Cancer Science

Japanese Cancer Association JCA

Open Access

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

Yosuke Ohno,[1,2] Yujiro Toyoshima,[1,2] Hideaki Yurino,[3] Norikazu Monma,[4] Huihui Xiang,[1] Kentaro Sumida,[1] Shun Kaneumi,[1] Satoshi Terada,[1] Shinichi Hashimoto,[3] Kazuho Ikeo,[4] Shigenori Homma,[2] Hideki Kawamura,[2] Norihiko Takahashi,[2] Akinobu Taketomi[2] and Hidemitsu Kitamura[1]

[1]Division of Functional Immunology, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University; [2]Department of Gastroenterological Surgery I, Hokkaido University Graduate School of Medicine, Sapporo; [3]Department of Integrative Medicine for Longevity, Graduate School of Medical Sciences, Kanazawa University, Kanazawa; [4]DNA Data Analysis Laboratory, National Institute of Genetics, Mishima, Japan

Key words

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

Correspondence

Hidemitsu Kitamura, Division of Immunoregulation, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan.
Tel: +81-11-706-5520; Fax: +81-11-706-5519;
E-mail: kitamura@igm.hokudai.ac.jp

Funding Information

This work was partially supported by Grants-in-Aid for Scientific Research (C) (25460584 and 15K08416 to H. K. and 16K10526 to N. T.) from the Japan Society for the Promotion of Science (JSPS), Grants-in-Aid for JSPS Research Fellows (14J06854 to S. T. and 13J01464 to S. K.), the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (H. K.), the Japan Agency for Medical Research and Development (AMED) (H. K. and A. T.), and the Joint Research Program of the Institute for Genetic Medicine, Hokkaido University (S. H. and A. T.).

Received March 15, 2017; Revised July 21, 2017; Accepted July 24, 2017

Cancer Sci **108** (2017) 1959–1966

doi: 10.1111/cas.13330

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.

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 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 phosphatidylinositide 3-kinase/

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

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). $IL\text{-}6^{-/-}$ 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 Biolegend (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 μg/mL streptomycin, 10 mM HEPES and 0.05 mmol/L 2-mercaptoethanol (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2. For flow cytometry, 2.5×10^5 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 (1×10^6) were injected intradermally into wild-type and $IL\text{-}6^{-/-}$ mice (day 0). The tumor size was measured by micrometer calipers from day 5. Tumor volumes were calculated using the following formula: volume (mm^3) = 0.2 × (length [mm] × width [mm]) × (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 μg/mouse) was injected intraperitoneally into wild-type and $IL\text{-}6^{-/-}$ mice at days −1 and 5, and then every 4 days thereafter. In therapeutic experiments, the anti-PD-L1 mAb (200 μg/mouse), control IgG (200 μg/mouse) or poly I:C (50 μg/mouse) were injected intraperitoneally into CT26 tumor-bearing wild-type and $IL\text{-}6^{-/-}$ mice at day 5 and then every 4 days thereafter.

Immunohistochemistry. Tumor tissues obtained from CT26 tumor-bearing wild-type and $IL\text{-}6^{-/-}$ 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-Hamstrer 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; Vecter Laboratories, Burlingame, CA, USA) at room temperature for 30 min. Protein expression was visualized using 3-3′-diaminobezidine-4HCL at room temperature for 5 min. Finally, all sections were counterstained with Mayer's H&E.

Flow cytometry. GFP-transduced CT26 cells (1×10^6) were intradermally injected into wild-type and $IL\text{-}6^{-/-}$ mice. Tumor

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

tissues were obtained from CT26 tumor-bearing wild-type and IL-$6^{-/-}$ mice at day 12. The retrieved tissues were minced using scissors and digested in a 1-mg/mL collagenase solution (Sigma, Tokyo, Japan). Cell suspensions were prepared and stained with fluorescent dye-conjugated mAbs after purified anti-FcγR mAb treatment. $GFP^{+}CD45^{-}$ and $GFP^{-}CD45^{+}$ populations collected from tumor tissues were determined as CT26 cancer cells and immune cells, respectively. A FACSCanto II (BD Bioscience) was used for flow cytometry. The results were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). The mean fluorescence intensity was calculated in paired samples.

Intracellular cytokine staining. For detection of cytoplasmic IFN-γ expression in T cells, single cell suspensions from tumors and regional lymph nodes (1×10^6 cells in 12-well culture dishes) 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 the anti-CD4 mAb, anti-CD8 mAb and 7-AAD, and fixed with 4% paraformaldehyde. After permeabilization, the fixed cells were stained with the anti-IFN-γ mAb. Data were acquired by flow cytometry.

Statistical analyses. *In vitro* experiments were repeated at least three to five times. *In vivo* experiments consisting of 5–10 mice in each group were independently performed two to three times. Single representative experiments are indicated in figures. Mean values and SD were calculated for each dataset. Significant differences in the results were determined by a one-way ANOVA and Dunnett's post-test. In some experiments, the two-tailed Student's *t*-test was used for evaluation of the difference between two groups. The *P*-values of $*P < 0.05$ were indicated as statistically significant in the figures, using the two-sided Student's *t*-test. $P < 0.05$ was considered as significant.

Results

Lack of interleukin-6 in the tumor-bearing host suppresses tumorigenesis and augments accumulation of immune cells *in vivo*. To address the relationship of IL-6 deficiency with tumor growth *in vivo*, murine CT26 colorectal cancer cells were intradermally injected into wild-type and IL$6^{-/-}$ mice. Tumor growth was significantly reduced in IL-$6^{-/-}$ mice compared with wild-type mice (Fig. 1a,b). Next, we evaluated the tumor-infiltrating cells by immunohistochemistry (IHC) to

Fig. 1. *In vivo* tumorigenesis and immune status 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) Tumor size was monitored for 13 days after injection. Means and SD ($n = 5$) of the tumor volume are indicated. $*P < 0.05$ by Student's *t*-test (b) Representative images of tumors at day 15. (c) H&E and immunohistochemistry (IHC) staining of tumor tissues collected at day 14 were performed using anti-CD3 and anti-CD11c antibodies. Representative images are shown. Bar represents 100 μm. (d) Tumor-infiltrating immune cells of wild-type and IL-$6^{-/-}$ mice at day 12 were evaluated by flow cytometry using anti-CD45, anti-CD4, anti-CD8, anti-CD11c and anti-I-A^{d} mAbs. Percentages of $CD45^{+}$, $CD4^{+}$ T, $CD8^{+}$ T and $CD11c^{+}$I-A^{dhigh} dendritic cells were calculated. Means and SD ($n = 5$) are indicated. $*P < 0.05$ by Student's *t*-test.

Fig. 2. Effect of $CD8^{+}$ T cells 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). An anti-CD8 mAb or control IgG (200 μg/mouse) was injected intraperitoneally into wild-type and IL-$6^{-/-}$ mice at days –1 and 5, and then every 4 days thereafter. (a) Tumor size was monitored for 13 days after injection. Means and SD ($n = 5$) of the tumor volume are indicated. $*P < 0.05$ by Dunnett's test. (b) Tumor volumes at day 20 are indicated. $*P < 0.05$ by Dunnett's test. (c) Representative images of tumors at day 15.

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

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^{dhigh} 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

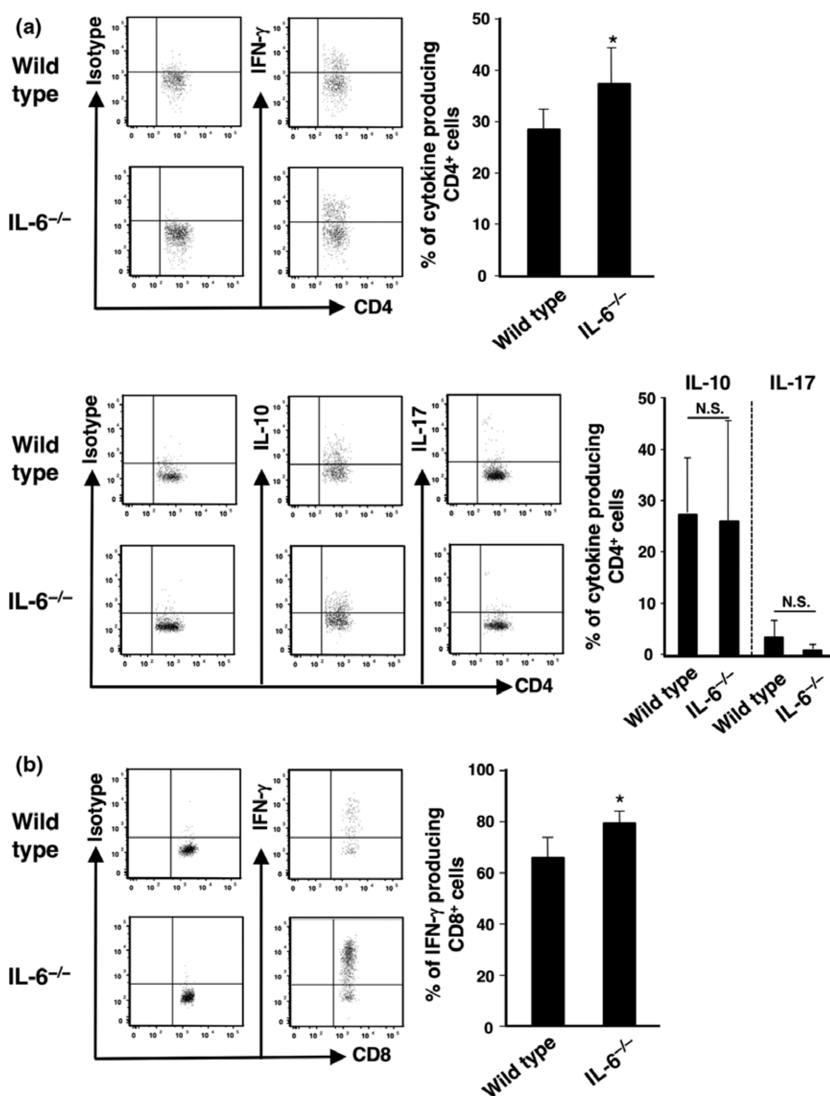

Fig. 3. Cytokine-producing ability of tumor infiltrating CD4$^+$ T and CD8$^+$ T cells under the interleukin (IL)-6-deficient condition. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-6$^{-/-}$ BALB/c mice (day 0). Cytokine production by CD4$^+$ T and CD8$^+$ 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.

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

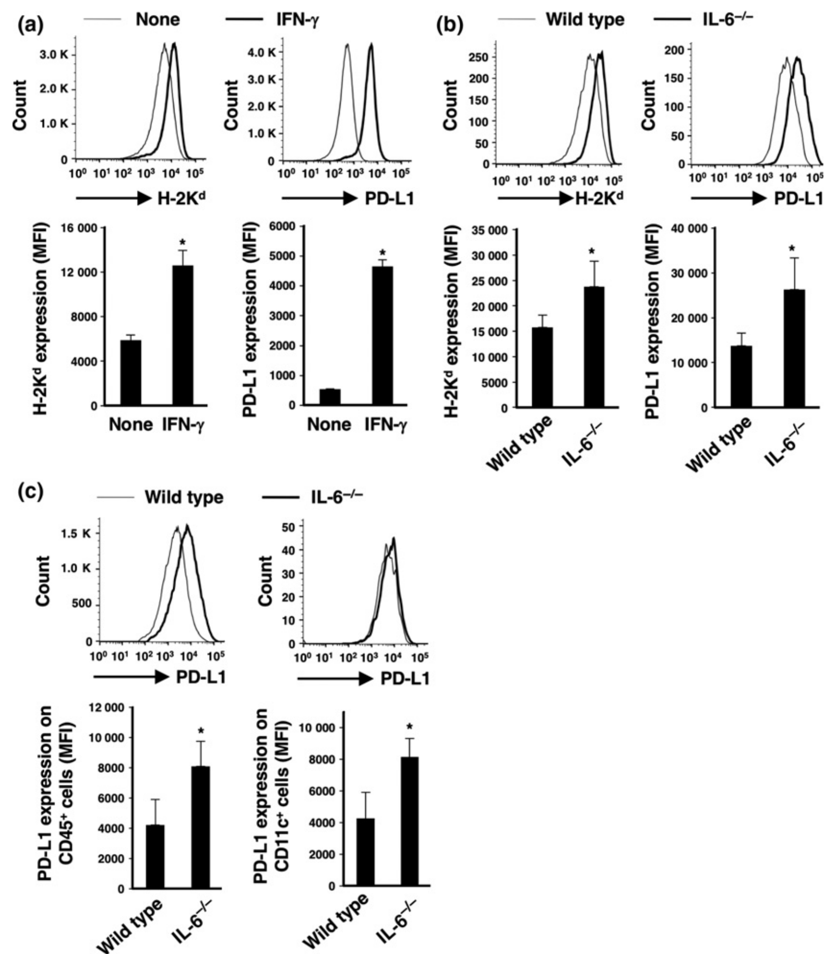

Fig. 4. Expression levels of MHC class I and PD-L1 on CT26 cells *in vitro* and *in vivo*. (a) CT26 cells were stimulated with IFN-γ (50 ng/mL) for 24 h *in vitro*. Surface expression levels of MHC class I and PD-L1 on CT26 cells were evaluated by flow cytometry using anti-H-2K^d and anti-PD-L1 mAbs. 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

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

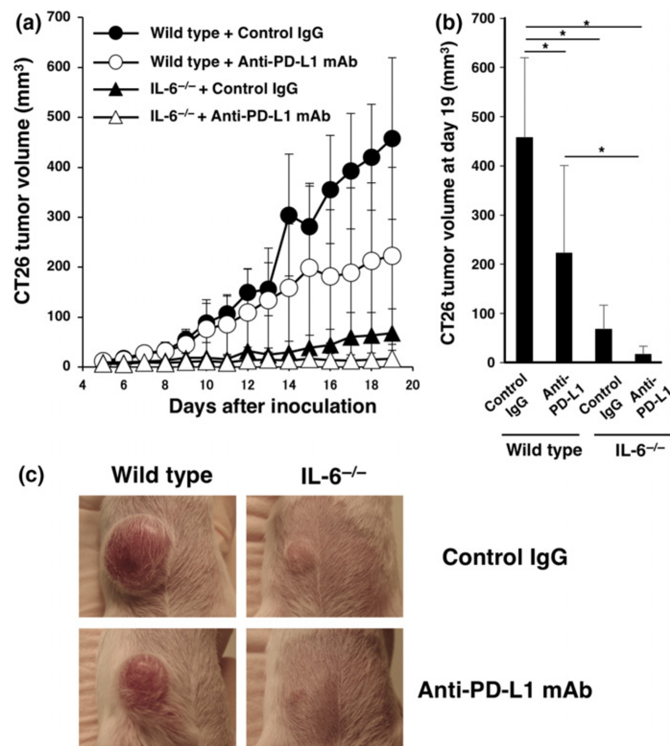

Fig. 5. Effect of PD-L1 blockade on tumorigenesis in interleukin (IL)-6-deficient mice. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-$6^{-/-}$ BALB/c mice (day 0). Anti-PD-L1 mAb or control IgG (200 μg/mouse) was injected intraperitoneally into wild type and 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.

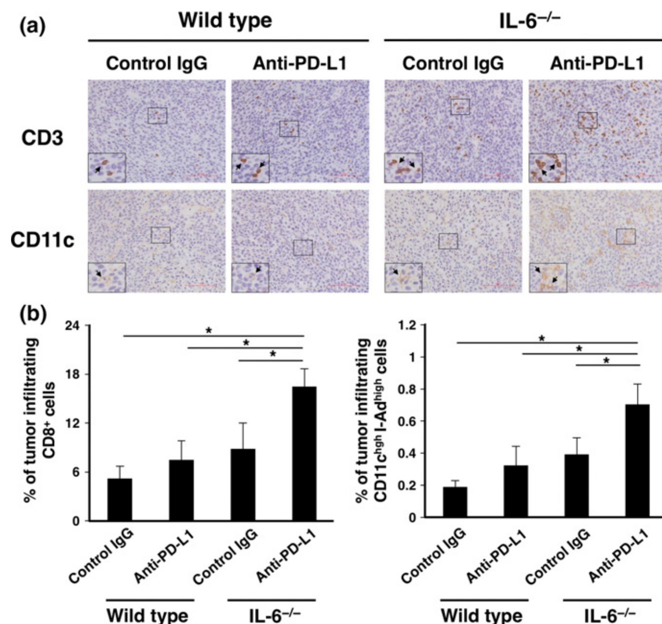

Fig. 6. Immune status of anti-PD-L1 mAb-treated tumor-bearing mice under the interleukin (IL)-6-deficient condition. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-$6^{-/-}$ BALB/c mice (day 0). (a) Immunohistochemistry (IHC) staining of tumor tissues collected at day 27 was performed using anti-CD3 and anti-CD11c mAbs. Representative images are shown. Bar represents 100 μm. (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^{dhigh} dendritic cells were calculated. Means and SD (n = 3) are indicated. *$P < 0.05$ by Dunnett's test.

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^{dhigh} 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, 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

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

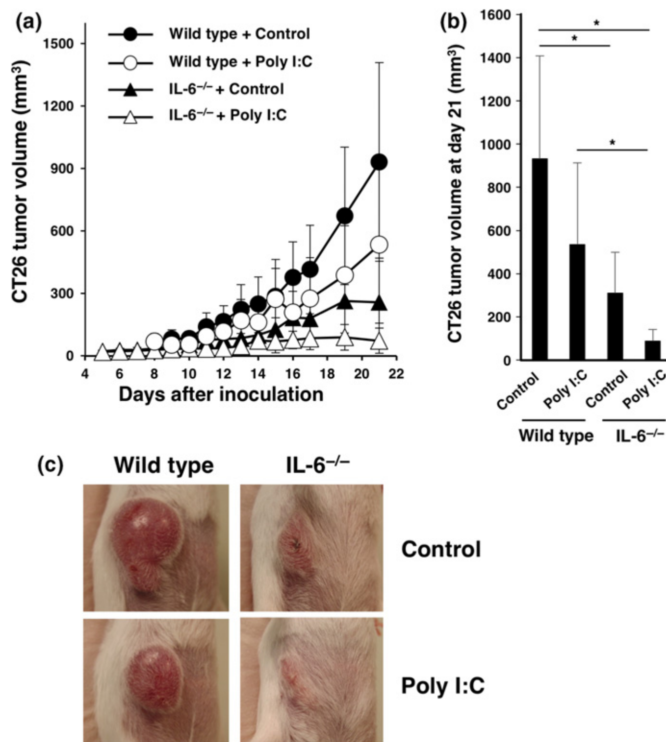

Fig. 7. Effect of poly I:C treatment on tumorigenesis in interleukin (IL)-6-deficient mice. CT26 murine colon cancer cells (1×10^6) were intradermally injected into wild-type and IL-$6^{-/-}$ BALB/c mice (day 0). Poly I:C (50 μg/mouse) or control PBS was injected intraperitoneally into wild-type and 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-kb 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-α2b 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.

Acknowledgments

We thank Ms A. Nishiuchi for her excellent technical and secretarial assistance and Dr T. Nishimura for thoughtful advice.

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

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 αβ 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 antitumour 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-κB 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-gamma production but not for cell proliferation. *Proc Natl Acad Sci USA* 2002; **99**: 12281–6.
23 Hsich 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-g-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-α2b 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.

© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.