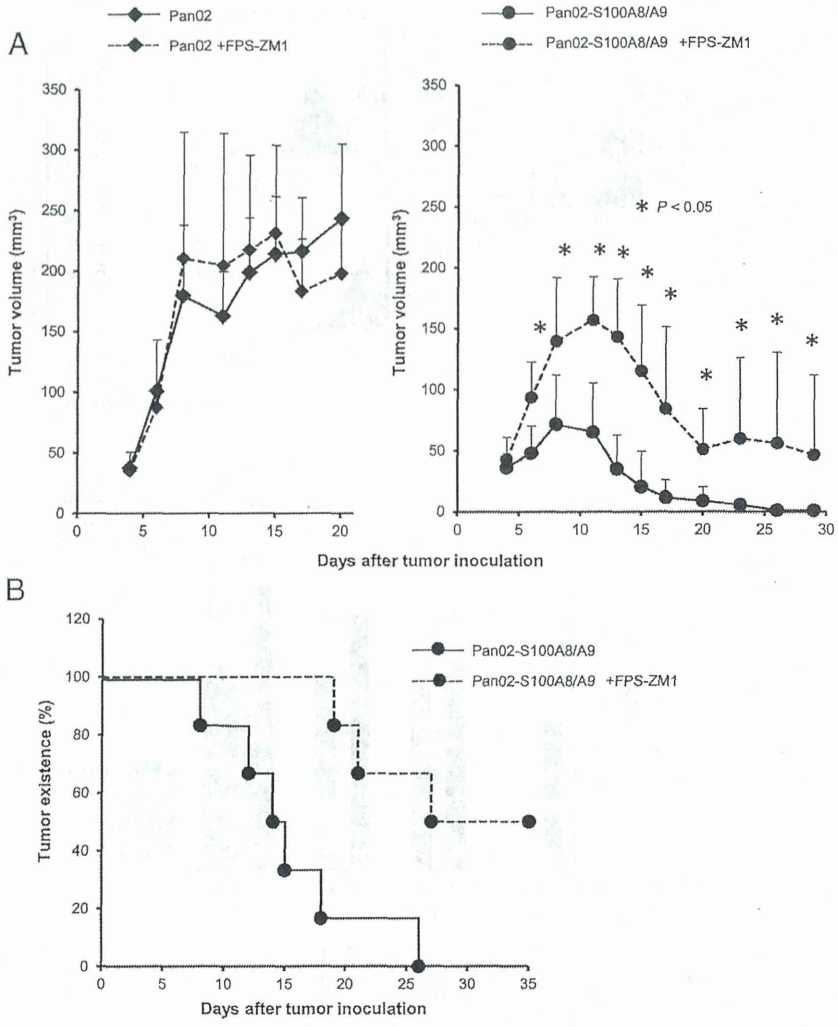

FIGURE 5. RAGE blockade formed Pan02-S100A8/A9 tumors in the immune-competent mice. (**A**) The growth of Pan02 s.c. tumors in RAGE-blocking model. Pan02 (*left panel*, $n = 6$) and Pan02-S100A8/A9 (*right panel*, $n = 6$) cells were inoculated into C57BL/6 mice, and FPS-ZM1 (1 mg/kg) was i.p. administered into the mice every other day. (**B**) Existence of Pan02-S100A8/A9 tumors in RAGE-blocking model. Tumor existence ratios of Pan02-S100A8/A9 in the same mice of (A) were plotted at the indicated days ($n = 6$).

S100A8–serum amyloid A–TLR4 establishes a premetastatic lung and that this inflammation-like state accelerated the migration of primary tumor cells to lung tissues in a mouse model. Cheng et al. (34) reported that S100A8/A9 plays important roles in the generation and recruitment of myeloid-derived suppressor cells in tumors. Both sides of immune stimulation or immune suppression on a tumor immune environment need consideration to understand the role of S100A8/A9 in cancer biology. The effect of S100A8/A9 on tumor growth may be determined based on the balance between the activation of innate immunity and the construction of an immune-tolerant microenvironment within tumors. We speculate that the level, location, and term of S100A8/A9 expression are related to the balance. Although the stimulation of innate immunity by S100A8/A9 may explain the better prognosis in some types of cancer, further studies are needed to understand the different manner of S100A8/A9-mediated in vivo effects.

In conclusion, we found a novel activation mechanism of NK cells via S100A8/A9–RAGE signaling, which may open a novel perspective on the in vivo interaction between inflammation and innate immunity. Furthermore, because S100A8/A9 strongly enhances the activity of NK cells, the S100A8/A9–NK cell axis may be useful for cancer immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

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ORIGINAL ARTICLE

Type I IFN gene delivery suppresses regulatory T cells within tumors

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Type I interferon (IFN) is a pleiotropic cytokine regulating the cancer cell death and immune response. IFN-α can, as we have also reported, effectively induce an antitumor immunity by the activation of tumor-specific T cells and maturation of dendritic cells in various animal models. Unknown, however, is how the type I IFN alters the immunotolerant microenvironment in the tumors. Here, we found that intratumoral IFN-α gene transfer significantly decreased the frequency of regulatory T cells (Tregs) per $CD4^+$ T cells in tumors. The concentration of a Treg-inhibitory cytokine, interleukin (IL)-6, was correlated with the IFN-α expression level in tumors, and intratumoral $CD11c^+$ cells produced IL-6 in response to IFN-α stimulation. To confirm the role of IL-6 in the suppression of Tregs in tumors, an anti-IL-6 receptor antibody was administered in IFN-α-treated mice. The antibody increased the frequency of Tregs in the tumors, and attenuated systemic tumor-specific immunity induced by IFN-α. Furthermore, the IFN-α-mediated IL-6 production increased the frequency of Th17 cells in the tumors, which may be one of the mechanisms for the reduction of Tregs. The study demonstrated that IFN-α gene delivery creates an environment strongly supporting the enhancement of antitumor immunity through the suppression of Tregs.

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INTRODUCTION

Interferon (IFN)-α is a pleiotropic cytokine regulating anti-proliferation, induction of cell death, anti-angiogenesis and immunomodulation. Although IFN-α was long thought to act mainly by suppressing tumor cell proliferation *in vivo*, more recently it has been established that type I IFNs have multiple immunoregulatory activities to innate and adaptive immunity.[1]

The exogeneous administration of recombinant IFN-α protein has been used for treatment in various types of cancer, including hematological malignancies such as hairy cell leukemia and some B- or T-cell lymphomas, and solid tumors such as melanoma, renal cell carcinoma and Kaposi's sarcoma.[2,3] However, conventional clinical studies with IFN-α protein showed a limited therapeutic efficacy because of the inability to target the cytokine to the right place at a sufficient dose because of the substantial toxicity with systemic administration. Therefore, safer and more effective delivery strategies have been investigated.[4] We previously showed that an intratumoral IFN-α gene transfer allowed an increased and sustained local concentration of IFN-α in tumors, with minimal leakage into the systemic circulation,[5–7] eliciting a systemic tumor-specific immunity in several animal models.[5–9] Owing to the effective induction of antitumor immunity and the lower toxicity, an intratumoral route of the IFN vector is considered to be superior to an intravenous systemic administration.[5]

Immune regulations by type I IFN are comprised of upregulation of major histocompatibility complex class I gene, promotion of the proliferation, survival and differentiation of T and B lymphocytes, stimulation of the cytotoxic activity of natural killer cells and activation of dendritic cells (DCs).[2,10–12] We also reported that DCs in tumors have critical roles: (i) intratumoral expression of IFN-α effectively induces cell death of cancer cells and exposes tumor-associated antigens in large quantity to DCs; (ii) IFN-α promotes the maturation of $CD11c^+$ cells, which facilitates the presentation of tumor-associated antigens on $CD11c^+$ cells; and (iii) $CD11c^+$ cells in tumors transduced with the IFN-α gene produce a large quantity of immune-stimulatory cytokines.[5,7,9]

It is widely known that the induction of tolerance by the tumor microenvironment is one of the critical mechanisms involved in tumor progression and resistance for immune therapy. Extensive studies have shown that $CD4^+Foxp3^+$ regulatory T cells (Tregs) are critical in controlling antitumor immune responses in the tumor microenvironment.[13] Therefore, the modulation or selective depletion of Tregs in tumors is a promising strategy for enhancing the efficacy of cancer immunotherapy.[14,15] As the effect of type I IFN on an immunotolerant microenvironment in tumors is unknown, we investigated the immunological effects of intratumoral IFN-α gene transfer on tumor-infiltrating Tregs in a mouse model. In this study, we found that IFN-α expression decreased the frequency of Tregs and that the production of interleukin (IL)-6 from DCs in the IFN-α-treated tumors may be attributed to this phenomenon.

MATERIALS AND METHODS

Tumor cell lines and recombinant adenovirus vectors

CT26 ($H\text{-}2^d$: American Type Culture Collections, Rockville, MD, USA) is a BALB/c-derived colon cancer cell line and was maintained in the RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum, 2 μM L-glutamine and 0.15% sodium bicarbonate (complete RPMI). The recombinant adenovirus vectors expressing mouse IFN-α (Ad-mIFN) and alkaline phosphatase cDNA (Ad-AP) were prepared as described.[5] The recombinant adenoviruses are based on serotype 5 with deletions of the entire E1 and a part of the E3 regions, and have the CAG promoter, which is a hybrid of the cytomegalovirus immediate early enhancer sequence

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and the chicken β-actin/rabbit β-globin promoter. A cesium chloride-purified virus was desalted using a sterile Bio-Gel P-6 DG chromatography column (Econopac DG 10; Bio-Rad, Hercules, CA, USA) and diluted for storage in a 13% glycerol/phosphate-buffered saline solution. All viral presentations were confirmed by PCR assay to be free of the $E1^+$ adenovirus.

Animals, tumor inoculation and IFN-α gene transfer

Six-to-eight-week-old female BALB/c ($H\text{-}2^d$) mice were purchased from Charles River Japan (Kanagawa, Japan). Animal studies were carried out according to the Guideline for Animal Experiments of the National Cancer Center Research Institute and approved by the Institutional Committee for Ethics in Animal Experimentation. CT26 cells (1×10^6) were injected subcutaneously into the right leg of BALB/c mice (day 0). At 10 days after inoculation (day 10), tumors were directly injected once with 1×10^8 plaque-forming unit of Ad-mIFN or control vector (Ad-AP). The tumor volume was calculated every 2–3 days using the formula: tumor volume = $1/2\times$([the shortest diameter]$^2\times$[the longest diameter]).

ELISpot assays

IFN-γ ELISpot kit (BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's instructions. Briefly, splenocytes (1×10^5) and mitomycin C-treated tumor cells (1×10^4) or syngenic lymphocytes (1×10^4) were co-cultured in 96-well plates pre-coated with mouse IFN-γ (BD Biosciences) for 20 h at 37 °C in complete RPMI medium in duplicate. The syngenic lymphocytes were used to examine whether the therapy induces an autoimmune reaction. After the wells were washed, biotinylated anti-mouse IFN-γ antibody (2 μg ml^{-1}) was added and incubated for 2 h at room temperature. A streptavidin-horseradish peroxidase solution was then added and incubated for 1 h at room temperature. After the addition of an aminoethyl carbazole substrate solution, spots were counted under a stereomicroscope.

Flow cytometric analysis

For surface staining, FITC-CD4, PE-CD4, PE-CD8 and PE-Cy7-CD8 (BD Biosciences) were used. Intracellular molecules were stained using the intracellular fixation/permeabilization kit (eBioscience, San Diego, CA, USA) with APC-Foxp3 (eBioscience), APC-IFN-γ and PE-IL-17A (BD Biosciences) according to the manufacturer's instructions. For intracellular cytokine staining with splenocytes, mechanically disaggregated cells were incubated with CT26 cells (1×10^5) for 24 h; brefeldin-A (10 μg ml^{-1}) was then added for 5 h of incubation at 37 °C in 96-well plates (1×10^6 cells per well). For intracellular cytokine staining with tumor-infiltrating T cells or tumor-draining lymph nodes, mechanically disaggregated cells were cultured at 37 °C for 5 h in 96-well plates in 200 μl in the presence of brefeldin-A (10 μg ml^{-1}), phorbol 12-myristate 13-acetate (100 ng ml^{-1}, Sigma-Aldrich, Poole, UK) and ionomycin (1.0 μg ml^{-1}, Sigma-Aldrich). All background intracellular responses (function-positive) were $<0.5\%$. The samples were measured using the FACSCalibur (BD Biosciences). Then, all of the analyses were carried out using FlowJo software (TreeStar, Inc., Ashland, OR, USA).

Immunohistochemistry

Immunostaining was performed using streptavidin-biotin-peroxidase complex techniques (Nichirei, Tokyo, Japan). Consecutive cryostat tissue sections (6 μm) were mounted on glass slides and fixed in 99.5% ethanol for 20 min. After blocking with normal rabbit serum, the sections were stained with anti-mouse CD4, CD8 and Foxp3 antibodies (BD Biosciences). The sections were counter-stained with methyl green. Positive cells were counted in 10 representative high-power-view fields (×400) under microscope.

RT-PCR and real-time PCR of IL-17, RORγt and Foxp3

PCR amplification of murine IL-17, RORγt (retinoic acid receptor-related orphan nuclear receptor gamma t), Foxp3 and β-actin was carried out using total RNA from tumors and tumor-draining lymph nodes in 50 μl of PCR mixture containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of recombinant Taq DNA polymerase and the following primer sets: IL-17 upstream (5′-TTTAACTCCCTTGGCGCAAAA-3′), IL-17 downstream (5′-CTTTCCCTCCG CATTGACAC-3′), RORγt upstream (5′-CCGCTGAGAGGGCTTCAC-3′), RORγt downstream (5′-TGCAGGAGTAGGCCACATTACA-3′), Foxp3 upstream (5′-CCCATCCCCAGGAGTCTTG-3′), Foxp3 downstream (5′-ACCATGACTA GGGGCACTGTA-3′), β-actin upstream (5′-CCTCTATGCCAACACAGTGC-3′) and β-actin downstream (5′-ATACTCCTGCTTGCTGATCC-3′) primers. RORγt and Foxp3 are Th17- and Treg-specific transcription factors, respectively. In total, 36 cycles (β-actin: 28 cycles) of the PCR were carried out at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. The PCR products were fractionated on 2.0% agarose gel.

To quantitatively analyze the expression of cytokine and transcription factors, the mRNAs of IL-17, RORγt, Foxp3 and β-actin were evaluated by a real-time PCR using the SYBR Green kit (Applied Biosystems Japan, Tokyo, Japan) with the Eco Real-Time PCR system (Illumina Inc., San Diego, CA, USA). Briefly, cDNA was added to a final volume of 10 μl per reaction containing 1× SYBR Green PCR Master Mix (Applied Biosystems) and 100 nM of the above primers. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, then 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min.

Isolation of DCs

DCs were isolated from tumors and spleens using mouse CD11c Microbeads and autoMACS magnetic sorter (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Flow cytometry showed that over 90% of isolated cells expressed CD11c and major histocompatibility complex class II, suggesting that a major population of isolated $CD11c^+$ cells were DCs.

Enzyme-linked immunosorbent assay

Tumors were isolated and lysed with RIPA buffer (10 mM Tris-HCl (pH 7.4), 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 0.2 mM PMSF, 1 μg ml^{-1} leupeptin). The amounts of cytokines in cell culture supernatants and tumors were assayed with antibodies for IL-6 and mouse IFN-α (Quantikine; R&D systems, Minneapolis, MN, USA) in accordance with the manufacturer's recommendations.

Statistical analysis

Comparative analyses of the data were performed by the Student's *t*-test, using SPSS statistical software (SPSS Japan Inc., Tokyo, Japan). $P<0.05$ was considered as a significant difference.

RESULTS

Antitumor effect of intratumoral IFN-α gene transfer

At 10 days after the subcutaneous inoculation of CT26 cells, tumors were directly injected once with 1×10^8 plaque forming unit of Ad-mIFN or Ad-AP. The injection of Ad-mIFN significantly suppressed the tumor growth as compared with the control Ad-AP injection (Figure 1a). All treated mice looked healthy during the course of the experiments, and the blood chemistry showed no abnormal values. ELISpot assay showed that the number of IFN-γ-producing cells in response to CT26 cells was increased by Ad-mIFN injection, whereas the spots in response to syngeneic lymphocytes were unchanged between the Ad-mIFN-injected mice and the control mice (Figure 1b). Intracellular cytokine staining also demonstrated that Ad-mIFN significantly increased the frequency of $IFN\text{-}\gamma^+CD8^+$ T cells than Ad-AP did (Figure 1c), indicating that intratumoral IFN-α gene transfer elicited a systemic tumor-specific immunity.

Intratumoral IFN-α gene transfer reduced the frequency of Tregs in tumors

To examine whether the expression of IFN-α affects the immunotolerant microenvironment in tumors, the frequency of Tregs in tumors was analyzed at serial time points after the virus injection. The injection of Ad-mIFN significantly reduced the frequency of Tregs within the total $CD4^+$ T cells in the tumors at days 16 and 22 by flow cytometric analysis (Figure 2a). Immunostaining of tumor tissues at day 16 also revealed that the number of tumor-infiltrating $CD4^+$ T cells was decreased in the Ad-mIFN-injected tumors, and that the number of Tregs was greatly reduced compared with that in Ad-AP-injected tumors (Figure 2b). The frequency of Tregs within the total $CD4^+$ T cells in the tumors was

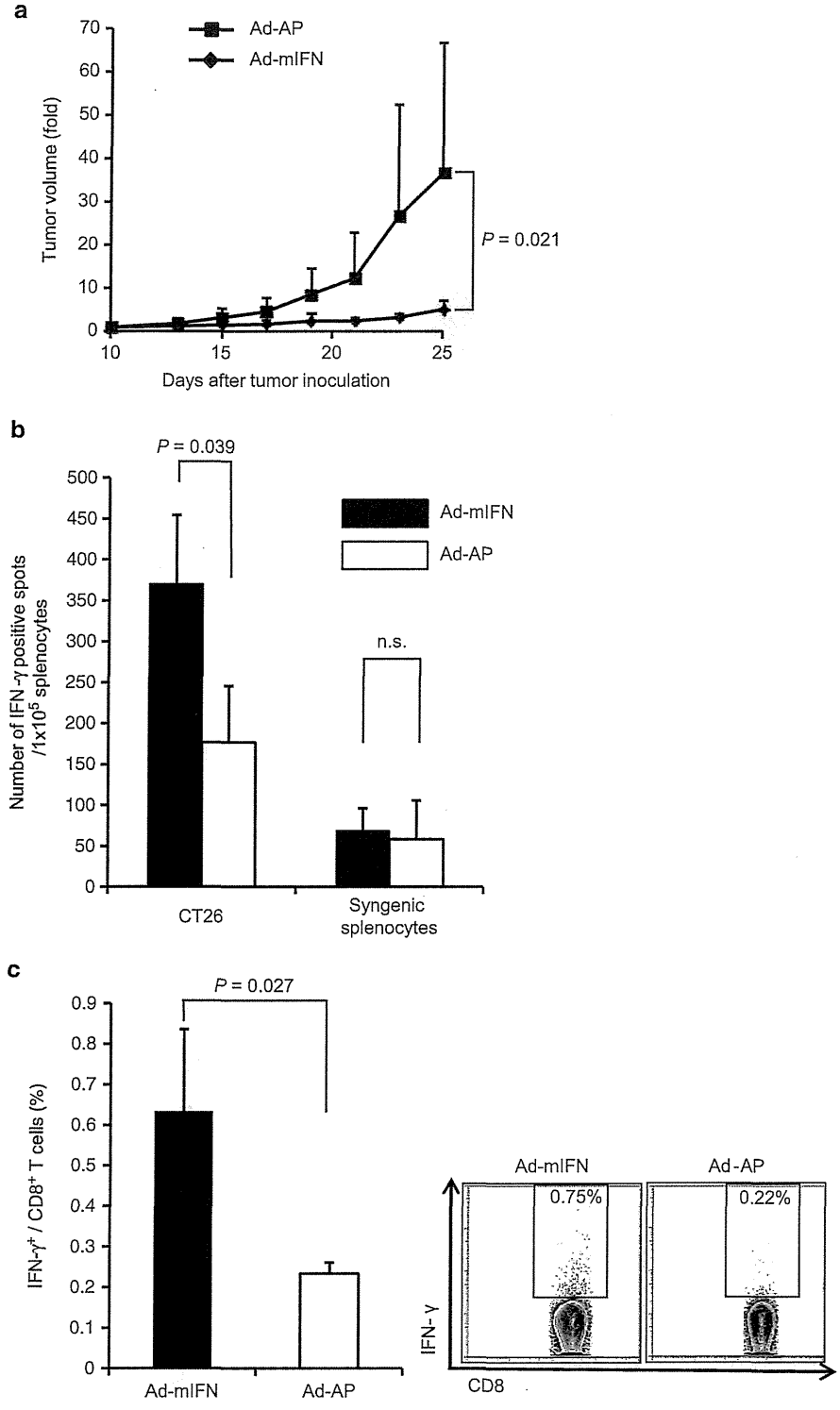

Figure 1. Antitumor effect of intratumoral IFN-α gene transfer. (**a**) Growth of tumors injected with Ad-mIFN. Tumor volumes were measured at indicated days following the intratumoral injection of Ad-mIFN ($n=6$) or Ad-AP ($n=8$). Relative tumor volumes compared with those at day10 were presented. Data are shown as means ± standard deviation (s.d.). (**b**) ELISpot assay of IFN-γ-producing cells in response to stimulation of CT26 cells. Twenty-two days after tumor inoculation, splenocytes were isolated from mice injected with Ad-mIFN ($n=4$) or Ad-AP ($n=3$), and were cultured with CT26 or syngeneic lymphocytes. Data are presented as means ± s.d. (**c**) Intracellular cytokine staining of IFN-γ-producing cells in response to CT26 cells. The splenocytes from mice injected with Ad-mIFN ($n=4$) or Ad-AP ($n=3$) at day 22 were incubated with CT26 cells and stained by anti-mouse IFN-γ antibody. The activated cell fractions were analyzed by staining with anti-mouse CD8 antibody. Representative FACS plots (right panel) are shown.

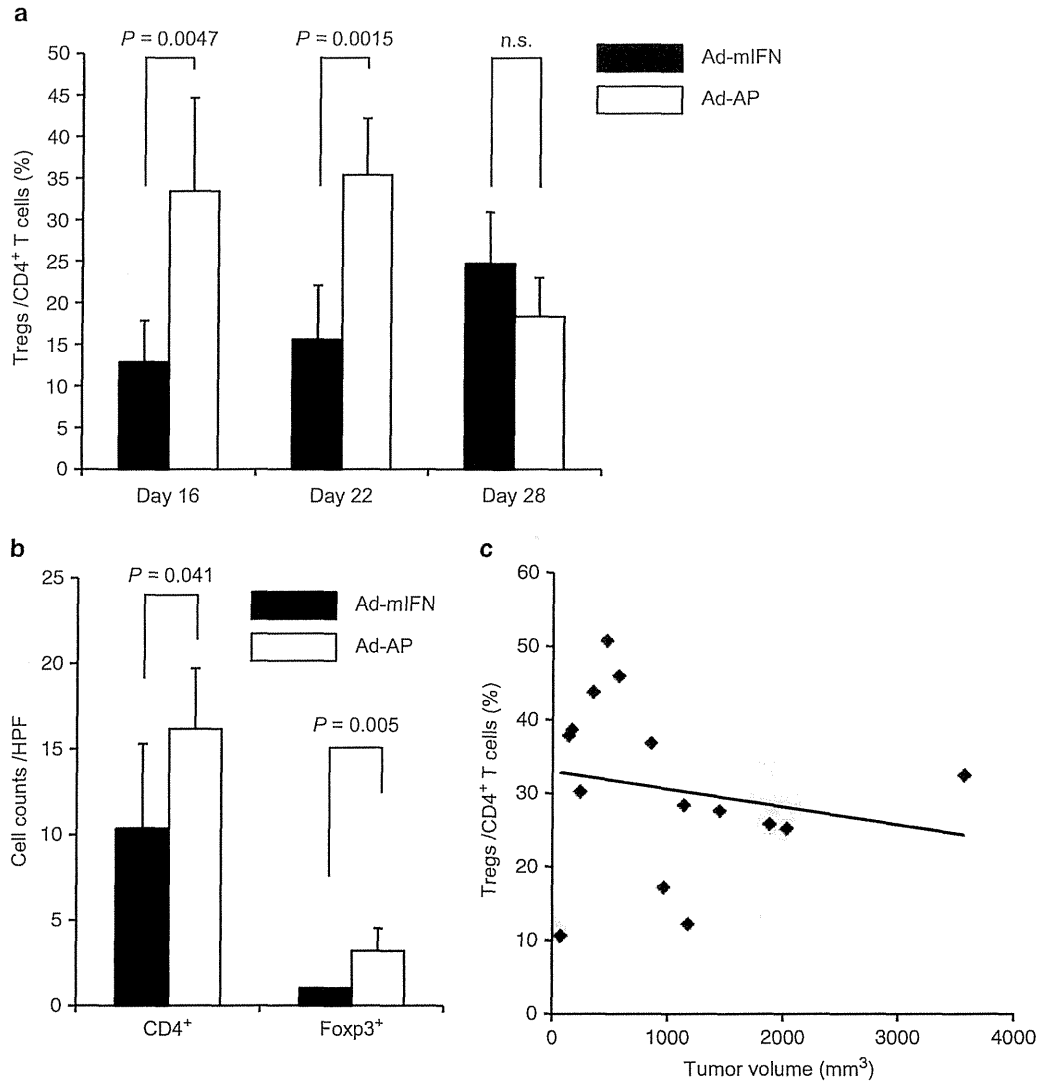

Figure 2. *Intratumoral IFN-α gene transfer reduced the frequency of Tregs in tumors.* (**a**) *Frequency of* $CD4^+Foxp3^+$ *Tregs per* $CD4^+$ *T cells in tumors.* Tumors injected with viruses were harvested at days 16, 22 and 28, and processed into single-cell suspension. The percentage of $CD4^+$ $Foxp3^+$ Tregs were analyzed by flow cytometry (Ad-mIFN: $n=5$, Ad-AP: $n=5$). (**b**) Immunohistochemical staining of $CD4^+$ and $Foxp3^+$ cells within tumors. The frozen sections of tumors injected with Ad-mIFN ($n=5$) or Ad-AP ($n=5$) were processed at day 16 for immunohistochemistry with anti-mouse CD4 and Foxp3 antibodies. (**c**) The frequency of Tregs within total $CD4^+$ T cells in tumors. The tumors injected with Ad-AP at serial time points from day 16 to 28 were harvested and the percentage of $CD4^+Foxp3^+$ Tregs were analyzed by flow cytometry ($n=5$).

not associated with tumor volume (Figure 2c), suggesting that the decrease of Treg frequency in tumors was not explained by tumor volume.

IFN-α gene transfer increased intratumoral IL-6 concentration

We hypothesized that cytokines are involved in the reduction of tumor-infiltrating Tregs by the Ad-mIFN injection. Among candidate cytokines, we focused on IL-6 because it has been reported that it has a crucial role in suppressing proliferation and differentiation of Tregs.[16] As shown in Figure 3a, intratumoral IL-6 concentration at day 16 was significantly increased by IFN-α gene transfer and then returned to the control level at day 22. In addition, there was a positive correlation between IL-6 concentration and IFN-α concentration in the tumors (Figure 3b). Although IL-6 is known to be released by various cells such as tumor-associated macrophages, myeloid-derived suppressor cells and cancer-associated fibroblasts, we especially focused on DCs because they are vital modulators of immune response and immune tolerance, and it was previously reported that type I IFN enhanced IL-6 production from $CD11c^+$ DCs.[17] The *in vitro* culture of DCs isolated from the Ad-mIFN-injected tumors exhibited a higher amount of IL-6 production compared with DCs isolated from the Ad-AP-injected tumors (Figure 3c). The *in vitro* culture of DCs isolated from the naïve splenocytes produced IL-6 in an IFN-α dose-dependent manner (Figure 3d).

IL-6 receptor blockade suppressed IFN-α-mediated Treg decrease in tumors

To examine whether the reduction in tumor-infiltrating Tregs is attributed to the elevation of intratumoral IL-6 concentration,

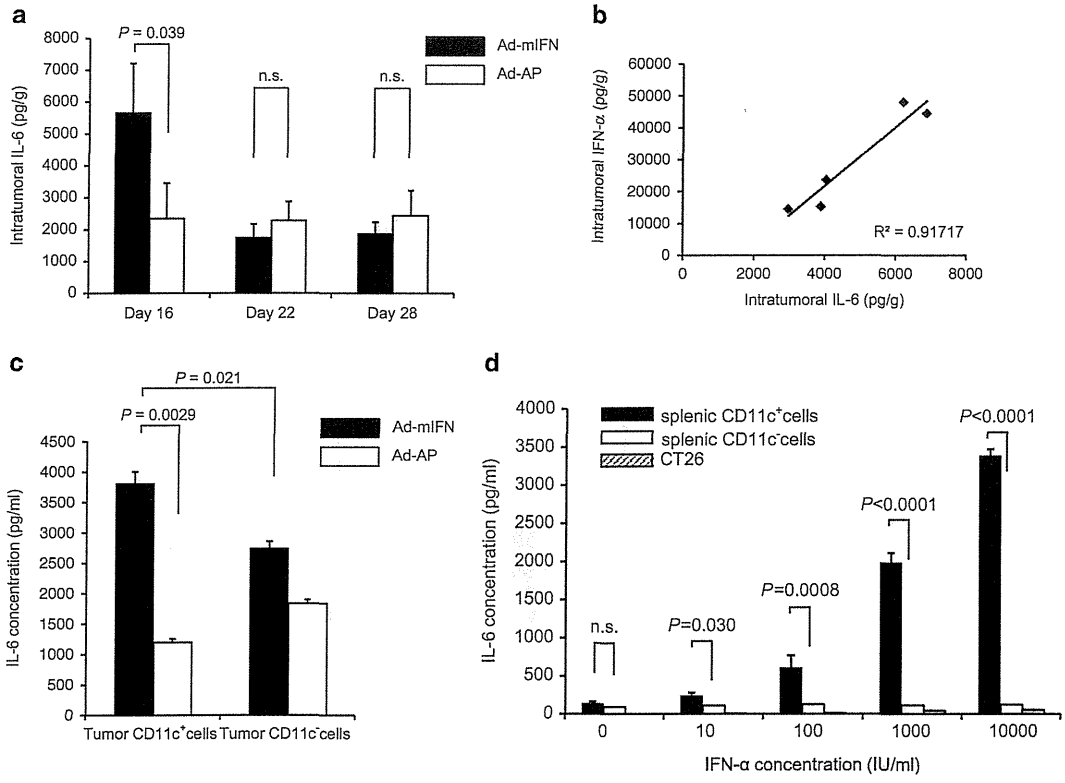

Figure 3. Intratumoral IL-6 concentration was significantly increased by IFN-α gene transfer. (**a**) IL-6 concentration in tumors. Tumors injected with Ad-mIFN ($n=5$) or Ad-AP ($n=5$) were harvested at days 16, 22 and 28, and IL-6 concentration was measured by ELISA. (**b**) Relationship between IL-6 and IFN-α concentration in tumors. Tumors injected with Ad-mIFN ($n=5$) or Ad-AP ($n=5$) were harvested at day 16, and the concentrations of IL-6 and IFN-α were compared by ELISA. (**c**) IL-6 production from tumor $CD11c^+$ cells. The $CD11c^+$ and $CD11c^-$ cells were isolated from tumors injected with Ad-mIFN ($n=2$) or Ad-AP ($n=2$) at day 16, and 5×10^4 cells were plated in 96-well plates. After 48 h, supernatants were assayed for the measurement of IL-6 concentration by ELISA. (**d**) IL-6 production from splenic $CD11c^+$ cells in response to a recombinant IFN-α protein. The $CD11c^+$ and $CD11c^-$ cells isolated from naïve splenocytes, and 5×10^4 of CT26 cells were cultured in 96-well plates with depicted concentration of recombinant mouse IFN-α (Miltenyi Biotech). After 48 h, supernatants were assayed for the measurement of IL-6 concentration by ELISA ($n=2$ for each group).

1000 μg of a monoclonal anti-IL-6 receptor antibody (clone MR16-1; Chugai Pharmaceutical Co., Gotemba, Japan) was intraperitoneally injected into the mice every 7 days after tumor inoculation, and Ad-mIFN or Ad-AP was injected once at day 10 after tumor inoculation (Figure 4a). As a control, rat IgG (Sigma-Aldrich, St Louis, MO, USA) or phosphate-buffered saline were injected in the same manner as anti-IL-6 receptor antibody treatment. The injection of anti-IL-6 receptor antibody significantly increased the frequency of Tregs within the total $CD4^+$ T cells in Ad-mIFN-injected tumors at day 22, whereas this increase was not recognized in the control rat IgG-treated mice (Figure 4b). There was no significant difference in tumor-infiltrating Tregs between rat IgG- and phosphate-buffered saline-injected mice. These results indicated that IFN-α suppressed Treg accumulation in tumors through an elevation of intratumoral IL-6 expression. Then, because a high ratio of effector T cells to Tregs is associated with a favorable prognosis for cancer patients,[18] we calculated the ratio of $CD8^+$ cells to $CD4^+Foxp3^+$ Tregs in the tumors. The ratio was significantly elevated in the Ad-mIFN-injected tumors, which was reduced by the treatment with anti-IL-6 receptor antibody (Figure 4c right). Although the differences in frequencies of $CD8^+$ T cells within whole tumor cells were not statistically significant among four groups, IFN-α had a tendency to increase the frequency of $CD8^+$ T cells (Figure 4c left), and IFN-α also reduced the frequency of Tregs within whole tumor cells at day 16 (Figure 4c middle), indicating that the markedly increased ratio of $CD8^+$ cells to $CD4^+Foxp3^+$ Tregs in the Ad-mIFN-injected tumors was resulted from both of increased $CD8^+$ T cells and decreased $CD4^+Foxp3^+$ Tregs.

IL-6 receptor blockade partially attenuated IFN-α-mediated tumor growth suppression

Next, we examined whether the reduction of tumor-infiltrating Tregs via IL-6 contributes to the induction of antitumor immunity by IFN-α gene transfer. As shown in Figure 5a, tumor growth was still suppressed in the IFN-α-injected mice after the administration of anti-IL-6 receptor antibody; however, the antibody treatment significantly reduced the antitumor effect of IFN-α gene transfer. ELISpot assay revealed that the number of IFN-γ-producing spots was decreased with anti-IL-6 receptor antibody in the Ad-mIFN-injected mice (Figure 5b). The number of IFN-γ^+ spots in response to CT26 cells was reduced by the administration of anti-IL-6 receptor antibody in Ad-AP-injected mice. As it was reported that IL-6 activates tumor-specific cytotoxic T lymphocytes, inducing a strong antitumor immunity,[45] the baseline expression of IL-6 in the tumors (Figure 3a) may activate the CT26-responsive T cells to a certain degree.

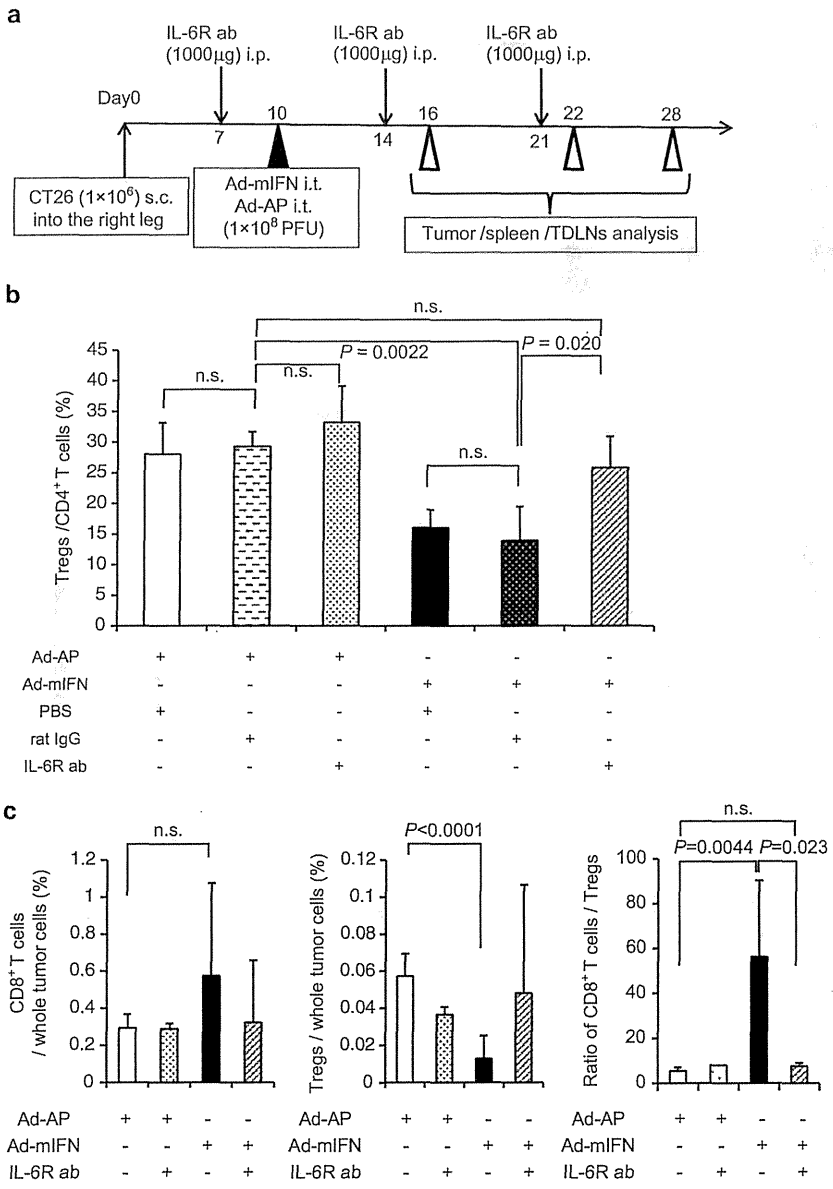

Figure 4. IL-6 receptor blockade suppressed IFN-α-mediated Treg reduction in tumors. (**a**) Schema of experiment. The 1000 μg of the monoclonal anti-IL-6 receptor antibody was intraperitoneally injected into the mice at days 7, 14 and 21 after tumor inoculation. Ad-mIFN or Ad-AP was injected once at day 10 after inoculation. (**b**) Frequency of $CD4^+Foxp3^+$ cells per $CD4^+$ T cells in tumors treated with IL-6R ab. Tumors were harvested at day 22, and $CD4^+$ T cells and $CD4^+Foxp3^+$ Tregs were analyzed by flow cytometry ($n=5$ for the group of Ad-AP i.t. +IL-6R ab i.p., $n=4$ for the other groups). (**c**) Ratio of $CD8^+$ T cells to $CD4^+Foxp3^+$ Tregs in tumors. Frequency of $CD8^+$ T cells within whole tumor cells (left panel). Frequency of $CD4^+Foxp3^+$ Tregs within whole tumor cells (middle panel). The number of $CD8^+$ T cells was compared with that of $CD4^+Foxp3^+$ Tregs in tumors at day 16 (right panel) ($n=4$ for the group of Ad-mIFN i.t.+IL-6R ab i.p., $n=6$ for the other groups). IL-6R ab, anti-IL-6 receptor antibody; i.t., intratumoral injection; i.p., intraperitoneal administration; TDLNs, tumor-draining lymph nodes.

Intratumoral IFN-α expression increased the number of Th17 cells in tumors

It has been previously reported that the upregulation of IL-6 within a tumor microenvironment converted Treg to Th17 phenotype in several murine models.[19,20] To examine whether the elevation of IL-6 concentration promotes differentiation of Tregs to Th17 cells in tumors, mRNA levels of Th17- and Treg-specific transcription factors were quantitatively analyzed by the real-time PCR method. The expression level of Foxp3 mRNA in Ad-mIFN-injected tumors was much lower than that in control Ad-AP-injected tumors, whereas the expression level of RORγt mRNA in Ad-mIFN-injected tumors was significantly higher than that in the control tumors (Figure 6a). Furthermore, the expression of IL-17 mRNA was detected by RT-PCR in the IFN-α injected tumors but not in the tumors treated with anti-IL-6 receptor antibody (Figure 6b). Intracellular cytokine staining also showed that the frequency of IL-17A-producing T cells per $CD4^+$ T cells in the tumors was elevated by the injection of Ad-mIFN, which was clearly inhibited by the anti-IL-6 receptor antibody (Figure 6c). The

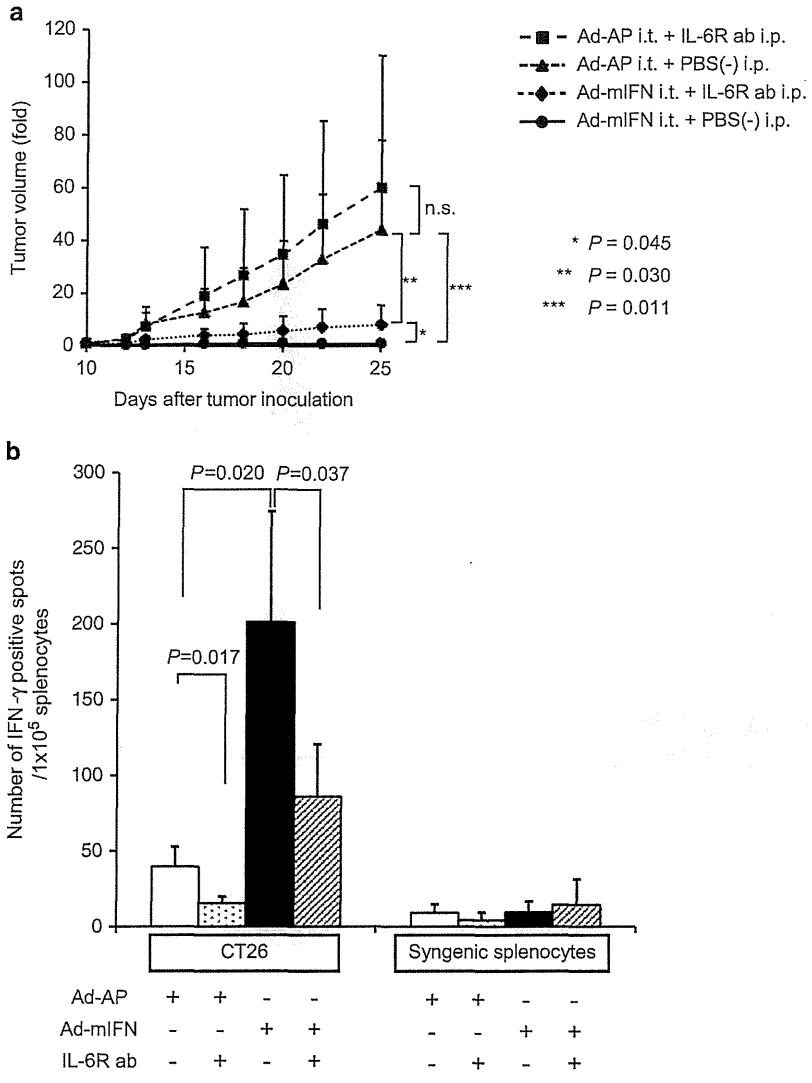

Figure 5. IL-6 receptor blockade partially attenuated IFN-α-mediated tumor growth suppression. (**a**) Growth of tumors treated with IL-6R ab. Tumor volumes in mice treated with the viruses and/or IL-6R ab were measured at the indicated days ($n=5$ for the group of Ad-AP i.t.+IL-6R ab i.p., $n=6$ for the other groups). Relative tumor volumes compared with those at day 10 were presented. (**b**) ELISpot assay of IFN-γ-producing cells in mice treated with IL-6R ab. The splenocytes were isolated from mice as shown in Figure 4a at day 28, and the cells were cultured with CT26 or syngeneic splenocytes ($n=5$ for the group of Ad-AP i.t.+IL-6R ab i.p., $n=6$ for the other groups).

frequency of IL-17A-producing $CD4^+$ T cells was not increased in the tumor-draining lymph nodes of Ad-mIFN-injected mice. These results suggested that the IFN-α-IL-6 pathway transdifferentiated Tregs to Th17 cells within the tumors but not within the tumor-draining lymph nodes.

DISCUSSION

In the present study, we examined the immunological activity of intratumoral IFN-α gene transfer on tumor-infiltrating Tregs to reveal the effects of type I IFN on an immunotolerant microenvironment. Our results showed that IFN-α expression promotes the production of IL-6 from DCs and reduced the frequency of Tregs in tumors. In Ad-mIFN-injected tumors, intratumoral IL-6 concentration returned to control level at day 22 (Figure 3a); however, the frequency of Tregs within the total $CD4^+$ T cells was still reduced (Figure 2a). A possible reason for this discrepancy was that the precedent elevation of IL-6 acted on the differentiation and proliferation of Tregs and persistently decreased the frequency of Tregs in tumors even at day 22.

There have been several reports demonstrating the suppressive effect of IFN-α on Tregs in the tumor microenvironment. Backer *et al.*[21] reported that IFN-α deactivates the suppressive function of human Tregs by downregulating their intracellular cAMP level independently of antigen-presenting cell. They also showed that IFN-α abolishes the tolerogenic phenotype of human DC induced by IL-10 and, thereby, prevents the induction of Tregs.[22] Pace *et al.*[23] demonstrated that IFN-α inhibits the suppressor capacity of murine natural Treg *in vitro* via the activation of APC. This study is the first report describing that IFN-α suppressed tumor-infiltrating Tregs through IL-6, focusing on the plasticity of Tregs within the tumor microenvironment. As IL-6 increases the methylation of upstream Foxp3 enhancer and represses the Foxp3 transcription in natural Tregs,[16] IFN-α-mediated IL-6 production may decrease

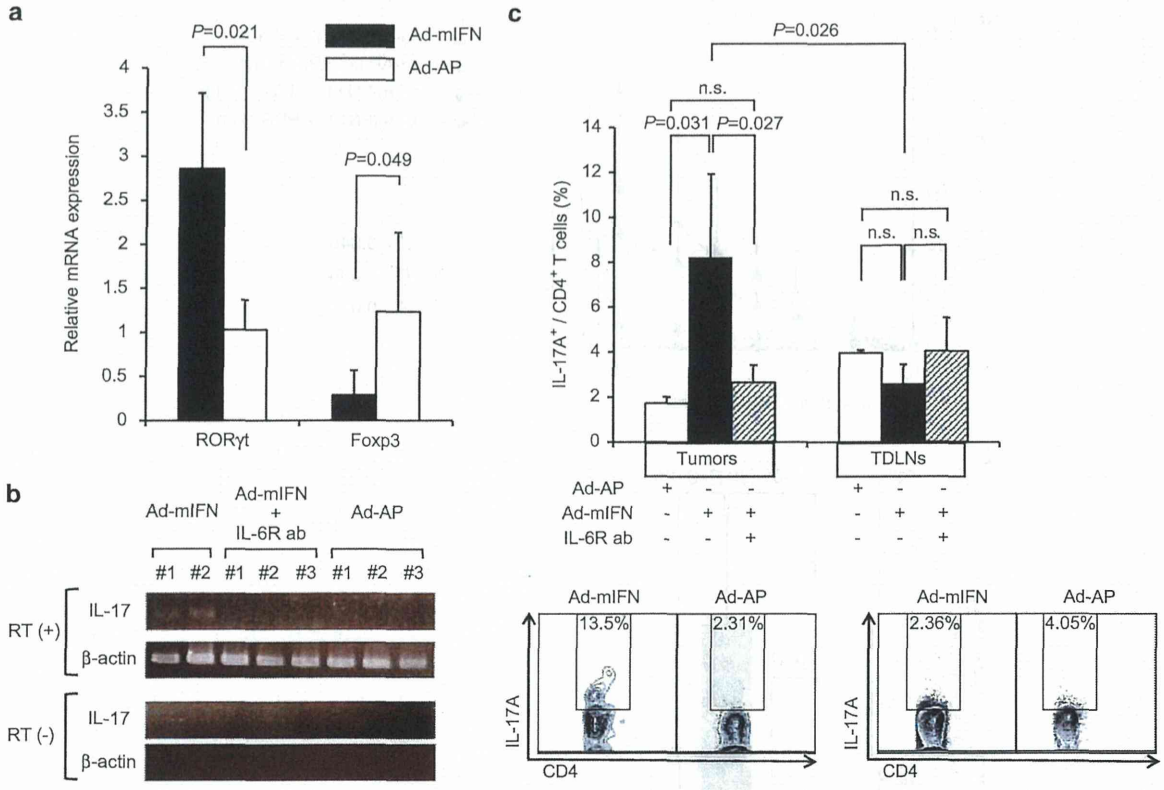

Figure 6. Intratumoral IFN-α expression increased the number of Th17 cells in tumors. (**a**) Expression of RORγt and Foxp3 genes in tumors. The tumors injected with viruses were harvested at day 16 and were subjected to real-time PCR analysis (Ad-mIFN: $n=4$, Ad-AP: $n=3$). (**b**) Expression of IL-17 in tumors. The tumors were harvested at day 28, and subjected to RT-PCR for IL-17 expression ($n=3$ for the group of Ad-AP i.t.+PBS i.p., $n=2$ for the group of Ad-mIFN i.t.+PBS i.p., $n=3$ for the group of Ad-mIFN i.t.+IL-6R ab i.p.). (**c**) Intracellular cytokine staining of IL-17A in $CD4^+$ T cells. The tumors and tumor-draining lymph nodes were harvested at day 22, and IL-17A expressions were analyzed by flow cytometry ($n=3$ for the group of Ad-AP i.t.+PBS i.p., $n=4$ for the group of Ad-mIFN i.t.+PBS i.p., $n=4$ for the group of Ad-AmIFN i.t.+IL-6R ab i.p.) (upper panel). Representative FACS plots of tumors (lower left panel) and tumor-draining lymph nodes (lower right panel) were shown.

$Foxp3^+$ cells in tumors. In addition, the number of $Foxp3^+$ Tregs may be further decreased by the transdifferentiation into Th17 cells.

Th17 cells are pro-inflammatory cells, which are characterized by the expression of several cytokines such as IL-17A, IL-17F, IL-21, IL-22 and IL-23R, and the cells express the specific transcription factors RORγt and RORα.[24–26] Basically, Th1, Th2, Th17 and Tregs can all be generated in the periphery from newly activated $CD4^+$ T cells. Importantly, it has recently been found that fully differentiated Tregs can themselves be converted into Th17 cells.[27–29] This process is accompanied by the loss of Foxp3 expression and upregulation of RORγt. IL-6 is known to be a crucial cytokine that turns off the Treg program, and Tregs can be reprogrammed and redifferentiated into Th17 cells in the presence of TGF-β and IL-6.[28]

Although we showed in the present study that IL-6 induced antitumor immunity, several studies have reported that IL-6 has tumor-promoting actions on both malignant and stromal cells in a range of experimental murine models.[30–34] Also, in several human cancers, IL-6 has been implicated as an important part of the cytokine network,[35–38] and therapeutic activity of IL-6 antagonists has been under investigation in several types of cancer.[35,39–41] These effects are mediated through IL-6 trans-signaling through gp130 activation with subsequent signaling through Janus kinases and signal transducer and activator of transcription 3 (STAT3). These data indicated the importance of IL-6 signaling pathways in inflammatory carcinogenesis due to chronic inflammation. On the other hand, IL-6 has been shown to activate tumor-specific cytotoxic T lymphocytes and induce a strong antitumor immunity.[42–45] The role of IL-6 in tumors is probably based on the balance between the positive and negative effect on tumor growth. In this study, DCs were stimulated to secrete IL-6 at early time points after IFN-α gene transfer into tumors; however, the IL-6 expression did not persist chronically. Therefore, the long-term expression of IL-6 may be related with carcinogenesis, whereas the transient high concentration of IL-6 may be crucial to strongly incline the immune balance of the tumor microenvironment in an antitumor direction. Alternatively, the involvement of other cytokines produced from DCs may co-operate with IL-6 to induce an effective tumor immunity after intratumoral IFN-α gene transfer.

The role of Th17 in the tumor microenvironment remains to be controversial. There are conflicting reports that Th17 is involved in the promotion of tumor growth[46] or has an antitumor role by promoting tumor-specific $CD8^+$ T-cell activation.[47] Gnerlich *et al.*[19] demonstrated that the addition of IL-6 to the tumor microenvironment skews the balance toward Th17 cells with delayed tumor growth and improvement of survival in a murine model of pancreatic cancer. However, what factors in the tumor microenvironment enhance the antitumor effect of Th17 cells has been under debate. As a next step, we are planning to examine the role of Th17 cells in inducing tumor immunity to clarify the full pictures of tumor immunity induced by intratumoral type I IFN gene delivery.