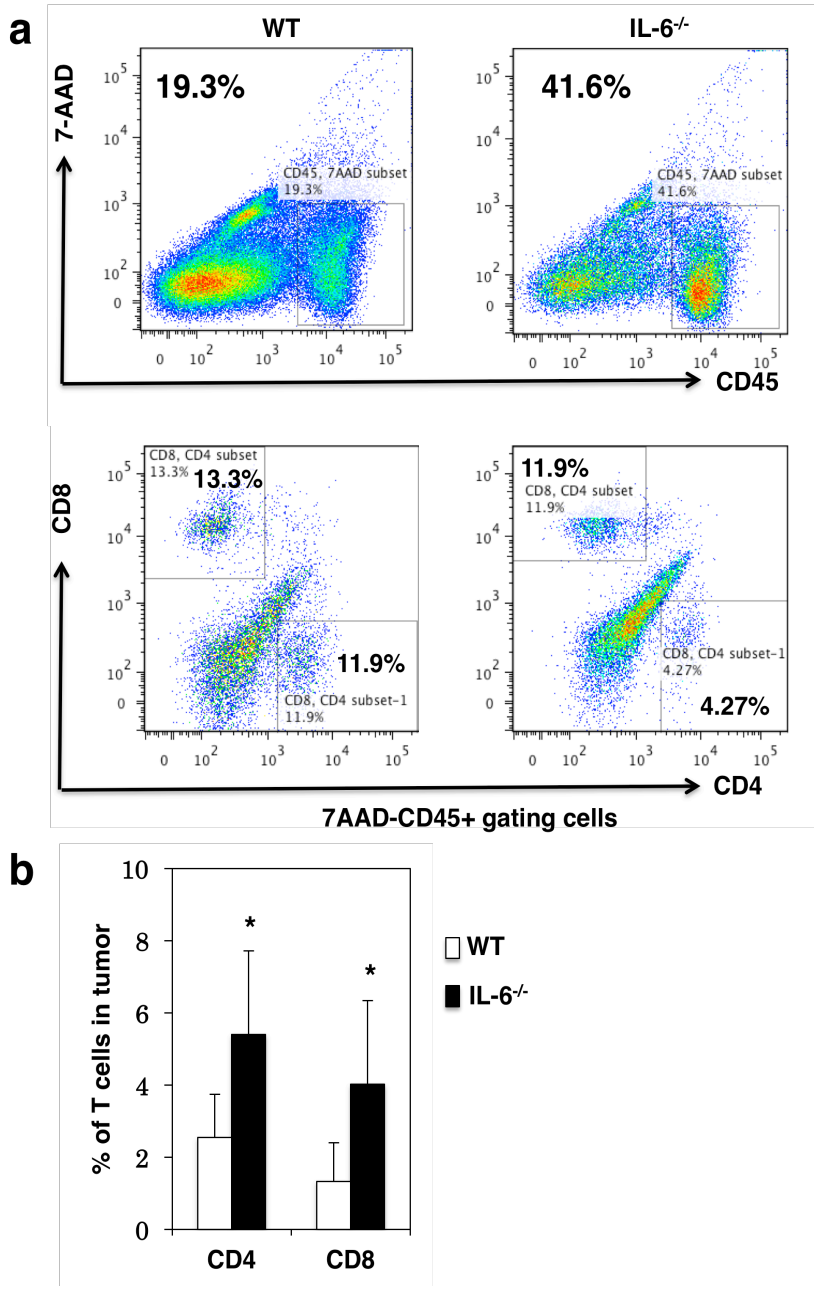

Figure 4. CD4$^+$ T and CD8$^+$ T cells were highly infiltrated in the tumor microenvironment of IL-6$^{-/-}$ mice compared with WT mice. CD45$^+$, CD4$^+$ or CD8$^+$ cells in tumor tissues of WT or IL-6$^{-/-}$ mice were evaluated by flow cytometry. Percentages of CD4$^+$ T and CD8$^+$ T cells were calculated using the following formula: (percentage of 7AAD$^-$CD45$^+$ cells) × (percentage of CD4$^+$ or CD8$^+$ cells). (a) Representative profiles are shown. (b) Means and SDs are indicated (n=5) (* P<0.05 *vs*. WT mice).

To confirm Th1 cytokine production by $CD4^+$ T cells in the tumor-bearing mice, cells from the tumor tissues and regional lymph nodes were collected and stimulated with PMA (25 ng/mL) and A23187 calcium ionophore (1 μg/mL) for 4 h. IFN-γ production by $CD4^+$ T cells was analyzed by flow cytometry. As a result, higher numbers of IFN-γ-producing $CD4^+$ T cells were present in both the tumor tissues and regional lymph nodes of IL-$6^{-/-}$ mice compared with WT mice **(Figure 5a and 5b)**.

These data indicated that IL-6 attenuated Th1 immunity in the tumor-bearing host, suggesting that IL-6 might be a therapeutic target to overcome immuno-suppression in cancer patients.

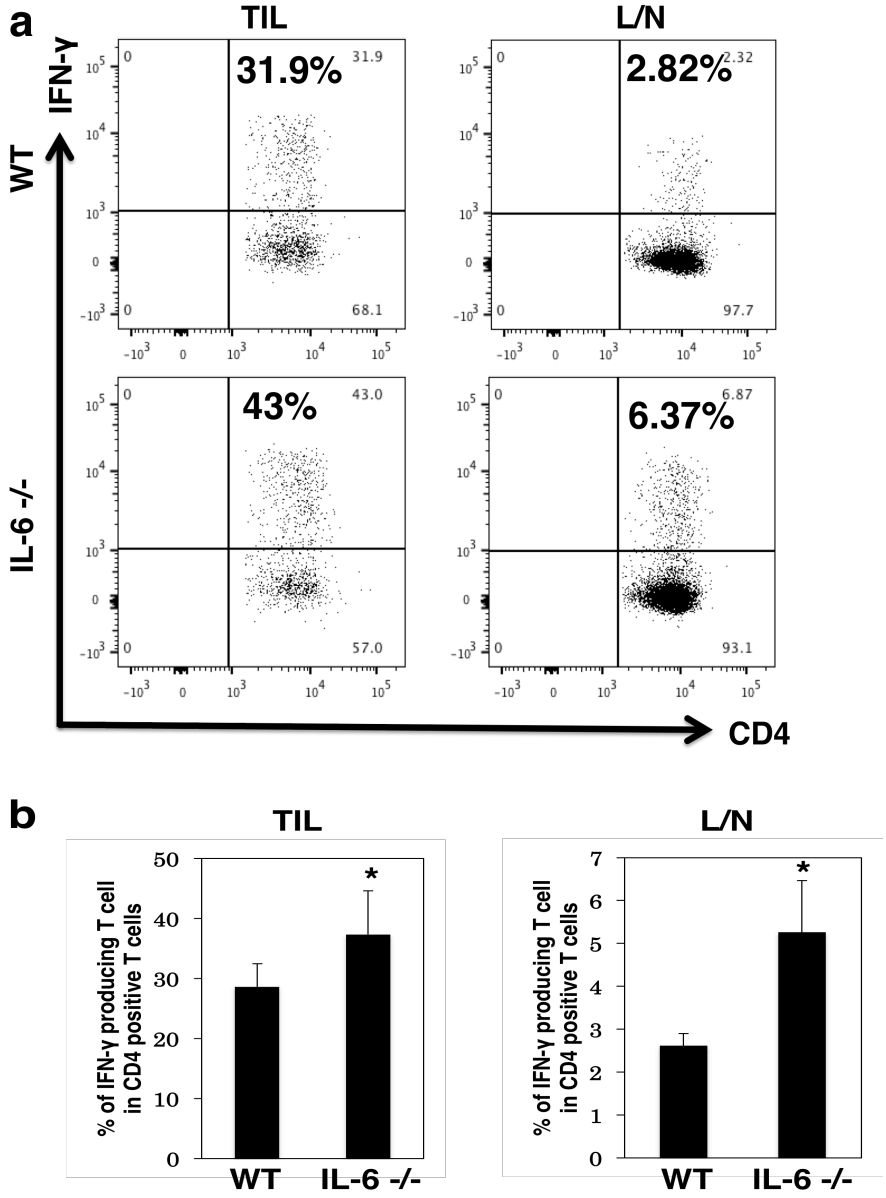

Figure 5. IL-6 suppressed Th1 immunity in the tumor tissues and regional lymph nodes of CT26 tumor-bearing mice. Percentages of IFN-γ producing $CD4^+$ T cells in tumor tissues (TIL) and regional lymph nodes (L/N) of WT or IL-$6^{-/-}$ mice were determined by flow cytometry. (a) Representative profiles are shown. (b) Means and SDs are indicated. (n=5) (* $P<0.05$ *vs.* WT mice).

IL-6 attenuates activation of cancer-related antigen-specific $CD4^+$ T cells by human MoDCs

Next, an investigation of whether IL-6 influenced function of human DCs and T cells was performed. Human MoDCs were induced from the monocytes of healthy donors in the presence or absence of IL-6. Then, the effect of IL-6 on the antigen-presenting ability of MoDCs was evaluated. Cancer-related antigen, MAGE-A4-, survivin-, or STEAP-specific $CD4^+$ T cells were induced by PBMCs of healthy donors and co-cultured with autologous MoDCs or IL-6-conditioned MoDCs (IL-6 MoDCs) in the presence of each antigen peptide. IFN-γ production levels were measured as a parameter of the antigen-presenting ability of MoDCs to activate effector Th cells. We found that IFN-γ production by MAGE-A4-, survivin-, and STEAP-specific $CD4^+$ T cells was reduced under co-culture with IL-6 MoDCs compared with control MoDCs (**Figure 6**).

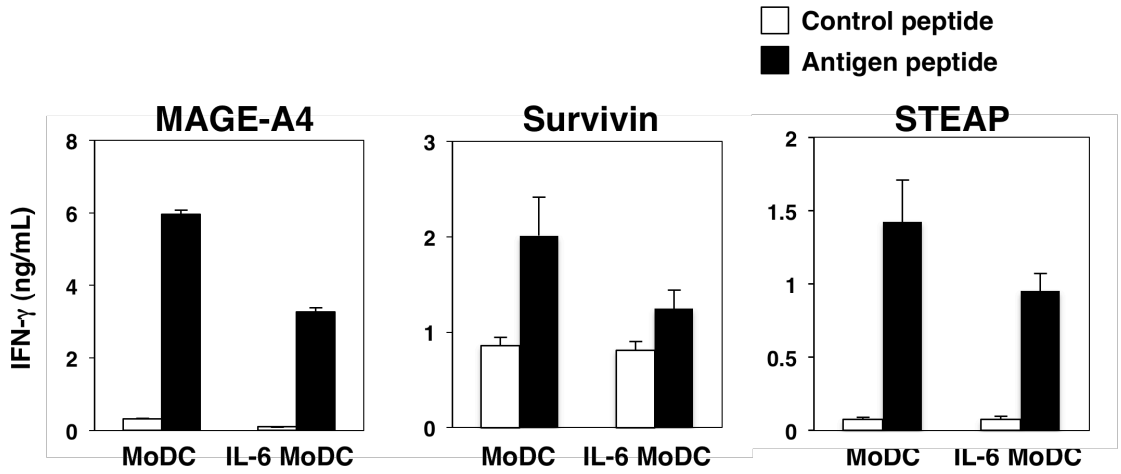

Figure 6. IL-6 reduced IFN-γ production by cancer related antigen-specific $CD4^+$ T cells after the stimulation with human MoDCs.

MAGE-A4-, survivin-, or STEAP-antigen-specific T cells (5×10^4 cells) were co-cultured with MoDCs or IL-6 MoDCs (5×10^3 cells) in the presence of control peptide or the corresponding antigen peptide for 24 h. IFN-γ production in the supernatant was determined by ELISA. Means and SDs are indicated.

In this study, cancer-related antigen-specific T cells were induced under Th1 stimulation as reported previously[21,22]. Data in this study confirmed IFN-γ production by the antigen-specific $CD4^+$ T cells was inhibited in the presence of anti-HLA-DP/DQ/DR mAbs (**Figure 7**).

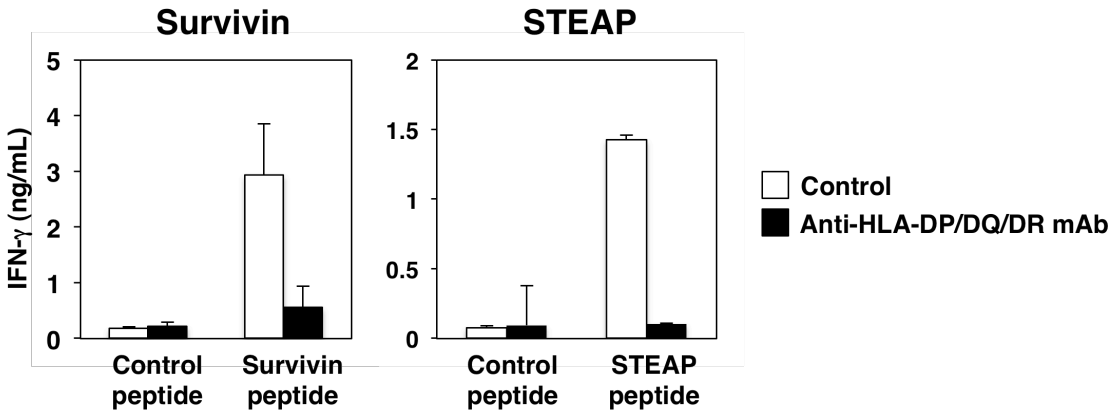

Figure 7. IFN-γ production by antigen-specific $CD4^+$ T cells was dependent on HLA-class II. Survivin- or STEAP-antigen-specific T cells (5×10^4 cells) were co-cultured with MoDCs or IL-6 MoDCs (5×10^3 cells) in the presence of each antigen peptide for 24 h. Control or anti-HLA-DP/DQ/DR mAbs were added to the co-culture. IFN-γ production in the culture supernatant was determined by ELISA. Means and SDs are indicated.

These data indicated that IFN-γ was produced from the antigen-specific Th cells in a HLA-class II-dependent manner. These findings suggest that IL-6 attenuates the antigen-presenting ability of MoDCs via MHC-class II and the subsequent activation of effector Th1 cells.

IL-6 attenuates Th1 immunity by the reduction of IL-12 production by MoDCs.

It is well known that Th1 immunity is essential for anti-cancer immunity. In addition, IL-12 is a very important cytokine for inducing Th1 immunity. Therefore, further investigations were pursued to determine whether IL-6 influenced IL-12 production by

MoDCs. *IL12A* and *IL12B* mRNA expression levels and cytokine production of IL-12 were evaluated after poly I:C stimulation. The results revealed that *IL-12* gene induction and protein levels were decreased in IL-6 MoDCs compared with the control MoDCs **(Figure 8a and 8b)**.

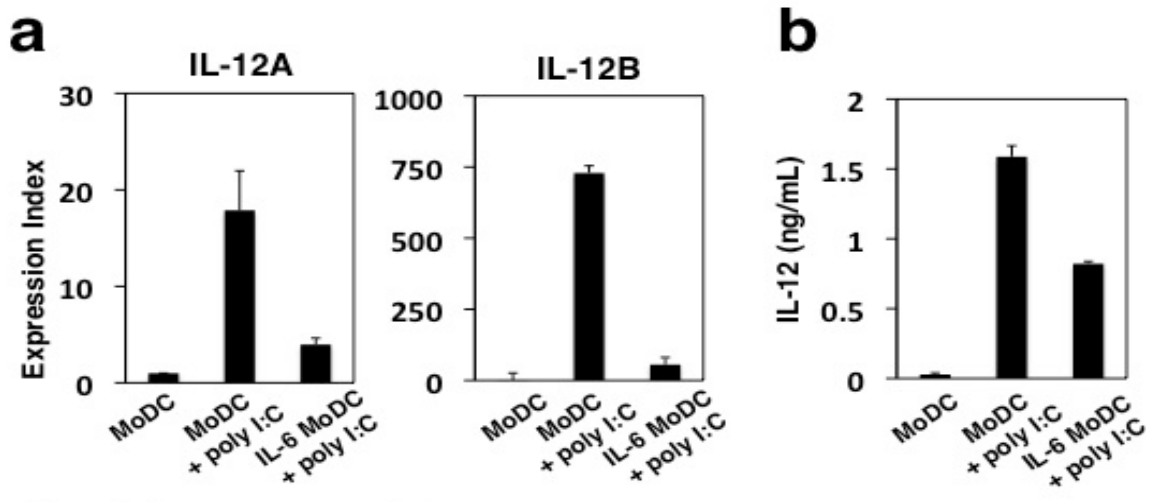

Figure 8. IL-6 inhibited IL-12 production by MoDCs stimulated with poly I:C. MoDCs were generated from PBMCs of healthy donors in the presence of IL-4 plus GM-CSF with or without IL-6 for 7 days. MoDCs and IL-6 MoDCs were stimulated with poly I:C (5 μg/mL) for 4 h, and *IL12A* and *IL12B* gene expression was measured by quantitative PCR. (b) MoDCs and IL-6 MoDCs were stimulated with poly I:C (5 μg/mL) for 24 h and IL-12 levels was measured by ELISA. Means and SDs are indicated.

Next, experiments were performed to assess whether IL-6-conditioned MoDCs, which decreased IL-12 production after polyI:C stimulation, attenuated induction of Th1 immunity. $CD4^+$ T cells isolated from healthy donors were co-cultured with autologous MoDCs or IL-6 MoDCs in the presence of agonistic anti-CD3 mAb. IFN-γ production levels from $CD4^+$ T cells co-cultured with IL-6 MoDCs were significantly

lower compared with WT MoDCs. In addition, IL-12 levels in the culture supernatant were significantly reduced in the case of IL-6 MoDCs. IL-4 production by $CD4^+$ T cells co-cultured with IL-6 MoDCs was enhanced compared with MoDCs.

These experiments confirmed that IFN-γ production by $CD4^+$ T cells co-cultured with MoDCs was decreased to a level equivalent to that of the IL-6 MoDCs in the presence of neutralizing anti-IL-12 mAb. IL-4 production was increased by the addition of anti-IL-12 mAb **(Figure 9a)**. Furthermore, STAT3 inhibitor recovered the IL-12-producing ability of IL-6 MoDCs and IFN-γ production by $CD4^+$ T cells **(Figure 9b)**. These data suggest that IL-6/STAT3 signaling pathway attenuates induction of Th1 cells through the decreasing IL-12 production by DCs.

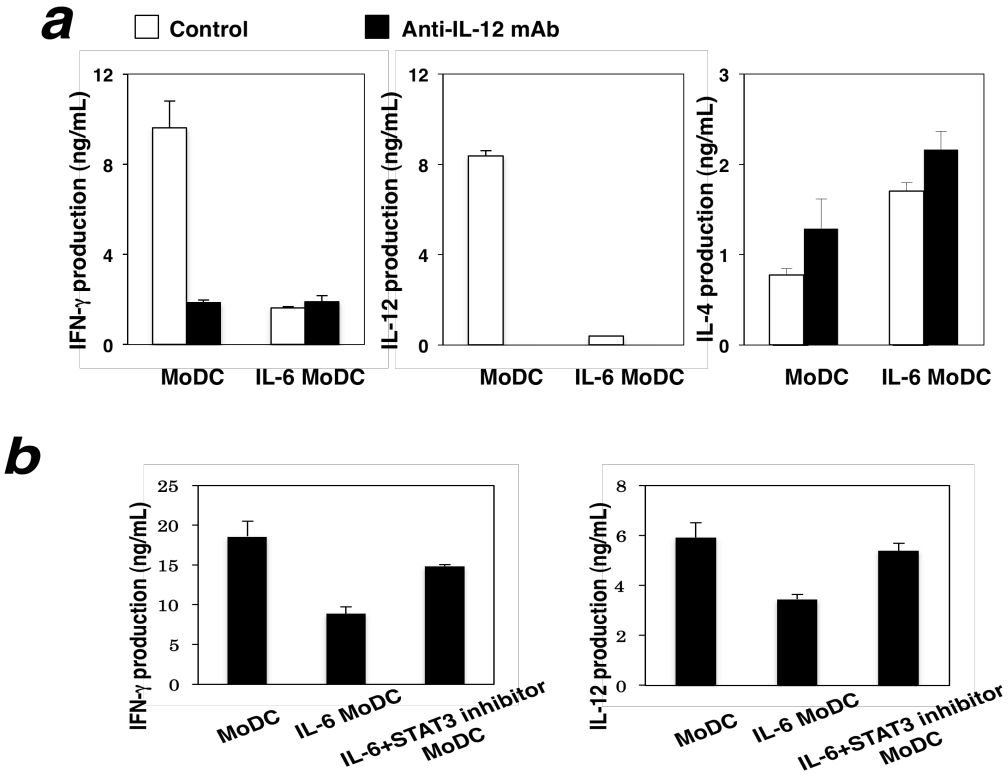

Figure 9. IL-6 attenuated Th1 immunity through reduced IL-12 production by MoDCs in a STAT3-dependent manner. MoDCs were generated from PBMCs of healthy donors in the presence of IL-4 plus GM-CSF with or without IL-6 for 7 days. (**a**) $CD4^+$ T cells (1×10^5 cells) were co-cultured with control MoDCs or IL-6 MoDCs (1×10^4 cells) in the presence of anti-CD3 mAb (2 μg/mL) for 24 h. Neutralizing anti-IL-12 mAb or control antibody (2 μg/mL) was added to the culture. IFN-γ, IL-12, and IL-4 levels were evaluated by ELISA. The means and SEs of representative data are indicated. (**b**) $CD4^+$ T cells (1×10^5 cells) were co-cultured with control MoDCs, IL-6 MoDCs or IL-6 MoDCs (1×10^4 cells) treated with STAT3 inhibitor in the presence of anti-CD3 mAb (2 μg/mL) for 24 h. IFN-γ and IL-12 levels were evaluated by ELISA. The means and SDs are indicated.

IL-6-mediated STAT3 activation inhibits maturation of human $CD11b^+C11c^+$ myeloid cells as antigen-presenting cells.

Previous reports indicated that IL-6 inhibited differentiation of murine DCs[17,18,20]. Therefore, experiments were undertaken to identify whether IL-6 influenced the functional maturation of human myeloid cells into professional antigen-presenting cells. We examined the surface expression levels of HLA class I, HLA class II, and co-stimulatory molecules as maturation markers of antigen-presenting cells. $CD11b^+CD11c^+$ adherent cells from PBMCs of healthy donors were cultured in the presence of IL-6. HLA-DR and CD86 expression on $CD11b^+CD11c^+$ cells were significantly reduced by IL-6 treatment, whereas HLA-A/B/C and CD80 were not altered. In addition, the reduction in HLA-DR and CD86 expression levels was blocked by the addition of STAT3 inhibitor (**Figure 10a and 10b**).

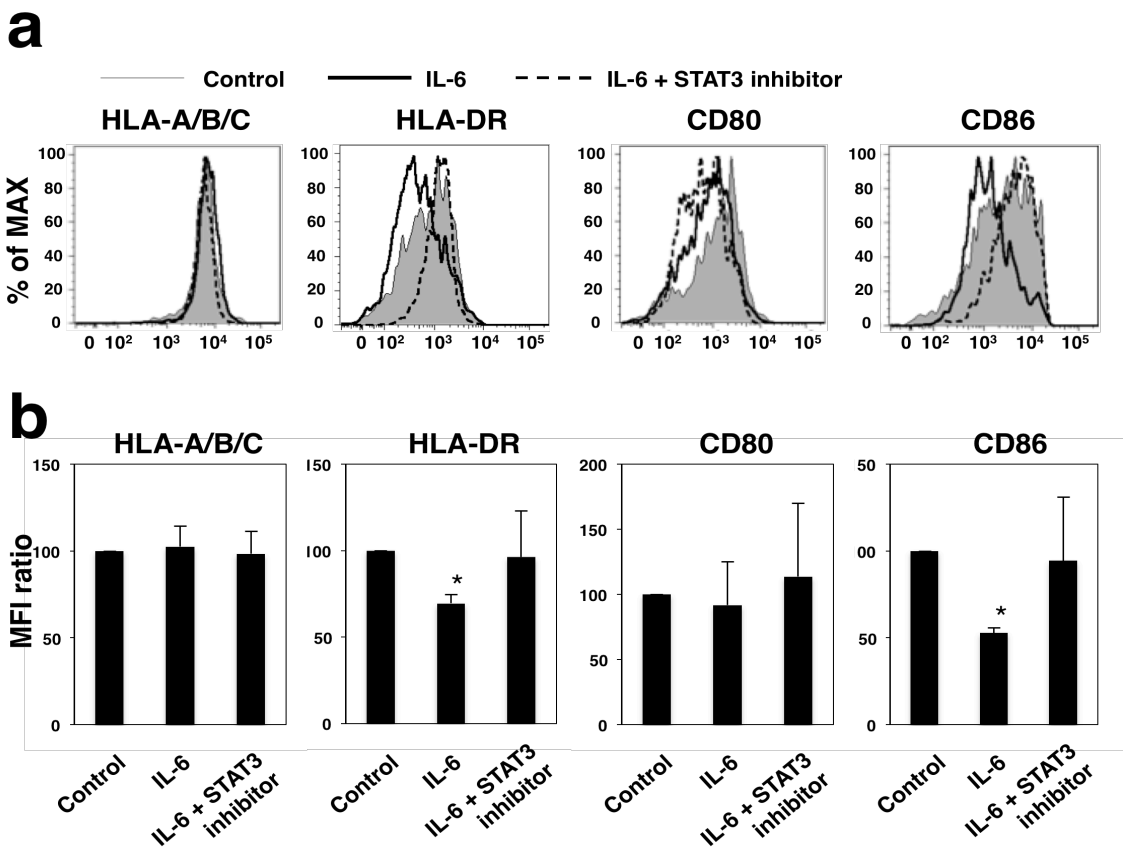

Figure 10. IL-6 reduced HLA class II and CD86 expression levels of $CD11b^+CD11c^+$ cells in a STAT3-dependent manner. Adherent cells were collected from PBMCs of healthy donors and cultured in the presence of IL-6 with or without STAT3 inhibitor for 72 h. Surface expression of $CD11b^+CD11c^+$ cells was determined by flow cytometry. (**a**) Representative profiles are shown. (**b**) MFI ratio was calculated and the mean and SD are indicated (n=5) (* $P<0.05$ *vs.* control).

We further investigated the T cell-stimulating ability of the IL-6-treated $CD11b^+CD11c^+$ cells in the presence of ant-CD3 mAb. IFN-γ and IL-2 levels in T cells co-cultured with IL-6-treated $CD11b^+CD11c^+$ cells were decreased compared with control $CD11b^+CD11c^+$ cells. These findings suggested that IL-6/STAT3 signaling in

$CD11b^+CD11c^+$ cells not only inhibited the maturation of human myeloid cells, but also attenuated their T cell-stimulating ability (**Figure 11**).

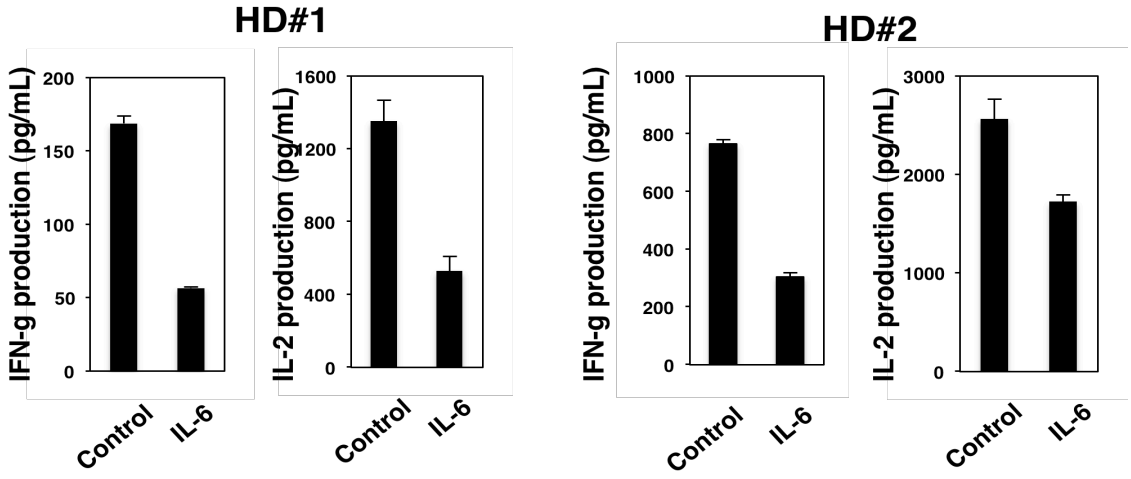

Figure 11. IL-6-treated $CD11b^+CD11c^+$ cells had attenuated T cell-stimulating ability. T cells isolated from PBMCs of healthy donors (1 x 10^5 cells) were co-cultured with antologous control $CD11b^+CD11c^+$ cells or IL-6-treated $CD11b^+CD11c^+$ cells (5 x 10^4 cells) for 24 h. IFN-γ and IL-2 levels in the culture supernatant were measured by ELISA.

COX2, lysosome protease and arginase activities are involved in the IL-6-mediated downregulation of surface HLA-DR expression levels on $CD11b^+CD11c^+$ cells

IL-6-induced effector molecules responsible for decreasing HLA class II and CD86 expression levels of $CD11b^+CD11c^+$ cells were examined. These experiments focused on lysosomal protease, arginase-1 and COX-2, because previous reports indicated that cathepsin and arginase activities are associated with the regulation of MHC-class II expression levels in murine DCs[17,20]. In addition, recent reports indicated that COX-2 is related to the generation of MDSCs and immunosuppressive functions[36,39]. IL-6 was

confirmed to enhance the gene expression levels of *COX2*, *CTSL* and *ARG1* in human $CD11b^{+}CD11c^{+}$ cells (**Figure 12**).

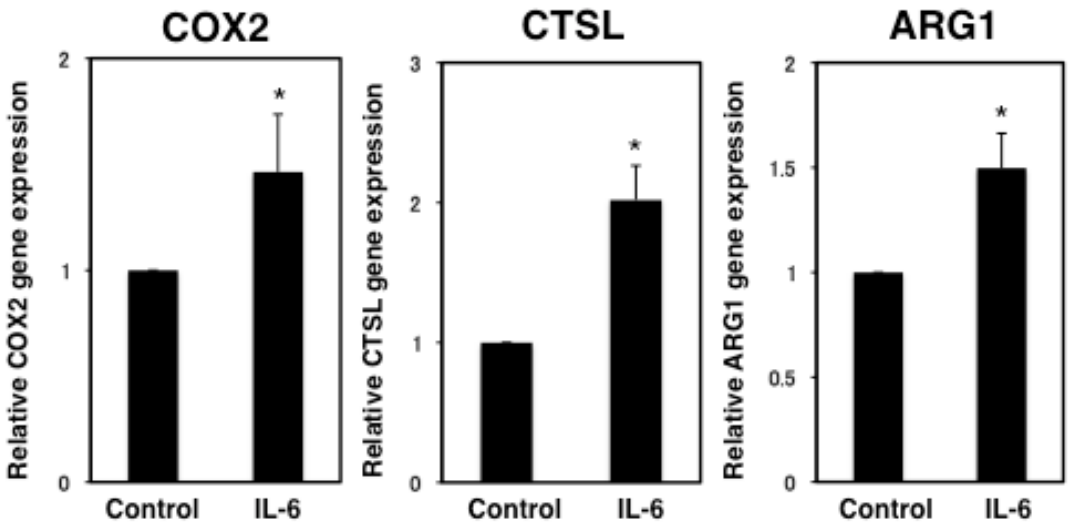

Figure 12. IL-6 enhanced *COX2*, *CTSL* and *ARG1* levels in human $CD11b^{+}CD11c^{+}$ myeloid cells. Adherent cells were collected from PBMCs of healthy donors and treated with IL-6 (50 ng/mL) for 24 h. *COX2*, *CTSL* and *ARG1* levels were determined by quantitative PCR. Means and SDs are indicated. (n=3) (* $P<0.05$ *vs.* control).

In addition, these experiments confirmed that IL-6-induced downregulation of HLA-DR expression was restored by the addition of inhibitors for COX-2, lysosomal proteases or arginase. COX-2 inhibitor partially restored the surface expression levels of CD86 (**Figure 13**).

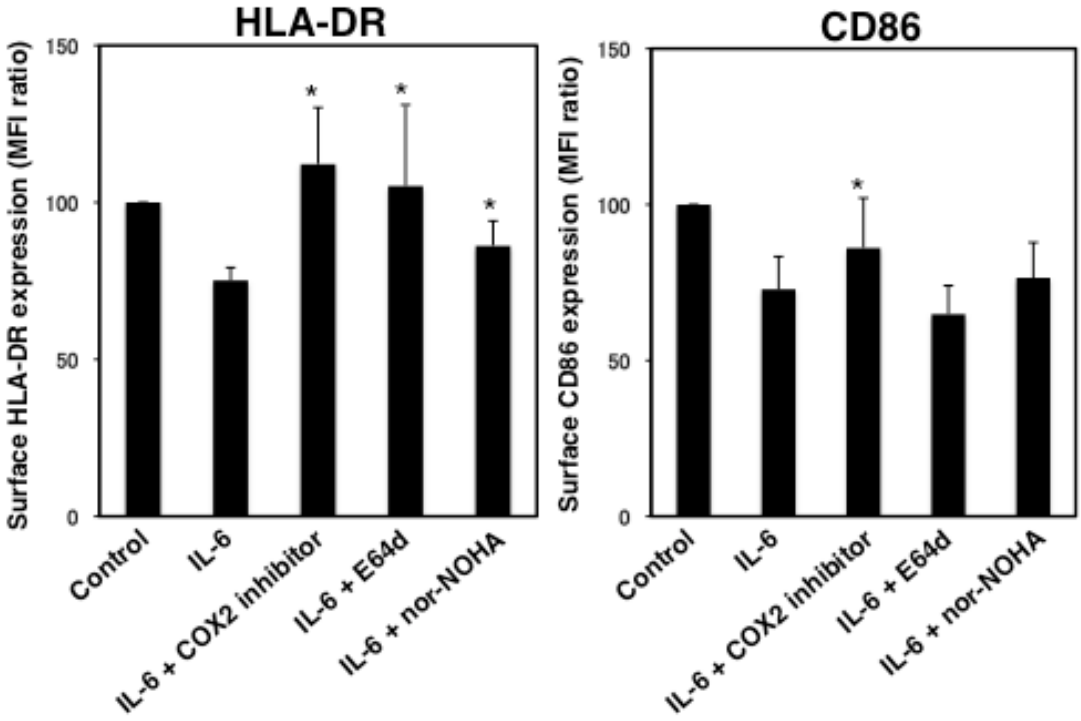

Figure 13. COX-2, lysosomal protease and arginase activity was involved in the regulation of surface expression levels of HLA-DR and/or CD86. Adherent cells were collected from PBMCs of healthy donors and treated with IL-6 (50 ng/mL) in the presence of COX-2 inhibitor, lysosomal protease inhibitor (E64d) or arginase inhibitor (nor-NOHA) for 24 h. Surface expression of HLA-DR and CD86 was determined by flow cytometry. MFI ratio was calculated and the mean and SD are indicated (n=5) (* $P<0.05$ *vs.* IL-6).

These data indicate that IL-6/STAT3 signaling may negatively regulate HLA-DR expression levels on human myeloid cells through the activation of COX-2, lysosomal protease, and/or arginase activity, suggesting that induction of these molecules by IL-6 might be related with maturation of human myeloid cells as antigen presenting cells.

IL-6/STAT3 signaling pathway is related to the function of tumor-infiltrating CD11b^{+}CD11c^{+} populations

The effects of IL-6 on antigen-presenting cells *in vitro* were next investigated, and IL-6/STAT3 signaling in human cancer specimens was examined. The expression of IL-6 and phosphorylated STAT3 at the tumor sites of colorectal cancers was evaluated by immunohistochemistry. As a result, IL-6 was confirmed to be produced by cancer-associated fibroblasts and tumor-infiltrating immune cells, and many tumor-infiltrating immune cells expressed phosphorylated STAT3 (**Figure 14**).

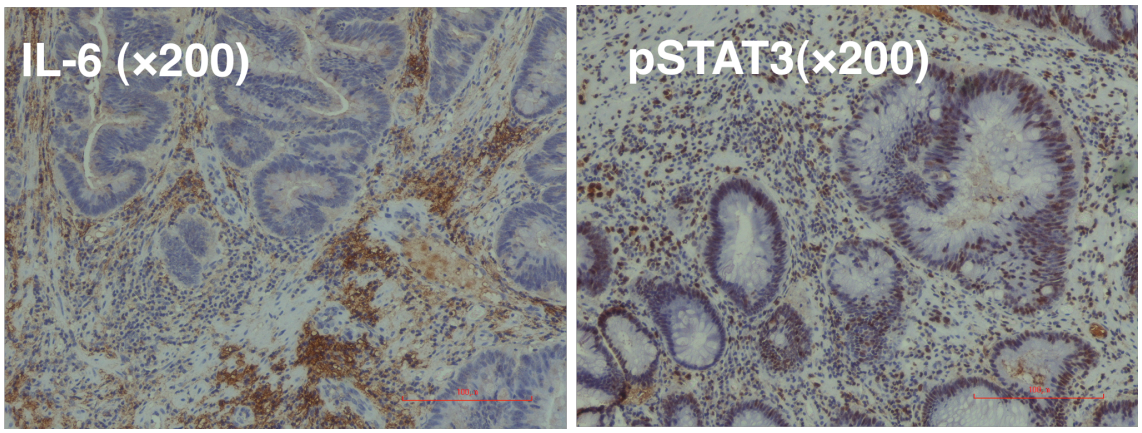

Figure 14. Immunohistochemistry of IL-6 and pSTAT3 in colorectal cancer. Cancer tissues obtained from colorectal surgical specimens were fixed in formalin and embedded in paraffin. Slides were treated with rabbit anti-human IL-6 or phospho-STAT3 antibodies over-night at 4 °C. All sections were incubated with peroxidase-labeled anti-mouse IgG polyclonal antibody or peroxidase labeled goat anti-rabbit immunoglobulin antibody as secondary antibody. Protein expression was visualized using DAB. Finally, sections were counterstained with Mayer's hematoxylin. Images are representative cases of colorectal cancers.

In this study, further investigations were undertaken to investigate whether IL-6 actually attenuated the antigen-presenting function of tumor-infiltrating myeloid cells. $CD11b^+CD11c^+$ cells were defined as myeloid cells. $CD11b^+CD11c^+$ cells were isolated from TILs and PBMCs of colorectal cancer patients by FACS **(Figure 15)**.

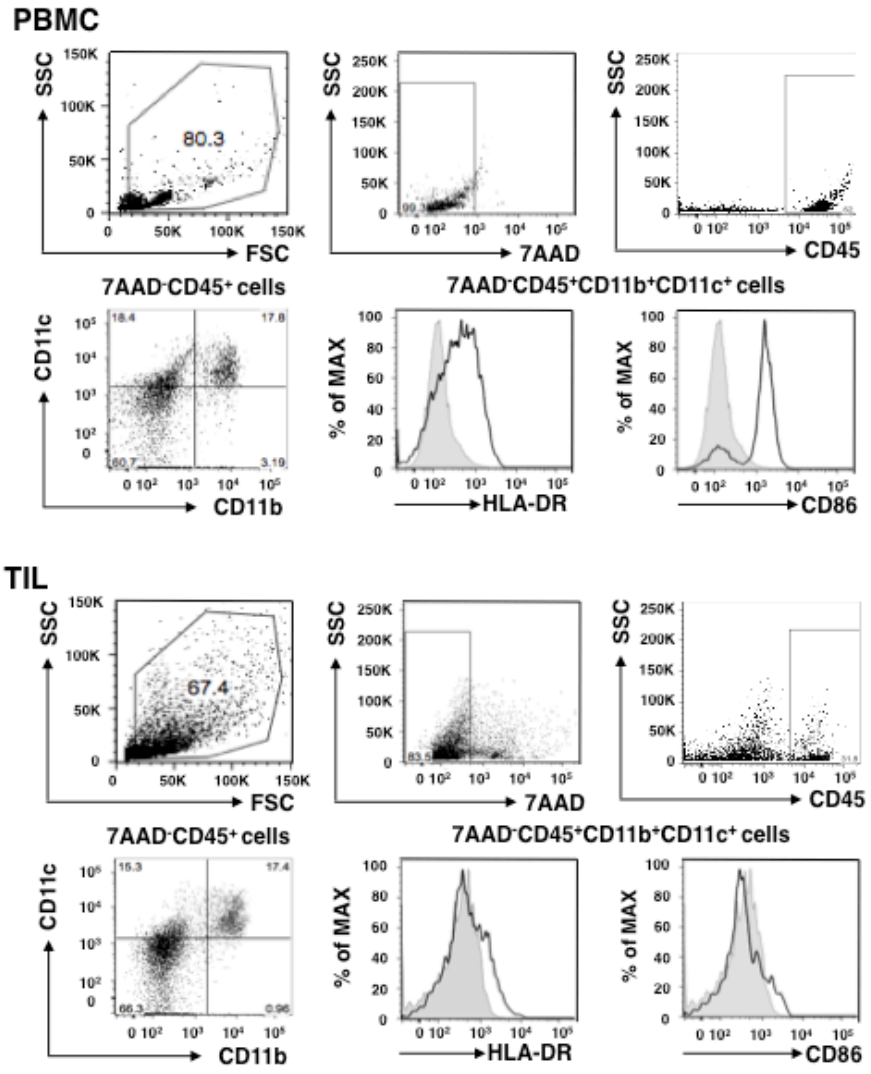

Figure 15. Gating strategy for isolation of $CD11b^+CD11c^+$ cells from PBMCs and TILs. $7AAD^+$ cells were gated out as dead cells, and $CD45^+CD11b^+CD11c^+$ cells were sorted as myeloid cells. Representative profiles are shown.

The surface expression levels of HLA-DR and CD86 were confirmed to be downregulated in tumor-infiltrating $CD11b^{+}CD11c^{+}$ cells. In addition, mRNA was retrieved from the isolated $CD11b^{+}CD11c^{+}$ cells, and the gene expression levels of *IL-6*, *COX2*, *CTSL* and *ARG1* were examined; all were much higher in $CD11b^{+}CD11c^{+}$ cells in TILs than in the PBMCs of colorectal cancer patients (**Figure 16a and 16b**). These data suggested that the influence of IL-6, which was investigated *in vitro*, is exerted in the human tumor microenvironment.

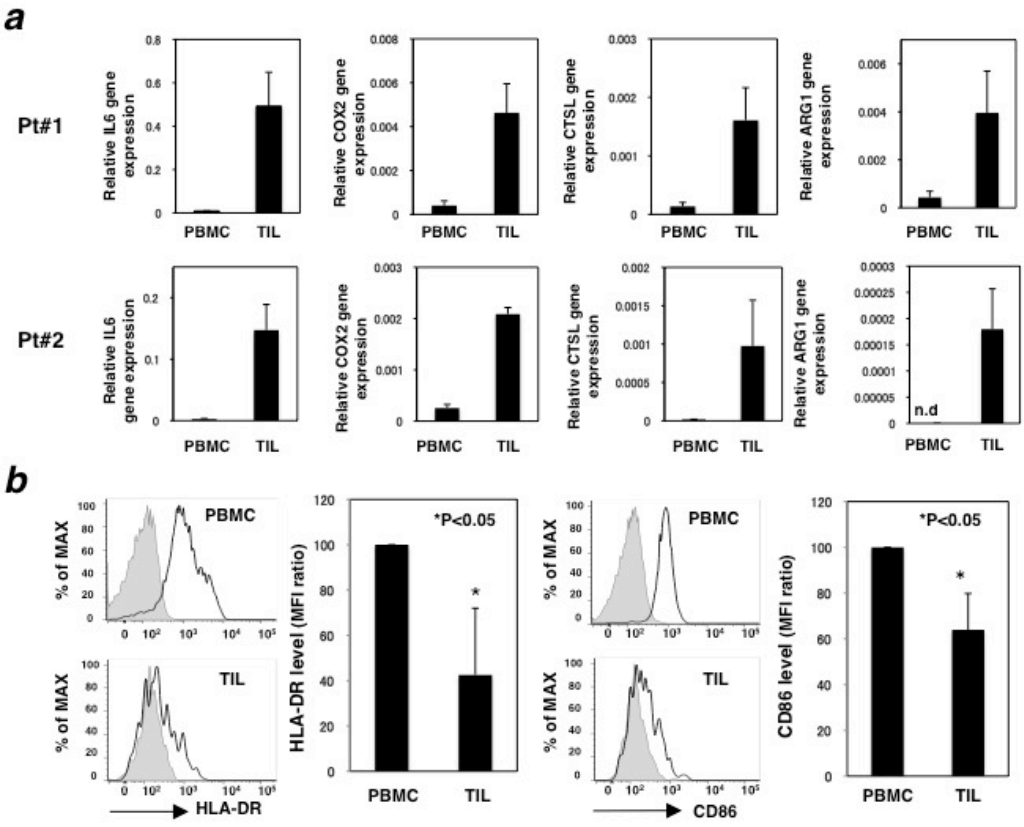

Figure 16. Tumor-infiltrating $CD11b^{+}CD11c^{+}$ cells enhanced *IL6*, *COX2*, *CTSL* and *ARG1* gene expression and decreased surface expression of HLA-DR and CD86 compared with that in PBMCs. $7AAD^{-}CD45^{+}CD11b^{+}CD11c^{+}$ cells were collected from PBMCs and tumor tissues of colorectal cancer patients. (**a**) *IL6*, *COX2*, *CTSL*, and *ARG1* levels in the

$CD11b^+CD11c^+$ cells were evaluated by quantitative PCR. Two representative datasets are shown. Means and SEs of relative gene expression are indicated. (**b**) HLA-DR and CD86 expression levels on the $CD11b^+CD11c^+$ cells in PBMCs and TILs were evaluated by flow cytometry. Representative profiles are shown. MFI ratio was calculated and the means and SDs are indicated (n=8).

In addition, studies were performed to determine whether $CD11b^+CD11c^+$ cells in TILs not only downregulated the surface expression of HLA-DR and CD86, but also impaired T cell-stimulating ability. As a result, both $CD4^+$ T and $CD8^+$ T cell proliferation were reduced in the presence of $CD11b^+CD11c^+$ cells from TILs compared with those from PBMCs. IFN-γ production by T cells was attenuated in the presence of $CD11b^+CD11c^+$ cells from TILs, whereas increased production was dependent on the number of $CD11b^+CD11c^+$ cells from TILs (**Figure 17**). These data suggested that tumor-infiltrating myeloid cells attenuated T cell-stimulating ability, and IL-6/STAT3 signaling may be related with the regulation of myeloid cells in the tumor microenvironment of colorectal cancer patients.

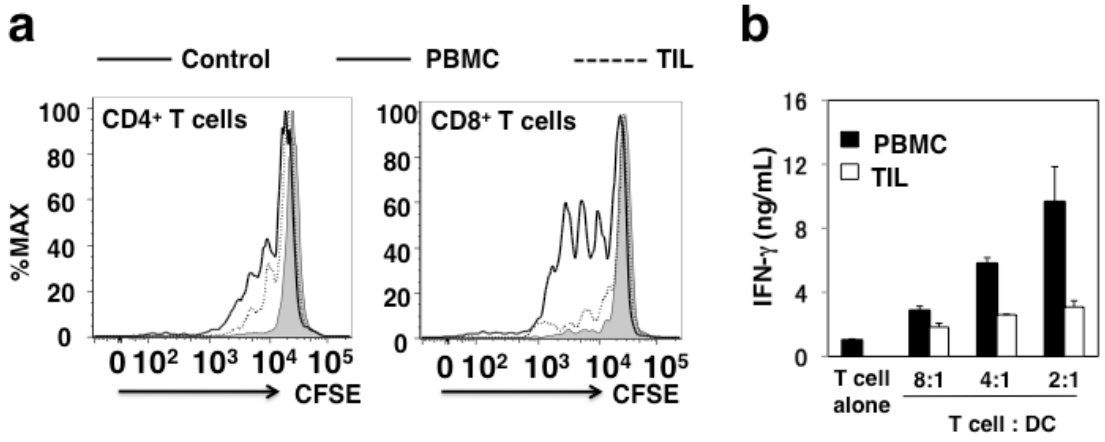

Figure 17. Tumor-infiltrating $CD11b^+CD11c^+$ cells attenuated T cell-stimulating ability. $7AAD^-CD45^+CD11b^+CD11c^+$ cells were collected from PBMCs and tumor tissues of colorectal

cancer patients. (a) CFSE-labeled $CD4^+$ T and $CD8^+$ T cells (1×10^5 cells) were stimulated with anti-CD3 mAb in the presence of isolated $CD11b^+CD11c^+$ cells (5×10^4 cells) from PBMCs or TILs for 72 h. Proliferation of the T cells was evaluated by flow cytometry. (b) IFN-γ production by $CD4^+$ T and $CD8^+$ T cells (1×10^5 cells) co-cultured with $CD11b^+CD11c^+$ cells (5×10^4 cells) from PBMCs and TILs at 24 h after TCR stimulation was measured by ELISA.

HLA-DR expression level is related to infiltration of $CD4^+$ T and $CD8^+$ T cells in the tumor sites of colorectal cancer patients

Finally, the relationship between tumor-infiltrating antigen presenting cells and tumor-infiltrating T cells was investigated. HLA-DR was used as a marker of antigen-presenting cells. Colorectal cancer tissues were used for this study. Both $CD4^+$ T and $CD8^+$ T cells were observed in colorectal tumor tissues with high HLA-DR expression (**Figure 18)**. These results suggest that the presence of antigen-presenting cells may be involved in the introduction of cancer-antigen specific T cells in the tumor microenvironment.

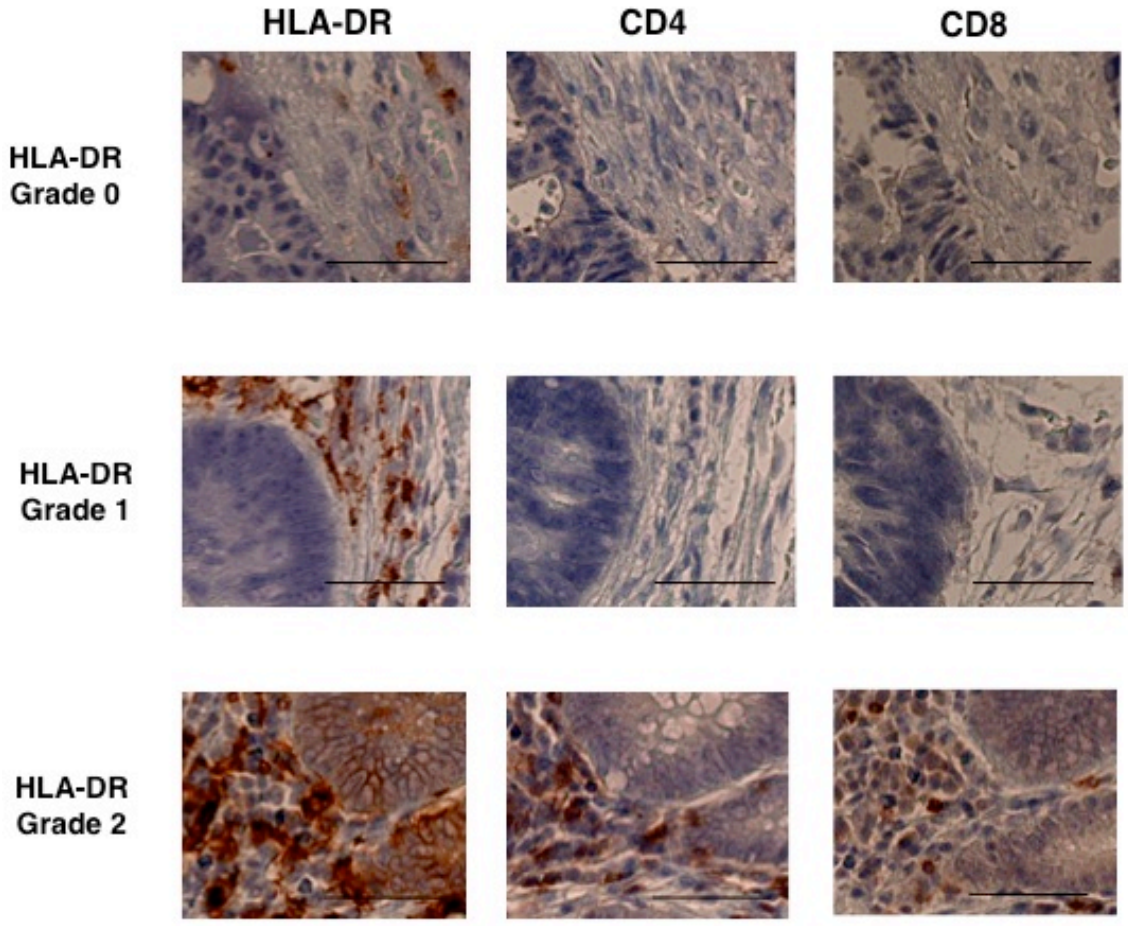

Figure 18. HLA-DR expression level is correlated with the frequency of $CD4^+$ T and $CD8^+$ T cells in tumor sites of colorectal cancer patients. Cancer tissues obtained from colorectal surgical specimens were fixed in formalin and embedded in paraffin. Slides were treated with rabbit anti-human HLA-DR, CD4 or CD8 antibodies. Protein expression was visualized using peroxidase-labeled secondary antibodies and DAB. Finally, sections were counterstained with Mayer's hematoxylin. Representative images (× 200) with grade 0 to 2 of HLA-DR-expressing tissues are indicated. Bars represent 200 μm.

Discussion

This study showed that the IL-6/STAT3 signaling pathway impaired the antigen-presenting function of human DCs via downregulation of HLA-class II expression, and attenuated Th1 immunity by decreasing IL-12 production by DCs. In addition, IL-6 inhibited the maturation of $CD11b^{+}CD11c^{+}$ myeloid cells into antigen-presenting cells. It is well known that patients with advanced cancer are immunocompromised, and cancer progression is closely related with chronic inflammation. IL-6 is a representative pro-inflammatory cytokine, and prospective and retrospective studies have shown that serum IL-6 levels are related to tumor stage and size, metastasis and survival in colon cancer patients[24], chemotherapy efficacy for advanced pancreatic cancer[25], advanced stage and metastasis-related morbidity of breast cancer[26], or clinical efficacy of personalized peptide vaccination for advanced biliary tract cancer[27]. These information suggest that IL-6 levels in cancer may not be simply promising prognostic biomarkers but also associated with tumorigenesis and antitumor immune responses. In this study, IL-6-conditioned MoDCs had reduced antigen-presenting function for Th cells. The helper function of antigen-specific Th cells such as production of Th1 cytokine was essential for inducing fully activated CTLs in tumor-bearing hosts[28,29]. A previous report demonstrated that cancer antigen-derived peptide containing helper epitope could induce CTLs efficiently *in vitro* according to the helper function of antigen-specific $CD4^{+}$ T cells[22]. In fact, cancer peptide vaccine containing helper epitope could induce CTLs in an advanced cancer

patient, and the patient experience clinical benefit[30]. In particular, activation of Th1 cells is crucial for anti-cancer immunity. IL-12 is an important cytokine for inducing Th1 immunity, because IL-12 activates STAT4 in $CD4^+$ T cells, inducing subsequent IFN-γ secretion[31]. This study showed that IL-6/STAT3 signaling attenuated IL-12 production of MoDCs, and actually impaired IFN-γ secretion of $CD4^+$ T cells *in vitro*. In previous reports, STAT3 was shown to regulate NF-κB recruitment to the IL-12p40 promoter in murine DCs[32] and activation of STAT3 inhibits *IL-12p35* gene expression in mice[33]. In fact, IL-12p35$^{-/-}$ mice easily developed tumors following carcinogen exposure compared with WT mice[34]. The report indicated that IL-12 was important for anti-cancer immunity via induction of Th1 immunity. Therefore, it may be that the IL-6/STAT3 signaling cascade attenuated anti-cancer immunity according to the impaired Th1 immunity by reduced IL-12 production.

Moreover, IL-6 stimulation also reduced HLA-DR and CD86 expression levels in $CD11b^+CD11c^+$ cells from PBMCs of healthy donors in a STAT3-dependent manner. In addition, $CD11b^+CD11c^+$ cells pretreated with IL-6 were confirmed to impair T cell-stimulating ability. In this study, activation of arginase, lysosome proteases and COX-2 were involved in the downregulation of HLA-DR on $CD11b^+CD11c^+$ cells. A previous study 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]. In addition, IL-6-induced activation of lysosome proteases caused reduction of MHC class II αβ-dimer levels in murine DCs[13]. Other previous reports indicated that activation of COX2/PEG2 cascade inhibited maturation

of DCs[35,36]. This information suggests that these functional molecules might be related with the IL-6-induced downregulation of HLA-class II expressed on human $CD11b^{+}CD11c^{+}$ cells.

IL-6 is produced by various cells including cancer cells, cancer-associated fibroblasts, and immune cells due to chronic inflammation in the tumor sites of colorectal cancers. In this study, $CD11b^{+}CD11c^{+}$ cells in colorectal tumor tissues reduced the surface expression levels of HLA-DR and CD86 compared with the PBMCs. *ARG1*, *CTSL* and *COX2* levels were increased in tumor-infiltrating $CD11b^{+}CD11c^{+}$ cells. In addition, tumor-infiltrating $CD11b^{+}CD11c^{+}$ cells impaired T cell-stimulating ability compared with PBMCs. These data suggest that tumor-infiltrating myeloid cells may downregulate the surface expression of HLA-DR and CD86 by arginase-1, lysosomal protease and COX-2. Thereby, IL-6/STAT3 signaling inhibits the maturation of tumor-infiltrating myeloid cells into antigen-presenting cells, and attenuates the subsequent T cell-stimulating ability. Although various immunosuppressive cytokines such as IL-10 and TGF-β are produced in the tumor microenvironment, but IL-6 may be one of the main cytokines that controls the function of myeloid cells in the tumor microenvironment.

In previous reports, $CD14^{-}HLA\text{-}DR^{low/-}$ cells were increased in the peripheral blood of advanced cancer patients, and these cells were defined as human MDSCs[37-39]. Thus, the IL-6/STAT3 signaling pathway might systemically influence peripheral myeloid cells, thereby surface expression of HLA-DR could be downregulated. It was reported that the presence of intra-tumoral T cells was a good prognostic factor in colorectal

cancers[40,41]. In this study, HLA class II expression levels of tumor-infiltrating immune cells were closely related to the invasion of $CD4^+$ T and $CD8^+$ T cells at tumor sites. This information suggested that appropriate and adequate antigen presentation is essential and important for the introduction of cancer antigen-specific T cells into the tumor microenvironment.

The present findings indicate that maintenance of the antigen-presenting ability of DCs and other myeloid cells is critical for the induction and activation of cancer antigen-specific T cells in the tumor microenvironment. Therefore, the therapeutic strategy for improving the function of DCs is effective in increasing good response to cancer immunotherapies such as cancer vaccines.

Therefore, IL-6 may be a promising target for cancer immunotherapy through both improving the antigen-presenting function of DCs and facilitating maturation of antigen-presenting cells into tumor-infiltrating cells. Recent clinical trials using a chimeric anti-IL-6 mAb have been conducted for patients with advanced cancer[42], and this treatment was well tolerated by patients. In addition, serum CRP was decreased and anemia caused by chronic inflammation was improved by administration of anti-IL-6 mAb in this study. It was reported that IL-6 is associated with cachexia in advanced cancer patients[43]. This information indicates that anti-IL-6 mAb could control the status of chronic inflammation in patients with advanced cancers. Therefore, blockade of IL-6/STAT3 signaling combined with other cancer immunotherapies such as peptide vaccines may show more clinical efficacy by restoring the T cell-stimulatory ability of antigen-presenting cells, which are represented by DCs, in cancer patients.

Summary and Conclusion

1. IL-6-induced STAT3 activation causes attenuation of antigen-presenting function and IL-12 production by human DCs, resulting in the subsequent suppression of Th1 immunity.
2. IL-6/STAT3 signaling pathway inhibits differentiation of antigen presenting cells into human myeloid cells.
3. IL-6/STAT3 signaling is one factor that causes inadequate T cell-mediated anti-tumor immunity via impairment of the antigen-presenting function of myeloid cells including DCs.

IL-6 causes immunosuppression in cancer patients by inducing dysfunction of antigen-presenting cells such as DCs, suggesting that inhibition of the IL-6/STAT3 signaling pathway will be a promising strategy for the development of novel cancer immunotherapy via improvements in antigen presentation to induce anti-tumor effector T cells in the tumor microenvironment **(Figure 19)**.

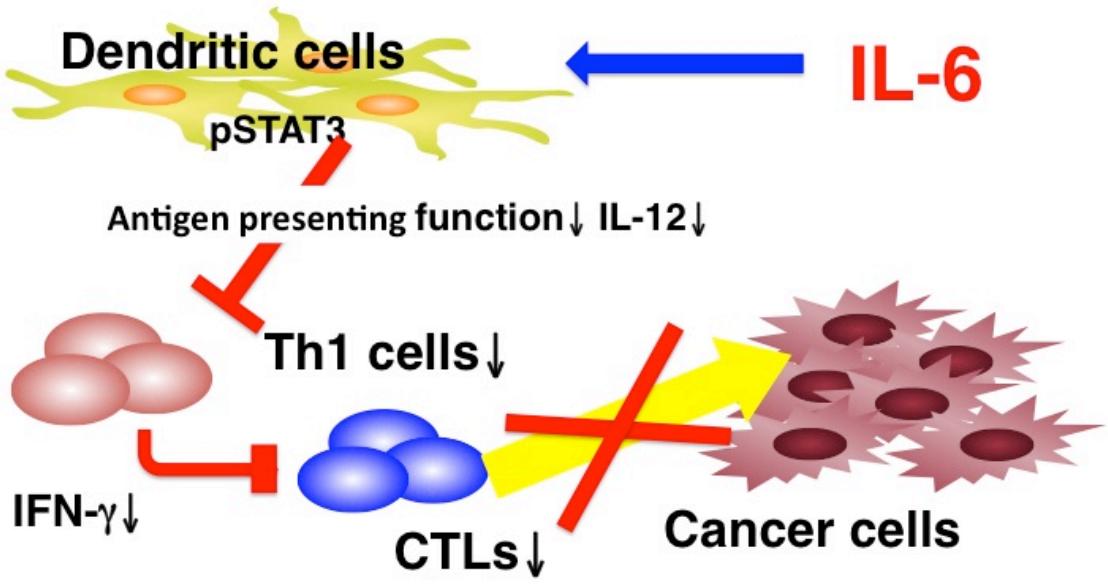

Figure 19. Outline for IL-6/STAT3-mediated dysfunction of dendritic cells in tumor microenvironment.

In this study, information from basic studies using murine models was applied to human subjects. To apply the present findings to the clinic, therapeutic models targeting the IL-6/STAT3 signaling pathway were established using experimental animals because it is necessary to reveal more detailed information about the role of IL-6 in the tumor microenvironment. The effects of the IL-6/STAT3 signaling pathway on antitumor immunity are still being investigated to develop effective cancer immunotherapies.

Acknowledgments

First, I would like to thank Prof. Akinobu Taketomi (Department of Gastroenterological Surgery I, Graduate School of Medicine, Hokkaido University) and Associate Prof. Hidemitsu Kitamura (Division of Functional Immunology, Institute of Genetic Medicine, Hokkaido University) for their thoughtful direction of all the experiments carried out in this dissertation.

I would like to thank Dr. Norihiko Takahashi and all members of the gastrointestinal group (Department of Gastroenterological Surgery I, Graduate School of Medicine, Hokkaido University) for the clinical specimens used in these studies.

I would like to thank all members of my laboratory (Division of Functional Immunology, Institute of Genetic Medicine, Hokkaido University) for their technical assistance.

References

1. van der Bruggen, P. *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643–1647 (1991).
2. Kono, K. Current status of cancer immunotherapy. *J Stem Cells Regen Med* **10**, 8–13 (2014).
3. Melero, I. *et al.* Therapeutic vaccines for cancer: an overview of clinical trials. *Nat Rev Clin Oncol* **11**, 509–524 (2014).
4. Hinrichs, C. S. & Rosenberg, S. A. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev* **257**, 56–71 (2014).
5. Savage, P. A., Malchow, S. & Leventhal, D. S. Basic principles of tumor-associated regulatory T cell biology. *Trends Immunol* **34**, 33–40 (2013).
6. Gabrilovich, D. I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* **12**, 253–268 (2012).
7. Motz, G. T. & Coukos, G. Deciphering and Reversing Tumor Immune Suppression. *Immunity* **39**, 61–73 (2013).
8. Hodi, F. S. *et al.* Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *N Engl J Med* **363**, 711–723 (2010).
9. Wolchok, J. D. *et al.* Nivolumab plus Ipilimumab in Advanced Melanoma. *N Engl J Med* **369**, 122–133 (2013).
10. Brahmer, J. R. *et al.* Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. *N Engl J Med* **366**, 2455–2465 (2012).

11. Palucka, K. & Banchereau, J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* **12**, 265–277 (2012).

12. Palucka, K. & Banchereau, J. Dendritic-Cell-Based Therapeutic Cancer Vaccines. *Immunity* **39**, 38–48 (2013).

13. Steinman, R. M. & Banchereau, J. Taking dendritic cells into medicine. *Nature* **449**, 419–426 (2007).

14. Steinman, R. M. & Mellman, I. Immunotherapy: bewitched, bothered, and bewildered no more. *Science* **305**, 197–200 (2004).

15. Hirano, T., Ishihara, K. & Hibi, M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene* **19**, 2548–2556 (2000).

16. Hirano, T. *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* **324**, 73–76 (1986).

17. Kitamura, H. *et al.* IL-6-STAT3 Controls Intracellular MHC Class II αβ Dimer Level through Cathepsin S Activity in Dendritic Cells. *Immunity* **23**, 491–502 (2005).

18. Park, S.-J. *et al.* IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. *J Immunol* **173**, 3844–3854 (2004).

19. Sumida, K. *et al.* Anti-IL-6 receptor mAb eliminates myeloid-derived suppressor cells and inhibits tumor growth by enhancing T-cell responses. *Eur J Immunol* **42**, 2060–2072 (2012).

20. Narita, Y. *et al.* The key role of IL-6-arginase cascade for inducing dendritic cell-dependent CD4(+) T cell dysfunction in tumor-bearing mice. *J Immunol* **190**, 812–820 (2013).

21. Ohkuri, T. *et al.* Identification of novel helper epitopes of MAGE-A4 tumour antigen: useful tool for the propagation of Th1 cells. *Br J Cancer* **100**, 1135–1143 (2009).

22. Ohtake, J. *et al.* Identification of novel helper epitope peptides of Survivin cancer-associated antigen applicable to developing helper/killer-hybrid epitope long peptide cancer vaccine. *Immunol Lett* **161**, 20–30 (2014).

23. Hayashi, S. *et al.* Six-transmembrane epithelial antigen of the prostate and enhancer of zeste homolog 2 as immunotherapeutic targets for lung cancer. *J Transl Med* **9**, 191 (2011).

24. Knüpfer, H. & Preiss, R. Serum interleukin-6 levels in colorectal cancer patients--a summary of published results. *Int J Colorectal Dis* **25**, 135–140 (2010).

25. Mitsunaga, S. *et al.* Serum levels of IL-6 and IL-1β can predict the efficacy of gemcitabine in patients with advanced pancreatic cancer. *Br J Cancer* **108**, 2063–2069 (2013).

26. Gupta, N., Goswami, B. & Mittal, P. Effect of standard anthracycline based neoadjuvant chemotherapy on circulating levels of serum IL-6 in patients of locally advanced carcinoma breast - a prospective study. *Int J Surg* **10**, 638–640 (2012).

27. Yoshitomi, M. *et al.* Personalized peptide vaccination for advanced biliary tract cancer: IL-6, nutritional status and pre-existing antigen-specific immunity as possible biomarkers for patient prognosis. *Exp Ther Med* **3**, 463–469 (2012).

28. Nishimura, T. *et al.* Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med* **190**, 617–627 (1999).

29. Hunder, N. N. *et al.* Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* **358**, 2698–2703 (2008).

30. Takahashi, N. *et al.* First clinical trial of cancer vaccine therapy with artificially synthesized helper/killer-hybrid epitope long peptide of MAGE-A4 cancer antigen. *Cancer Sci* **103**, 150–153 (2012).

31. Morinobu, A. *et al.* STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. *Proc Natl Acad Sci U S A* **99**, 12281–12286 (2002).

32. Hoentjen, F. STAT3 regulates NF- B recruitment to the IL-12p40 promoter in dendritic cells. *Blood* **105**, 689–696 (2005).

33. Kortylewski, M. *et al.* Regulation of the IL-23 and IL-12 Balance by Stat3 Signaling in the Tumor Microenvironment. *Cancer Cell* **15**, 114–123 (2009).

34. Langowski, J. L. *et al.* IL-23 promotes tumour incidence and growth. *Nature* **442**, 461–465 (2006).

35. Harizi, H., Juzan, M., Grosset, C., Rashedi, M. & Gualde, N. Dendritic cells issued in vitro from bone marrow produce PGE(2) that contributes to the

immunomodulation induced by antigen-presenting cells. *Cell Immunol* **209**, 19–28 (2001).

36. Obermajer, N., Muthuswamy, R., Lesnock, J., Edwards, R. P. & Kalinski, P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood* **118**, 5498–5505 (2011).

37. Lin, Y. *et al.* Immunosuppressive CD14+HLA-DR(low)/- monocytes in B-cell non-Hodgkin lymphoma. *Blood* **117**, 872–881 (2011).

38. Huang, A. *et al.* Increased CD14(+)HLA-DR (-/low) myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to chemotherapy in non-small cell lung cancer patients. *Cancer Immunol Immunother* **62**, 1439–1451 (2013).

39. Mao, Y. *et al.* Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. *Cancer Res* **73**, 3877–3887 (2013).

40. Laghi, L. *et al.* CD3+ cells at the invasive margin of deeply invading (pT3–T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. *Lancet Oncol* **10**, 877–884 (2009).

41. Huh, J. W., Lee, J. H. & Kim, H. R. Prognostic significance of tumor-infiltrating lymphocytes for patients with colorectal cancer. *Arch Surg* **147**, 366–372 (2012).

42. Angevin, E. *et al.* A phase I/II, multiple-dose, dose-escalation study of siltuximab, an anti-interleukin-6 monoclonal antibody, in patients with advanced solid tumors. *Clin Cancer Res* **20**, 2192–2204 (2014).

43. Fujii, S.-I., Liu, K., Smith, C., Bonito, A. J. & Steinman, R. M. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* **199**, 1607–1618 (2004).