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Lack of interleukin-6 in the tumor microenvironment augments type-1 immunity and increases the efficacy of cancer immunotherapy

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Key words

Cytotoxic T cells, dendritic cells, interferon- γ , interleukin-6, programmed death-ligand 1

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Recently, effective immune checkpoint therapy using anti-programmed cell death protein 1 (PD-1), -programmed death-ligand 1 (PD-L1), and/or -cytotoxic T lymphocyte-associated protein 4 antibodies to activate effector T cells in cancer patients has been reported for various solid tumors.^(1–3) These results indicate that cancer antigen-specific T cells, which eliminate cancer cells, potentially exist in tumor microenvironments. As a result, blocking negative signals against tumor-infiltrating T cells can restore the cytotoxic function toward their target cancer cells. Thus, introduction of cancer-specific T cells to the tumor microenvironment is required as the first step for more effective cancer immunotherapies.

Dendritic cells, which are representative antigen-presenting cells, strongly induce antigen-specific immune responses

Conquering immunosuppression in tumor microenvironments is crucial for effective cancer immunotherapy. It is well known that interleukin (IL)-6, a pleiotropic cytokine, is produced in the tumor-bearing state. In the present study, we investigated the precise effects of IL-6 on antitumor immunity and the subsequent tumorigenesis in tumor-bearing hosts. CT26 cells, a murine colon cancer cell line, were intradermally injected into wild-type and IL-6-deficient mice. As a result, we found that tumor growth was decreased significantly in IL-6-deficient mice compared with wild-type mice and the reduction was abrogated by depletion of CD8⁺ T cells. We further evaluated the immune status of tumor microenvironments and confirmed that mature dendritic cells, helper T cells and cytotoxic T cells were highly accumulated in tumor sites under the IL-6-deficient condition. In addition, higher numbers of interferon (IFN)- γ -producing T cells were present in the tumor tissues of IL-6-deficient mice compared with wild-type mice. Surface expression levels of programmed death-ligand 1 (PD-L1) and MHC class I on CT26 cells were enhanced under the IL-6-deficient condition *in vivo* and by IFN- γ stimulation *in vitro*. Finally, we confirmed that *in vivo* injection of an anti-PD-L1 antibody or a Toll-like receptor 3 ligand, polyinosinic-polycytidylic acid, effectively inhibited tumorigenesis under the IL-6-deficient condition. Based on these findings, we speculate that a lack of IL-6 produced in tumor-bearing host augments induction of antitumor effector T cells and inhibits tumorigenesis *in vivo*, suggesting that IL-6 signaling may be a promising target for the development of effective cancer immunotherapies.

through activation of CD4⁺ T and CD8⁺ T cells. In cancer patients, mature dendritic cells highly expressing MHC class I, MHC class II and co-stimulatory molecules on their cell surface are crucial to induce cancer antigen-specific effector T cells. Therefore, proper regulation of dendritic cells in the tumor microenvironment is important for induction of antitumor immunity.^(4–7)

Interleukin (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. IL-6 first binds to the IL-6 receptor (IL-6R), inducing dimerization of gp130, a signal transducer. Gp130 dimerization rapidly activates the JAK family and several signaling pathways, including phosphatidylinositol 3-kinase/

ERK/MAPK and signal transducer and activator of transcription 3 (STAT3). STAT3 activation induces numerous effector genes involved in cell proliferation, differentiation and survival.^(8,9) Various cell types, including cancer cells, cancer-associated fibroblasts and immune cells, are well known to produce IL-6 in the tumor-bearing host. A previous study has reported that serum IL-6 levels are related to the tumor stage and size, metastasis, and survival in colon cancer patients.⁽¹⁰⁾ Clinical studies have demonstrated that the IL-6 level might be a good predictive biomarker for the clinical benefit of peptide vaccines.^(11,12)

Previously, it was reported that IL-6 signaling suppresses MHC class II expression on murine dendritic cells through STAT3 activation and attenuates CD4⁺ T cell-mediated immune responses.^(13,14) Furthermore, a previous study using tumor-bearing mouse models indicated that administration of a mAb antagonizing IL-6R enhances T cell responses and inhibits tumor growth *in vivo*.^(15,16) In tumor-bearing mice, IL-6 suppresses CD4⁺ T cell-mediated immunity through downregulation of MHC class II by enhancing the arginase activity of dendritic cells. Thus, IL-6-mediated STAT3 activation appears to be a critical mechanism to induce dysfunctional immune system responses in the tumor microenvironment through regulation of antigen-presenting cells. Recent studies have revealed that IL-6 produced in tumor environments suppresses differentiation of interferon (IFN)- γ -producing helper T cells and promotes subsequent tumor formation.⁽¹⁷⁻¹⁹⁾ Furthermore, we found that IL-6 is widely produced and STAT3 is activated in tumor microenvironments of colorectal cancer patients, and the CD11b⁺CD11c⁺ population isolated from tumor tissues shows higher IL-6 gene expression compared with the same phenotypic population isolated from PBMC.⁽²⁰⁾ Blockade of IL-6/STAT3 signaling cascades may, therefore, be a novel approach to overcome the dysfunctional antitumor immunity of cancer patients.

In this study, we further investigated the precise effects of IL-6 on antitumor immunity and the subsequent tumorigenesis in tumor-bearing hosts, and revealed that a lack of IL-6 in tumor microenvironments augments type-1 immunity, including induction of antitumor cytotoxic T cells, and inhibits tumorigenesis *in vivo*. These findings suggest that blockade of IL-6 signaling may enhance immunotherapies, such as immune checkpoint inhibition and administration of immunological adjuvants, in cancer patients.

Materials and Methods

Mice and cells. BALB/c mice were obtained from Charles River Japan (Kanagawa, Japan). IL-6^{-/-} mice were obtained from the Center for Animal Disease Models, Research Institute for Biomedical Sciences, Tokyo University of Science (Chiba, Japan). All mice were maintained in specific pathogen-free conditions and used at 6–8 weeks of age. Murine colon cancer cell line CT26 was obtained from the American Type Culture Collection (CRL-2638; VA). All mouse experiments were approved by the Animal Ethics Committee of Hokkaido University (No. 14-0062) and conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University.

Antibodies, cytokines and chemicals. Fluorescent dye-conjugated anti-CD45 (30-F11), anti-CD11c (N418), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-H-2K^d (AF6-88.5), anti-I-A^d (AF6-120.1), anti-IFN- γ (XMG1.2) and anti-IL-4 (11B11) mAbs were purchased from Biologend (San Diego, CA, USA) or BD Biosciences (San Diego, CA, USA). A mAb for CD8

depletion (clone 53.6.7) and an antagonistic mAb against PD-L1 (clone 10F.9G2) were purchased from Bio X Cell (West Lebanon, NH, USA). Recombinant murine IFN- γ was purchased from PeproTech EC (London, UK). 7-AAD Viability Dye was purchased from BECKMAN COULTER (Marseille Cedex, France). 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).

Cell culture. CT26 cells were maintained in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FCS, 200 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES and 0.05 mmol/L 2-mercaptoethanol (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO₂. For flow cytometry, 2.5 \times 10⁵ CT-26 cells were treated with 50 ng/mL IFN- γ in 12-well culture plates for 24 h.

Tumor-bearing mouse model. CT26 cells (1 \times 10⁶) were injected intradermally into wild-type and IL-6^{-/-} mice (day 0). The tumor size was measured by micrometer calipers from day 5. Tumor volumes were calculated using the following formula: volume (mm³) = 0.2 \times (length [mm] \times width [mm]) \times (height [mm])². For *ex vivo* analysis, CT26 cells were transfected with pMX-IRES-GFP, obtained from Dr T Kitamura (The University of Tokyo), by Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA, USA) and the GFP-transduced CT26 cells were used for the subsequent flow cytometry. The anti-CD8 mAb or control IgG (200 μ g/mouse) was injected intraperitoneally into wild-type and IL-6^{-/-} mice at days -1 and 5, and then every 4 days thereafter. In therapeutic experiments, the anti-PD-L1 mAb (200 μ g/mouse), control IgG (200 μ g/mouse) or poly I:C (50 μ g/mouse) were injected intraperitoneally into CT26 tumor-bearing wild-type and IL-6^{-/-} mice at day 5 and then every 4 days thereafter.

Immunohistochemistry. Tumor tissues obtained from CT26 tumor-bearing wild-type and IL-6^{-/-} mice at day 14 were fixed in 4% paraformaldehyde PBS and then embedded in paraffin. After deparaffinization, antigen retrievals for CD3 and CD11c were performed with a reagent kit (pH 9.0) (415211; Nichirei Bioscience, Tokyo, Japan) at 95°C for 10 min and with proteinase K solution (S3004; Dako, Hamburg, Germany) at room temperature for 5 min, respectively. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide at room temperature for 10 min. After washing with TBS, sections were treated with anti-CD3 (ab134096; Abcam) or anti-CD11c (GTX74940; GeneTex, Inc., Irvine, CA, USA) antibodies overnight at 4°C. Sections for CD3 and CD11c were incubated at room temperature for 30 min with Histofine Simple Stain, MAX PO (R) (424144; Nichirei Bioscience) at room temperature for 30 min or with rabbit Anti-Hamster IgG (6215-01; Southern Biotechnology Associates, Birmingham, AL, USA) at room temperature for 30 min, Histofine Simple Stain, MAX PO (R) (424144; Nichirei Bioscience) at room temperature for 30 min, TSA PLUS Biotin KIT (NEL749A001; PerkinElmer, Waltham, MA, USA) at room temperature for 5 min and VECTASTAIN Elite ABC Reagent (PK6100; Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min. Protein expression was visualized using 3-3'-diaminobenzidine-4HCL at room temperature for 5 min. Finally, all sections were counterstained with Mayer's H&E.

Flow cytometry. GFP-transduced CT26 cells (1 \times 10⁶) were intradermally injected into wild-type and IL-6^{-/-} mice. Tumor

evaluate the immune status of the tumor environment in CT26 tumor-bearing wild-type and IL-6^{-/-} mice. CD3⁺ T cells and CD11c⁺ dendritic cells had accumulated in tumor tissues of IL-6^{-/-} mice compared with wild-type mice (Fig. 1c). We further investigated the ratios of tumor-infiltrating immune cells by flow cytometry. As a result, CD45⁺ immune cells were more highly infiltrated into the tumor tissues of IL-6^{-/-} mice compared with wild-type mice. In addition, CD4⁺ T, CD8⁺ T and CD11c⁺I-A^{high} mature dendritic cells were more infiltrated in tumor tissues of IL-6-deficient mice (Fig. 1d). These data suggest that a lack of IL-6 in the tumor-bearing host may facilitate introduction of effector T cells and dendritic cells into the tumor microenvironment.

Interleukin-6 produced in the tumor microenvironment may suppress antitumor effects of cytotoxic T cells *in vivo*. Because our previous reports demonstrated that IL-6 attenuates T cell immune responses through the reduction of mature dendritic cells,^(13,14) we investigated whether the antitumor effect of cytotoxic T cells was involved in this mouse model. As a result, depletion of CD8⁺ T cells enhanced tumor growth in wild-type mice (Fig. 2a-c), indicating that CD8⁺ T cells killed CT26 cancer cells of this model *in vivo*. We further confirmed that depletion of CD8⁺ T cells significantly augmented tumor growth in IL-6^{-/-} mice (Fig. 2a-c). Therefore, these data

suggest that IL-6 produced in a tumor-bearing host may promote *in vivo* tumorigenesis through suppression of antitumor effector T cells.

Interferon-γ-producing CD4⁺ T and CD8⁺ T cells accumulate in tumor tissues of interleukin-6-deficient mice. We further evaluated the cytokine production abilities of tumor-infiltrating CD4⁺ T cells and CD8⁺ T cells in wild-type and IL-6^{-/-} mice. As a result, higher numbers of IFN-γ-producing CD4⁺ T cells were present in the tumor tissues of IL-6^{-/-} mice compared with wild-type mice (Fig. 3a,b). IL-10- and IL-17-producing CD4⁺ T cells were not altered between IL-6^{-/-} and wild-type mice (Fig. 3a,b). Furthermore, we confirmed that IFN-γ-producing CD8⁺ T cells were present in the tumor tissues of IL-6^{-/-} mice compared with wild-type mice (Fig. 3a,b). IFN-γ, but not IL-10-producing or IL-17-producing CD4⁺ T cells had increased in the draining lymph nodes of IL-6^{-/-} mice compared with wild-type mice (Fig. S1). These data indicate that IL-6 produced in the tumor-bearing host attenuates both Th1 and Tc1 types of immunity not only in the tumor sites but also in total body, suggesting that IL-6 might be a promising target to introduce antitumor effector T cells into tumor microenvironments.

In this model, we found significant differences between the anti-CD8 mAb-treated wild-type and IL-6^{-/-}, suggesting the

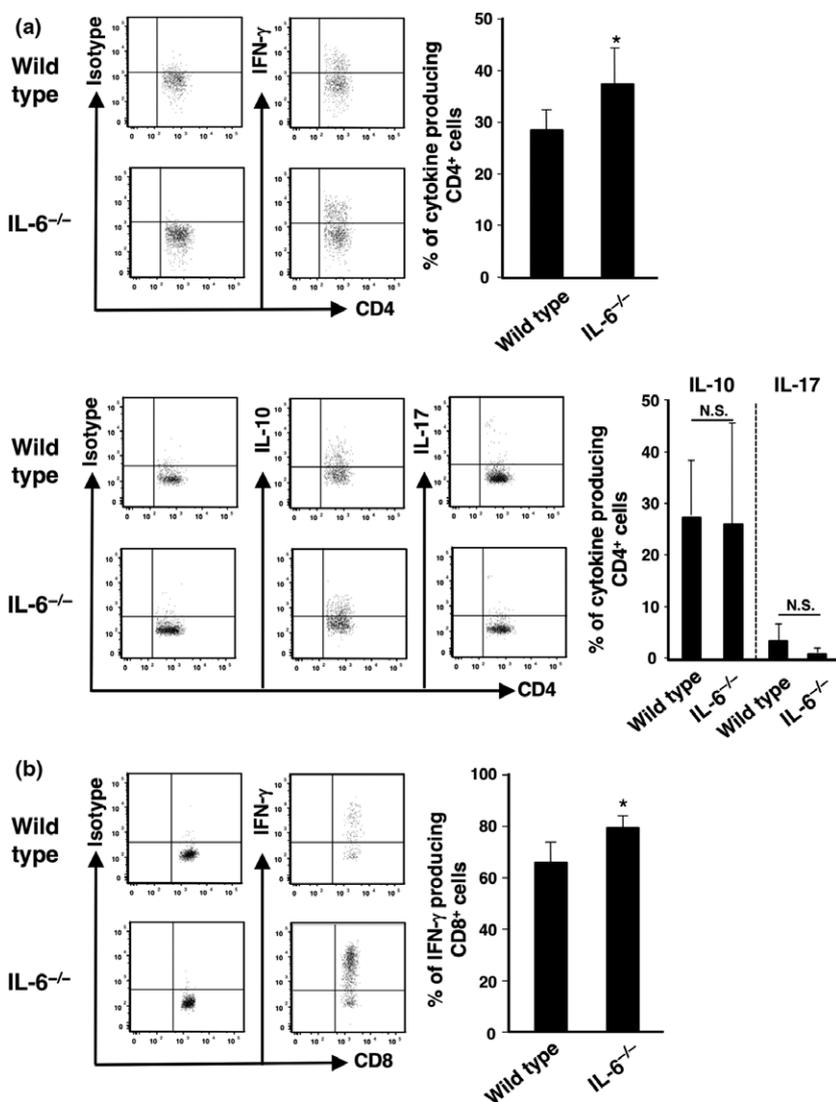


Fig. 3. Cytokine-producing ability of tumor-infiltrating CD4⁺ T and CD8⁺ T cells under the interleukin (IL)-6-deficient condition. CT26 murine colon cancer cells (1 × 10⁶) were intradermally injected into wild-type and IL-6^{-/-} BALB/c mice (day 0). Cytokine production by CD4⁺ T and CD8⁺ T cells in tumor tissues of wild-type and IL-6^{-/-} mice at day 12 were evaluated by flow cytometry using anti-IFN-γ, anti-IL-10, anti-IL-17, anti-CD4 and anti-CD8 mAbs. (a) Representative intracellular staining profiles of CD4⁺ T cells. Percentages of IFN-γ-producing, IL-10-producing or IL-17-producing CD4⁺ T cells were calculated. Means and SD (n = 5) are indicated. *P < 0.05 by Student's *t*-test. N.S. indicates not statistically significant. (b) Representative intracellular staining profiles of CD8⁺ T cells. Percentages of IFN-γ-producing CD8⁺ T cells were calculated. Means and SD (n = 5) are indicated. *P < 0.05 by Student's *t*-test.

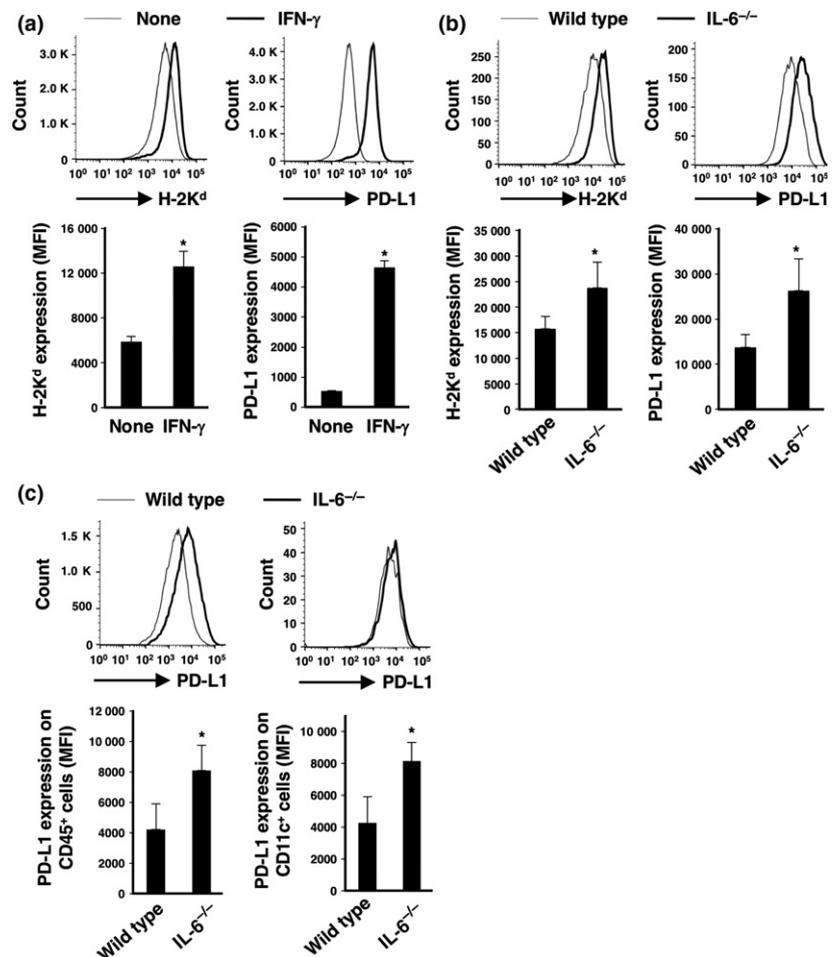


Fig. 4. Expression levels of MHC class I and PD-L1 on CT26 cells *in vitro* and *in vivo*. (a) CT26 cells were stimulated with IFN- γ (50 ng/mL) for 24 h *in vitro*. Surface expression levels of MHC class I and PD-L1 on CT26 cells were evaluated by flow cytometry using anti-H-2K^d and anti-PD-L1 mAbs. Representative profiles are shown. Mean fluorescence intensities (MFI) for the expression levels of H-2K^d and PD-L1 were calculated. Means and SD ($n = 3$) are indicated. * $P < 0.05$ by Student's *t*-test. (b) GFP-transduced CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-6^{-/-} BALB/c mice (day 0). Surface expression levels of MHC class I and PD-L1 on GFP⁺CD45⁻ CT26 cells at day 27 were evaluated by flow cytometry using anti-H-2K^d and anti-PD-L1 mAbs. Representative profiles are shown. MFI for the expression levels of H-2K^d and PD-L1 were calculated. Means and SD ($n = 3$) are indicated. * $P < 0.05$ by Student's *t*-test. (c) Surface expression levels of MHC class I and PD-L1 on GFP⁺CD45⁺ cells and GFP⁺CD45⁺CD11c⁺ cells at day 27 were evaluated by flow cytometry using anti-H-2K^d and anti-PD-L1 mAbs. Representative profiles are shown. MFI for the expression levels of H-2K^d and PD-L1 were calculated. Means and SD ($n = 3$) are indicated. * $P < 0.05$ by Student's *t*-test.

existence of a CD8⁺ T-independent beneficial effect of IL-6 deficiency (Fig. 2b). To address the involvement of CD4⁺ T cells in the tumorigenesis, we performed depletion of CD4⁺ T cells by using anti-CD4 mAb in this model. In the early phase, such as at day 9 after inoculation of CT26 cells, the tumor growth was enhanced by *in vivo* injection of anti-CD4 mAb; however, we found that depletion of CD4⁺ T cells significantly suppressed CT26 tumor growth at a later phase under IL-6-deficient condition (Fig. S2a–c). These data not only indicate that IL-6 will suppress anti-tumor function of CD4⁺ T cells at induction phase but also suggest that suppression of anti-tumor immunity by Tregs may be involved in this model.

We further investigated the effect of both CD4⁺ T and CD8⁺ T depletions and compared with CD8⁺ T single depletion in IL-6-deficient condition. As the result, we found that there was no significant difference between the both depletions and CD8⁺ T single depletion (Fig. S2a–c). These data suggest that IL-6 will suppress CD8⁺ T cells and other immune cells, but not CD4⁺ T cells, or may also directly stimulate cancer cells to augment the tumor growth in this model mice.

Type-1 condition enhances expression levels of MHC class I and PD-L1 on CT26 cells *in vitro* and *in vivo*. We examined the characteristic features of CT26 cancer cells under the type-1 immune condition. IFN- γ stimulation enhanced surface expression levels of H-2K^d, MHC class I and PD-L1 on CT26 cells *in vitro* (Fig. 4a). In addition, we found that both MHC class I and PD-L1 expression levels on CT26 cells were significantly

augmented in tumor-bearing IL-6^{-/-} mice compared with wild-type mice (Fig. 4b).

We further investigated the PD-L1 expression levels on CD45⁺ immune cells and CD11c⁺ dendritic cells in tumor tissues of wild-type and IL-6^{-/-} mice. As a result, we confirmed that PD-L1 expression levels on both populations in IL-6^{-/-} mice were significantly enhanced compared with those in wild-type mice (Fig. 4c).

These data suggest that CT26 cells and immune cells including dendritic cells attenuated antitumor responses by effector T cells through the PD-L1 expressions under the IL-6-deficient condition *in vivo*, whereas MHC class I-restricted recognition by cytotoxic CD8⁺ T cells was increased.

Interleukin-6 deficiency facilitates antitumor effects of immune checkpoint therapy using the anti-PD-L1 mAb. To investigate the effect of immune checkpoint inhibition under the IL-6-deficient condition, we injected the anti-PD-L1 mAb into CT26 tumor-bearing wild-type and IL-6^{-/-} mice. We found that *in vivo* injection of the anti-PD-L1 mAb into IL-6-deficient mice significantly reduced tumorigenesis compared with wild-type mice (Fig. 5a,b).

Moreover, we performed PD-L1 combination therapy on IL-6^{-/-} mice using 4T1, murine breast cancer cells. As a result, we confirmed that tumor growth of the 4T1 model mice was significantly reduced under IL-6-deficient condition. The therapy using anti-PD-L1 mAb under IL-6-deficient condition was very effective as well as CT26, murine colon cancer cells (Fig. S3a–c). In this study, we further evaluated the effect of

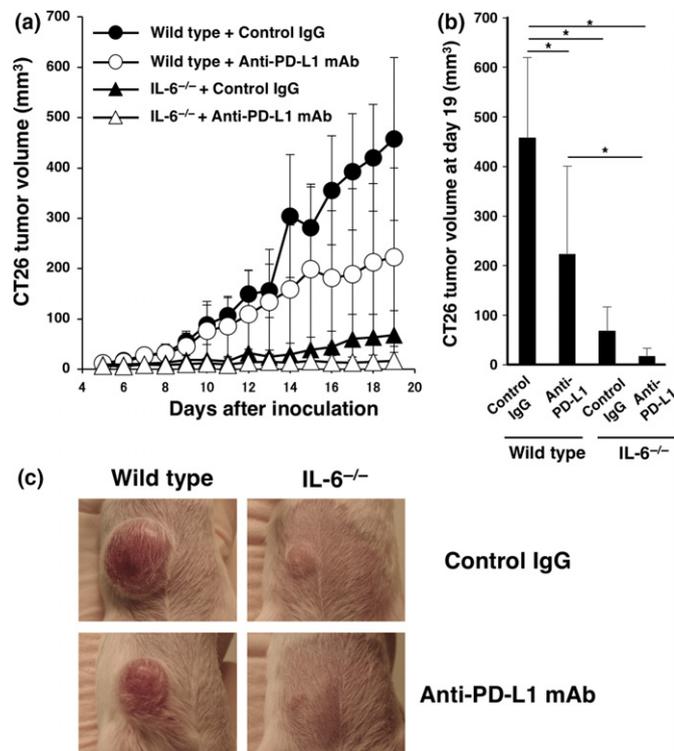


Fig. 5. Effect of PD-L1 blockade on tumorigenesis in interleukin (IL)-6-deficient mice. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-6^{-/-} BALB/c mice (day 0). Anti-PD-L1 mAb or control IgG (200 μ g/mouse) was injected intraperitoneally into wild type and IL-6^{-/-} mice at day 5 and then every 4 days thereafter. (a) Tumor size was monitored for 19 days after injection. Means and SD ($n = 5$) of the tumor volume are indicated. (b) Tumor volumes at day 19 are indicated. * $P < 0.05$ by Dunnett's test. (c) Representative images of tumors at day 15.

in vivo injection anti-IL-6R mAb into CT26 tumor-bearing wild-type mice. As a result, we confirmed that the administration of anti-IL-6R mAb significantly suppressed CT26 tumor growth in this therapy model (Fig. S4a-c).

These data suggest that the lack of IL-6, which enhanced type-1 immunity in the tumor-bearing state, enhances the antitumor effect of immune checkpoint therapy using an anti-PD-L1 mAb.

***In vivo* injection of the anti-PD-L1 mAb into interleukin-6-deficient mice promotes accumulation of T cells and mature dendritic cells in the tumor microenvironment.** We evaluated immune cells in the tumor environment of CT26 tumor-bearing wild-type and IL-6^{-/-} mice injected with the anti-PD-L1 mAb by IHC. CD3⁺ T cells and CD11c⁺ dendritic cells were highly accumulated in the tumor tissues of anti-PD-L1 mAb-injected IL-6^{-/-} mice (Fig. 6a). We further investigated the ratios of tumor-infiltrating immune cells by flow cytometry. As a result, cytotoxic CD8⁺ T cells and CD11c⁺I-A^{high} mature dendritic cells were more infiltrated in the tumor tissues of IL-6-deficient mice injected with the anti-PD-L1 mAb compared with wild-type mice (Fig. 6b). These data suggest that a lack of IL-6 in the tumor-bearing host may facilitate the introduction of antitumor effector T cells and mature dendritic cells into the tumor microenvironment by anti-PD-L1 mAb treatment.

Poly I:C administration under the interleukin-6-deficient condition shows a strong antitumor effect *in vivo*. Administration of immunological adjuvants occasionally induces IL-6 production *in vivo*.⁽²¹⁾ As a therapeutic experiment, we injected poly I:C,

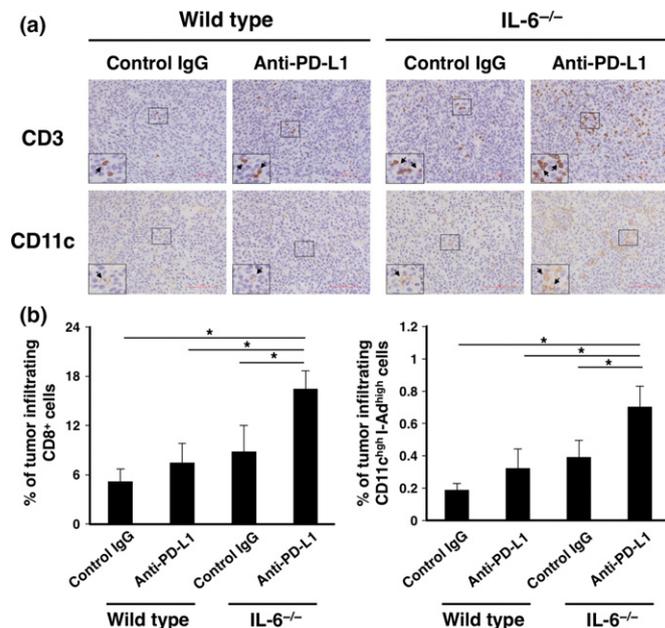


Fig. 6. Immune status of anti-PD-L1 mAb-treated tumor-bearing mice under the interleukin (IL)-6-deficient condition. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-6^{-/-} BALB/c mice (day 0). (a) Immunohistochemistry (IHC) staining of tumor tissues collected at day 27 was performed using anti-CD3 and anti-CD11c mAbs. Representative images are shown. Bar represents 100 μ m. (b) Tumor-infiltrating immune cells of wild-type and IL-6^{-/-} mice at day 27 were evaluated by flow cytometry using anti-CD8, anti-CD11c and anti-I-Ad mAbs. Percentages of CD8⁺ T cells and CD11c⁺I-A^{high} dendritic cells were calculated. Means and SD ($n = 3$) are indicated. * $P < 0.05$ by Dunnett's test.

a TLR3 ligand, into tumor-bearing wild-type and IL-6^{-/-} mice. The poly I:C injection significantly reduced tumorigenesis in both IL-6-deficient mice and wild-type mice (Fig. 7a,b).

In this study, we further investigated IL-12 production by CD11c⁺ cells from tumor tissues of polyI:C- or control PBS-treated wild-type or IL-6^{-/-} mice. As a result, we confirmed that the IL-12p35 gene expression levels of control-stimulated and poly I:C-stimulated CD11c⁺ cells of wild-type mice were significantly low compared with those of IL-6^{-/-} mice, respectively. Furthermore, we found that the poly I:C stimulation in CD11c⁺ cells induce IL-12 production in both IL-6-free condition and in IL-6-sufficient condition (Fig. S5). These data suggest that poly I:C-induced IL-6 production suppresses antitumor immunity and may promote *in vivo* tumorigenesis, whereas the polyI:C injection induces IL-12 production by dendritic cells in tumor-bearing host.

Discussion

Persisting induction and maintenance of tumor-specific cytotoxic T cells in the tumor microenvironment are crucial to overcome cancer. The present study showed that IL-6 produced by the tumor-bearing host augmented tumorigenesis by decreasing introduction of antitumor effector cells, such as cytotoxic T cells and mature dendritic cells, into the tumor microenvironment. Recently, we demonstrated that IL-6 treatment attenuates the surface expression level of MHC class II and IL-12 production of human dendritic cells, and, in fact, impairs IFN- γ production by CD4⁺ T cells *in vitro*.⁽²⁰⁾ IL-12 is an important cytokine for induction of Th1 immunity, because

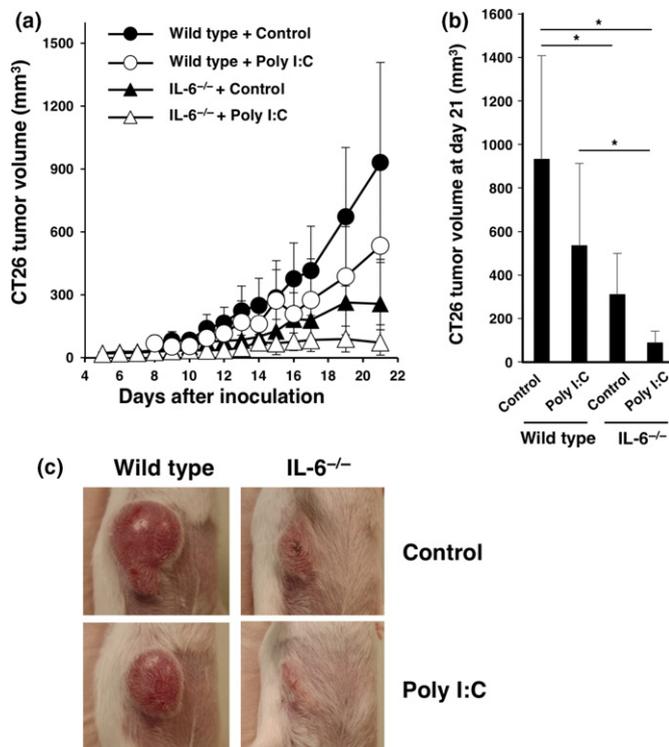


Fig. 7. Effect of poly I:C treatment on tumorigenesis in interleukin (IL)-6-deficient mice. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-6^{-/-} BALB/c mice (day 0). Poly I:C (50 μ g/mouse) or control PBS was injected intraperitoneally into wild-type and IL-6^{-/-} mice at day 5 and then every 4 days thereafter. (a) Tumor size was monitored for 21 days after injection. Means and SD ($n = 5$) of the tumor volume are indicated. (b) Tumor volumes at day 21 are indicated. * $P < 0.05$ by Dunnett's test. (c) Representative images of tumors at day 15.

IL-12 activates STAT4 in CD4⁺ T cells, inducing subsequent IFN- γ secretion.^(22–24) The helper function of antigen-specific Th1 cells is essential to induce fully activated cytotoxic T cells in tumor-bearing hosts.⁽²⁵⁾ In this study, CT26 tumor growth was enhanced by *in vivo* injection of anti-CD4 mAb at an early phase; however, we found that depletion of CD4⁺ T cells significantly suppressed CT26 tumor growth at the later phase under IL-6-deficient condition. These data suggest that involvement of Tregs exists in our experiment using anti-CD4 mAb. Furthermore, we speculate that there is a possibility that anti-tumor effects of CD4⁺ T cells that can be rescued by IL-6 deficiency may be exerted through CD8⁺ T cells at the later phase in our tumor-bearing model. Generally, certain responses of CD8⁺ T cells, such as long-lasting proliferation/survival or recruitment into draining LN and tumor site, are known to be helped by CD4⁺ T cells, which may also be mediated through a mutual interaction with dendritic cells. Therefore, we speculated that IL-6 produced in the tumor-bearing host suppresses type-1 antitumor immunity involving the activation of helper and cytotoxic T cells by causing dysfunction of dendritic cells.

We isolated CD11c⁺ dendritic cells from tumor tissues and performed transcriptome analysis by next generation sequencing. We found 2121 genes changed between wild-type and IL-6^{-/-} dendritic cells ($q < 0.2$) and confirmed that STAT1 gene expression (FPKM) in wild-type and IL-6^{-/-} dendritic cells were 66882.5 and 84507.7, respectively, suggesting that the IL-6-deficient condition in the tumor-bearing state augments

the STAT1-mediated signaling pathway in CD11c⁺ dendritic cells. This result is consistent with the augmented IFN- γ production by Th1 and Tc1 cells in the IL-6-deficient tumor-bearing host.

Previous studies have reported that PD-L1 expression is induced by IFN- γ production from lymphocytes and STAT-1 activation based on type-1 immunity.^(26–28) In general, PD-L1 expressed on cancer cells reduces antitumor immune responses by PD-1-expressing effector cytotoxic T cells. In this study, we revealed that the IL-6-deficient condition increased Th1 and Tc1 immunity that caused augmentation of the PD-L1 expression level on CT26 cancer cells and immune cells including dendritic cells *in vivo*, suggesting negative feedback regulation. Based on these findings, we blocked PD-1/PD-L1 signaling under the IL-6-deficient condition by administration of the anti-PD-L1 mAb *in vivo*. As expected, injection of the anti-PD-L1 mAb into CT26 tumor-bearing IL-6^{-/-} mice strongly inhibited tumorigenesis *in vivo*. These data suggest that the mechanism of IL-6-dependent immunosuppression might be different from the PD1/PD-L1-mediated dysfunction of antitumor immunity.

A TLR3 ligand, poly I:C, among immunological adjuvants is a powerful agent to induce antitumor immunity by increasing IL-12 production from dendritic cells in tumor-bearing hosts.⁽²⁹⁾ However, the TLR3/TRIF/MyD88-NF- κ b signaling pathway in dendritic cells induces both IL-6 and IL-12 production.⁽²¹⁾ We confirmed that the IL-6-deficient condition promoted IL-12 induction and the antitumor effect of immunoadjuvant therapy using poly I:C.

Generally, cancer cells as well as immune cells produced IL-6 in tumor-bearing hosts.⁽¹⁶⁾ Therefore, we have to block the immunosuppressive effects of IL-6 from both tumor cells and the other host cells to develop more effective cancer immunotherapy. We confirmed that blockade of IL-6 signaling by *in vivo* injection of anti-IL-6R mAb significantly suppressed tumor growth in the present study and other models as previously reported.^(15,16) A recent clinical trial using an anti-IL-6R mAb, tocilizumab, with carboplatin/doxorubicin and IFN- α 2b has been conducted for patients with recurrent epithelial ovarian cancer.⁽³⁰⁾ This clinical study reported that myeloid cells in the IL-6R mAb-treated patients had produced more IL-12, while T cells were more activated and secreted higher amounts of effector cytokines including IFN- γ . Therefore, antibodies for IL-6R may be a good tool to block IL-6-signaling cascade caused by IL-6 produced in tumor-bearing hosts.

In this study, we found that the lack of IL-6 in a tumor-bearing host suppresses tumorigenesis and augments the accumulation of immune cells *in vivo*. Furthermore, we confirmed that the IL-6-deficient condition significantly promotes the antitumor effects of *in vivo* injection of an anti-PD-L1 mAb or poly I:C, an immunological adjuvant. Based on the present data, we speculate that blockade of the IL-6 signaling pathway may promote introduction of antitumor immunity into the tumor-bearing host. Such an approach may be a promising strategy for the development of more effective immune checkpoint blockade therapies using anti-PD-1/PD-L1 mAbs and immunological adjuvants such as poly I:C for cancer patients.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

- 1 Hodi FS, O'Day SJ, McDermott DF *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711–23.
- 2 Wolchok JD, Kluger H, Callahan MK *et al.* Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* 2013; **369**: 122–33.
- 3 Brahmer JR, Tykodi SS, Chow LQM *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; **366**: 2455–65.
- 4 Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* 2012; **12**: 265–77.
- 5 Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity* 2013; **39**: 38–48.
- 6 Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* 2007; **449**: 419–26.
- 7 Steinman RM, Mellman I. Immunotherapy: bewitched, bothered, and bewildered no more. *Science* 2004; **305**: 197–200.
- 8 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* 2000; **19**: 2548–56.
- 9 Hirano T, Yasukawa K, Harada H *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 1986; **324**: 73–6.
- 10 Knpfer H, Preiss R. Serum interleukin-6 levels in colorectal cancer patient—A summary of published results. *Int J Colorectal Dis* 2010; **25**: 135–40.
- 11 Makuuchi Y, Honda K, Osaka Y *et al.* Soluble interleukin-6 receptor is a serum biomarker for the response of esophageal carcinoma to neoadjuvant chemoradiotherapy. *Cancer Sci* 2013; **104**: 1045–51.
- 12 Hazama S, Takenouchi H, Tsunedomi R *et al.* Predictive biomarkers for the outcome of vaccination of five therapeutic epitope peptides for colorectal cancer. *Anticancer Res* 2014; **34**: 4201–5.
- 13 Kitamura H, Kamon H, Sawa SI *et al.* IL-6-STAT3 controls intracellular MHC class II $\alpha\beta$ dimer level through cathepsin S activity in dendritic cells. *Immunity* 2005; **23**: 491–502.
- 14 Park SJ, Nakagawa T, Kitamura H *et al.* IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. *J Immunol* 2004; **173**: 3844–54.
- 15 Sumida K, Wakita D, Narita Y *et al.* Anti-IL-6 receptor mAb eliminates myeloid-derived suppressor cells and inhibits tumor growth by enhancing T-cell responses. *Eur J Immunol* 2012; **42**: 2060–72.
- 16 Narita Y, Kitamura H, Wakita D *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* 2013; **190**: 812–20.
- 17 Tsukamoto H, Nishikata R, Senju S *et al.* Myeloid-derived suppressor cells attenuate TH1 development through IL-6 production to promote tumor progression. *Cancer Immunol Res* 2013; **1**: 64–76.
- 18 Tsukamoto H, Senju S, Matsumura K *et al.* IL-6-mediated environmental conditioning of defective Th1 differentiation dampens antitumor immune responses in old age. *Nat Commun* 2015; **6**: 6702.
- 19 Tsukamoto H, Fujieda K, Hirayama M *et al.* Soluble IL-6R expressed by myeloid cells reduces tumor-specific Th1 differentiation and drives tumor progression. *Cancer Res* 2017; **77**: 2279–91.
- 20 Ohno Y, Kitamura H, Takahashi N *et al.* 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 Immunol Immunother* 2016; **65**: 193–204.
- 21 Hu Y, Cong X, Chen L *et al.* Synergy of TLR3 and 7 ligands significantly enhances function of DCs to present inactivated PRRSV antigen through TRIF/MyD88-NF- κ B signaling pathway. *Sci Rep* 2016; **6**: 23977.
- 22 Morinobu A, Gadina M, Strober W *et al.* STAT4 serine phosphorylation is critical for IL-12-induced IFN- γ production but not for cell proliferation. *Proc Natl Acad Sci USA* 2002; **99**: 12281–6.
- 23 Hsieh CS, Macatonia SE, Tripp CS *et al.* Development of TH1 CD4+ T cells through IL-12 produced by listeria-induced macrophage. *Science* 1993; **260**: 547–9.
- 24 Kalinski P, Hilkens CM, Snijders A *et al.* IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 1997; **159**: 28–35.
- 25 Nishimura T, Iwakabe K, Sekimoto M *et al.* Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med* 1999; **190**: 617–27.
- 26 Bellucci R, Martin A, Bommarito D *et al.* Interferon- γ -induced activation of JAK1 and JAK2 suppresses tumor cell susceptibility to NK cells through upregulation of PD-L1 expression. *Oncoimmunology* 2015; **4**: e1008824.
- 27 Kharma B, Baba T, Matsumura N *et al.* STAT1 drives tumor progression in serous papillary endometrial cancer. *Cancer Res* 2014; **74**: 6519–30.
- 28 Abiko K, Matsumura N, Hamanishi J *et al.* IFN- γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. *Br J Cancer* 2015; **112**: 1501–9.
- 29 Azuma M, Takeda Y, Nakajima H *et al.* Biphasic function of TLR3 adjuvant on tumor and spleen dendritic cells promotes tumor T cell infiltration and regression in a vaccine therapy. *Oncoimmunology* 2016; **5**: e1188244.
- 30 Dijkgraaf EM, Santegoets SJ, Reyners AK *et al.* A phase I trial combining carboplatin/doxorubicin with tocilizumab, an anti-IL-6R monoclonal antibody, and interferon- α 2b in patients with recurrent epithelial ovarian cancer. *Ann Oncol* 2015; **26**: 2141–9.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Cytokine producing ability of CD4⁺ cells in draining lymph nodes under interleukin (IL)-6-condition.

Fig. S2. Effect of CD4⁺ T and CD8⁺ T cells on CT26 tumorigenesis in interleukin (IL)-6-deficient mice.

Fig. S3. Effect of PD-L1 blockade on tumorigenesis of 4T1 murine breast cancer cells in interleukin (IL)-6-deficient mice.

Fig. S4. Effect of IL-6R and PD-L1 blockade on CT26 tumorigenesis in wild-type mice.

Fig. S5. Effect of poly I:C treatment on IL-12p35 gene induction in tumor-infiltrating CD11c⁺ dendritic cells.