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Systemic Administration of IL-23 Induces Potent Antitumor Immunity Primarily Mediated through Th1-Type Response in Association with the Endogenously Expressed IL-12[1]

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IL-23, a cytokine, which is composed of the p40 subunit shared with IL-12 and the IL-23-specific p19 subunit, has been shown to preferentially act on Th1 effector/memory CD4$^+$ T cells and to induce their proliferation and IFN-γ production. The IL-23 is also reported to act on Th17-CD4$^+$ T cells, which are involved in inducing tissue injury. In this study, we examined the antitumor effects associated with systemic administration of IL-23 and their mechanisms in mouse tumor system. Systemic administration of high-dose IL-23 was achieved using in vivo electroporation of IL-23 plasmid DNA into the pretibial muscles of C57BL/6 mice. The IL-23 treatment was associated with significant suppression of the growth of pre-existing MCA205 fibrosarcoma and prolongation of the survival of treated mice without significant toxicity when compared with those of the mice treated with EGFP. Although the therapeutic outcomes were similar to those with the IL-12 treatment, the IL-23 treatment induced characteristic immune responses distinctive to those of IL-12 treatment. The IL-23 administration even at the therapeutic levels did not induce detectable IFN-γ concentration in the serum. In vivo depletion of CD4$^+$ T cells, CD8$^+$ T cells, or NK cells significantly inhibited the antitumor effects of IL-23. Furthermore, the CD4$^+$ T cells in the lymph nodes in the IL-23-treated mice showed significant IFN-γ and IL-17 response upon anti-CD3 mAb stimulation in vitro. These results and the ones in the IFN-γ or IL-12 gene knockout mice suggest that potent antitumor effects of IL-23 treatment could be achieved when the Th1-type response is fully promoted in the presence of endogenously expressed IL-12. *The Journal of Immunology,* **2007, 178: 7571–7580.**

The final goal of cancer immunotherapy is to induce long-lasting tumor-specific immunity that can suppress the growth and metastasis of malignant tumor cells in tumor-bearing host. However, this goal has been difficult to be achieved because of multiple and critical obstacles, including low immunogenicity of tumor-associated Ags and immunosuppression associated with tumor. Some of these situations might be improved if Th1 immune reaction could be developed at the tumor site (1–5). Recently, development of Th17 has been shown to have possibility to induce favorable situation for immune responses to give damages to the tumor tissue as well (6). One of the strategies to improve tumor local environment is to provide appropriate cytokines systemically or locally. Among the cytokines used for this strategy, IL-12 has been attracted many attentions. The IL-12 was originally identified as a factor that stimulates NK cells and promotes maturation of CTLs. Further studies of IL-12 have shown that IL-12 has other important functions, including the induction of Th1 development and suppression of angiogenesis (7–9). Furthermore, systemic or local administration of IL-12 has been shown to be associated with significant antitumor effects in mouse tumor systems (10, 11). However, systemic administration of IL-12 protein has not been proven clinically useful because of the significant adverse effects in human even at the very low dose (12).

The IL-23 cytokine, which is composed of the IL-23-specific p19 subunit and the IL-12 p40 subunit, has been shown to preferentially act on Th1 effector or memory CD4$^+$ T cells and induce their proliferation and IFN-γ production (13). The IL-23 is expressed by activated dendritic cells of mouse and human (14) and binds to the receptor complex composed of IL-12Rβ1 subunit and specific IL-23R subunit. The IL-23R complex is detected on Th1 and Th2 cells, bone marrow-derived dendritic cells, and macrophages in mouse (15, 16). The IL-23 activates JAK-STAT signaling molecules, including JAK2, TYK2, and STAT1, STAT3, STAT4, and STAT5, as does IL-12. However, STAT4 is activated with IL-23 to the level substantially weaker than that with IL-12 and forms DNA-binding complexes different from the ones induced with IL-12 (17). Furthermore, it has been proposed recently that CD4$^+$ T cells producing IL-17, which is known to be induced with IL-23, represent a distinct inflammatory Th-cell lineage termed Th17 (18–20). Because IL-17 has crucial roles in regulating tissue inflammation and the development of disease in several animal models of autoimmunity (21–23), IL-23 could be involved in antitumor immunity as well.

These findings suggest that IL-23 might modulate certain antitumor immune responses but in the manner somewhat different from those of IL-12. Recently, multiple reports have been published regarding the roles of IL-23 in regulating antitumor immune

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responses in vivo. Although the endogenous IL-23 expression has been reported to promote tumor incidence and growth (24), the studies using local (25–27) and systemic (28) administration of IL-23 have shown that application of IL-23 at the excessive amount induces antitumor immune responses. The magnitude of the reported studies are somewhat different among these reports partially due to the difference of the model systems, including the mouse strain, transplanted tumor cell line, and methods of administration, but they agree that IL-23 administration is associated with positive antitumor effects. However, further studies are still required to learn more about the roles and mechanism of the immune responses induced with the administration of IL-23, which is now known to induce important IL-17 responses (21–23). In this study, we evaluated the in vivo antitumor effects of the IL-23 treatment in mouse tumor systems using systemic administration of IL-23 achieved by in vivo electroporation (IVE)[3] of IL-23 plasmid DNA in the pretibial muscles of mice in the present study. Systemic administration of IL-23 was associated potent antitumor effects with the characteristics immune responses mediated by the functions of $CD4^+$ T cells. These results suggest that IL-23 could be used as a therapeutic agent to treat cancer.

Materials and Methods

Tumor cell lines

MCA205, a methylcholanthrene-induced murine fibrosarcoma cell line, and MC38, a murine colon adenocarcinoma cell line, were gifts from Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD). EL-4, a murine T cell leukemia cell line, was purchased from the American Type Culture Collection (ATCC). These tumor cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, sodium pyruvate, nonessential amino acids, 100 mg/ml streptomycin, 100 IU/ml penicillin, and 5×10^{-5} M 2-ME (all from Invitrogen Life Technologies), referred to henceforth as complete medium. They were demonstrably free of mycoplasma contamination with the mycoplasma PCR ELISA kit (Roche Diagnostic) periodically.

Animals

C57BL/6 (H-$2K^b$) mice were purchased from CLEA Japan. Female IFN-γ gene knockout (IFN-γ KO) mice with the C57BL/6 background have been previously prepared by Iwakura. Female IL-12 KO ($p35^{-/-}$) mice with the C57BL/6 background (IL-12 KO mice) were purchased from The Jackson Laboratory.

All mice in this study were female, maintained in specific pathogen-free conditions in our animal facility at Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, and used at 6–8 wk of age.

Construction of expression plasmids

The expression plasmid used in this study were made inserting cDNAs into pCMV-intronA plasmid (a gift from Dr. J. Baar, University of Pittsburgh Cancer Institute, Pittsburgh, PA), which express the genes under the control of CMV early promoter with an intron A with or without internal ribosomal entry site (IRES) obtained from pCITE-1 (EMD Biosciences). EGFP and pIRES2-DsRed2 cDNAs were purchased from BD Clontech. The mouse IL-12 cDNAs were gifts from Dr. S. Schoenhaut (Hoffman-LaRoche, Basel, Switzerland). The cDNA encoding the p19 chain of mouse IL-23 was generated with RT-PCR using the transcripts of macrophage cell line J774 that constitutively express p19 transcripts as the template. The full-length p19 cDNA was subjected to PCR amplification using p19-3-specific primers, followed by another round of amplification using second p19-1-specific primers. These PCR primer sequences were as follows: mp19-1, sense, 5′-GCTCTAGATGCTGGATTGCAGAGCAG-3′, and antisense, 5′-ACGCGTCGACTGGTAGATGTCTGGGCTGATAGA-3′; and mp19-3, sense, 5′-ACAGGCCTGGTGCAGATCACAGAG-3′, and antisense, 5′-TGCCCTTCACGCAAAACAAA-3′. The sequence of the product was confirmed to be identical to the published sequence (29).

[3] Abbreviations used in this paper: IVE, in vivo electroporation; BFA, brefeldin A; i.d., intradermal(ly); IRES, internal ribosomal entry site; KO, knockout; MLTR, mixed lymphoid cell and tumor cell reaction; PLSD, protected least significant difference.

Animal experiments using IVE

Mice were inoculated intradermally (i.d.) in the left flank with 1×10^5 of MCA205 tumor cells on day 0. Each experimental group consisted of five to eight animals. On day 9, when tumor size reached ~6–8 mm^2, IVE was performed as described below (30). Briefly, mice were anesthetized with pentobarbital sodium and injected with plasmid DNA diluted in sterile saline (50 μl, 1 μg/μl) into the bilateral pretibial muscles using a tuberculin syringe with 27-gauge needles. A pair of electrode needles was inserted into the muscle to a depth of 5 mm to the DNA injection sites, and electric pulses were delivered using an electric pulse generator (CUY-21; BEX). The shape of the pulse was a square wave, i.e., the voltage remained constant for the duration of the pulse. Electrodes consisted of a pair of tungsten needles of 5 mm in length and 0.4 mm in diameter. We set up the standard of electroporation as four electric pulses (1 time/s) of 100-ms duration at 50 V (100 V/cm).

To deplete $CD4^+$ or $CD8^+$ T cells in vivo, 300 μg in total of Abs produced by hybridomas for anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 53-6.72) (ATCC) was administrated i.p. 1, 3, and 5 days before tumor inoculation, 1 day before IVE, and once every week after IVE. For in vivo NK cell depletion, 200 μg of rabbit anti-asialo GM1 antiserum (WAKO) was injected i.v. on 4 days before tumor inoculation, on 1, 5, and 8 days before IVE, and every 4 days after IVE. Standard two-color flow cytometric analysis using FACSCalibur (BD Biosciences) was performed on the PBMC before tumor challenge to verify successful depletion of specific cell subsets. Tumor size (mm^3) was measured twice a week and calculated using the following formula: $a \times b \times 3.14/4$ (a = largest diameter, b = smallest diameter). Each set of experiments were performed for at least three times to confirm reproducibility. The information of the representative experiments were shown in *Results* only when the consistent results were obtained in each set of the experiments.

Confirmation of gene expression

The NIH3T3 cells (ATCC) were transiently transfected with 2 μg of pCMV-A-IL-23 using Effecten (Qiagen), according to the manufacturer's instructions. After 48 h of incubation, the supernatant was harvested for the test. Total RNA from pretibial muscle was isolated with the Isogen kit (Nippon Gene), according to the manufacturer's recommendations. Expression of p19, p35, and p40 mRNA in muscle was determined by RT-PCR. The DNA contamination in RNA samples was removed using deoxyribonuclease (Invitrogen Life Technologies), according to the manufacturer's instructions. One microgram of total RNA was applied for the synthesis of cDNA with SuperScript II RNase H Reverse Transcriptase (Invitrogen Life Technologies). PCR was performed with a DNA Thermal Cycler (Gene Amp PCR system 9600; PerkinElmer) using AmpliTaq DNA polymerase (Roche). It consisted of 30 cycles under the following conditions: 20 s at 94°C for denaturation, 20 s at 55°C for primer annealing, and 30 s at 72°C for primer extensions. As a control, we also measured mRNA levels of β-actin. The primer sequence of the oligonucleotides used for PCR were as follows: β-actin, sense, 5′-ATGGATGACGATATCGCT-3′, and antisense, 5′-ATGAGGTAGTCTGTCAGGT-3′; mp40, sense, 5′-AGATGACATCACCTGGACCT-3′, and antisense, 5′-GCCATGAGCACGTGAACCGT-3′; mp35, sense, 5′-ACCAGCACATTGAAGACCTG-3′, and antisense, 5′-GACTGCATCAGCTCATCGAT-3′; mp19-1, sense, 5′-GCTCTAGATGCTGGATTGCAGAGCAG-3′, and antisense, 5′-ACGCGTCGACTGGTAGATGTCTGGGCTGATAGA-3′; mp19-2, sense, 5′-AATGTGCCCCGTATCCAGTGTG-3′, and antisense, 5′-GGCTCCCCTTTGAAGAT-3′; mp19-3, sense, 5′-ACAGGCCTGGTGCAGATCACAGAG-3′, and antisense, 5′-TGCCCTTCACGCAAAACAAA-3′; and IFN-γ, sense, 5′-CTTGGCTTTGCAGCTCTTCCT-3′, and antisense, 5′-CCTTTTGCCAGTTCCTCCAGA-3′. The PCR products were visualized with ethidium bromide staining under UV light following electrophoresis on 2% agarose gels.

Measurement of cytokine levels

The levels of mouse IL-23 concentration was examined in the supernatants or serum samples using the IL-23(p19/p40) ELISA (Ready-SET-Go; eBioscience). Biological activity of IL-23 was measured using 1×10^6 of Con A (Sigma-Aldrich)-blast T cells cultured for 72 h in the presence of the culture supernatants of pCMV-A-IL-23- or p40-IRES-DsRed2-transfected 293 cells or recombinant mouse IL-23 (R&D Systems). Concentration of IL-17 in the culture supernatants was determined using IL-17-specific ELISA kit (OptEIA; BD Pharmingen). The concentration of mouse p40, IL-12, or IFN-γ were measured in the serum obtained from C57BL/6 mice 4, 11, and 25 days after IVE of EGFP, IL-12, or IL-23 using respective ELISA kits (OptEIA; BD Pharmingen). Every experiment was performed in triplicate.

Mixed lymphoid cells and tumor cells reactions (MLTR)

Lymphoid cells (1×10^6) were isolated from lymph nodes of the treated mice and cocultured with gamma ray-irradiated MCA205, MC38, or EL-4 tumor cells (1×10^5) in the presence of rIL-2 (20 U/ml) in 48-well plates. Four days after initiation of culture, all cells were harvested and used for immunological assays.

Cytotoxicity assay

The cytotoxic activities mediated by Ag specific CTL was measured by 4-h ^{51}Cr release assays. Briefly, the effector cells were incubated with ^{51}Cr-labeled MCA205 or EL-4 target cells at different E:T ratios at 37°C for 4 h, and target cell lysis was calculated. Tumor-specific cytotoxicity in this study was determined using MCA205 tumor cells as target cells. As a control, parental EL-4 tumor cells were used. The percentage of cytotoxicity was calculated as described previously (31).

Cytoplasmic cytokine expression

Lymphoid cells isolated from lymph nodes of treated mice were stimulated with immobilized anti-CD3 mAb (10 μg/ml) for 6 h in the presence of brefeldin A (BFA; Sigma-Aldrich) and stained with FITC-conjugated anti-CD4 mAb (clone L3T4) or anti-CD8α mAb (clone Ly-2). For the detection of cytoplasmic cytokine, these cells were fixed with 4% paraformaldehyde, treated with permeabilizing solution (50 mM NaCl, 5 mM EDTA, 0.02% NaN_3, and 0.5% Triton X-100 (pH 7.5)), and then treated with blocking solution (0.5% BSA and 0.02% NaN_3 in PBS). These treated cells were incubated with PE-conjugated anti-IFN-γ mAb (clone XMG1.2) or anti-IL-17 mAb for 45 min at 4°C. The percentage of cells expressing cytoplasmic IFN-γ or IL-17 was determined by FACSCalibur with BD CellQuest Pro (BD Biosciences). All FITC- and PE-conjugated mAbs were purchased from BD Pharmingen.

Cytokine expression upon in vitro stimulation using soluble anti-CD3 mAb

The lymphoid cells separated from the lymph nodes were also tested for cytokine responses upon the stimulation with soluble anti-CD3 mAb (0.1 μg/ml; BD Pharmingen) for 36 h with or without anti-CD4 mAb (clone GK1.5, 20 μg/ml; ATCC).

Statistical examination

Tumor sizes of the animals in the respective groups were compared with Two-way Repeated-Measures ANOVA by Fisher's protected least significant difference (PLSD) test. The results were considered to be statistically significant when p values were <0.05.

Results

Biologically active IL-23 was expressed with pCMV-A-IL-23

To examine IL-23 and IL-12 expression of pCMV-A-IL-23, the NIH3T3 cells were transfected using with pCMV-A-IL-23 or control vectors such as pCMV-A-EGFP and pCMV-A-IL-12, and the culture supernatant was harvested 48 h after the transfection to measure the concentration of IL-23 and IL-12 (Fig. 1*A*). The 30 ng/ml immunoreactive IL-23 was detected only in the supernatant of the cells transfected with pCMV-A-IL-23, and 800 pg/ml immunoreactive IL-12 was detected only in the supernatant of the cells transfected with pCMV-A-IL-12. The biological activity of the protein expressed by the pCMV-A-IL-23 was also confirmed. The culture supernatant of NIH3T3 cells transfected with pCMV-A-IL-23 was tested for its ability to induce IL-17 secretion from Con A-blast T cells. As shown in Fig. 1*B*, significant levels of IL-17 was detected in the culture supernatant of Con A-blast T cells in a dose-dependent manner when the supernatant of NIH3T3 cells transfected with pCMV-A-IL-23 was added. In contrast, IL-17 was not detected on either of the culture supernatants of Con A-blast T cells stimulated with the supernatant of NIH3T3 cells transfected with p40-IRES-DsRed2 as a negative control. These results indicate that biologically active IL-23 can be expressed by the transfection of pCMV-A-IL-23.

Systemic administration of IL-23 protein at high levels was achieved using IVE of IL-23 plasmid DNA into the pretibial muscles

To study in vivo effects associated with systemic administration of IL-23, we used IVE into the bilateral pretibial muscles, which allows the prolonged production and release to systemic blood circulation of large amount of proteins (30). To confirm the transgene expression of IVE, the plasmids were transfected into the pretibial muscles of mice, and the expression of each subunit was examined using RT-PCR. In IL-23-transfected mice, p19 and p40 were detected, but p35 was not detected. In contrast, only the p35 and p40 were detected in IL-12-transfected mice (Fig. 1*C*). We also examined the concentration of IL-23 and IL-12 protein in the serum after IVE using pCMV-A-IL-23 or control vectors such as pCMV-A-EGFP and pCMV-A-IL-12. Significantly high IL-23 protein expression was also confirmed in the serum of the animals treated with IVE using pCMV-A-IL-23 4 days prior. However, significantly high IL-12 protein expression was detected only in the serum of the animals treated with IVE using pCMV-A-IL-12 (Fig. 1*D*). Furthermore, biological activity of immunoreactive IL-23 in the serum was examined (Fig. 1*E*). The mice were treated with IVE using 100 μg of IL-23 and sacrificed 4 days after the treatment to harvest the serum. Then, Con A-blast T cells (1×10^6) were cultured for 72 h in the presence of the serum adjusted to contain 0.5 ng/ml total p40 defined by ELISA. A significant amount of IL-17 was detected only in the supernatant of the culture stimulated with the serum harvested from the mice treated with IL-23- IVE, while IFN-γ was not detected in the same serum. These results clearly indicate that the administration of pCMV-A-IL-23 using IVE results in the expression of biologically active IL-23 in vivo at high levels. This system was used throughout the following studies.

IL-23 treatment resulted in significant CTL responses to suppress the growth of MCA205 tumor cells without including the high serum IFN-γ concentration

To study whether systemic administration of IL-23 using IVE can inhibit tumor growth in vivo, we inoculated 1×10^5 cells of MCA205 tumor cells i.d. into C57BL/6 mice on day 0 and treated the mice with increasing doses of IL-23 (20, 40, and 100 μg of plasmid DNA) on day 9. As shown in Fig. 2*A*, IL-23 treatment was associated with significant suppression of tumor growth in a dose-dependent manner. The final outcome of the IL-23 treatment at the highest dose (100 μg/mouse) was equivalent to that of IL-12 treatment (100 μg/mouse). However, the effects of IL-23 treatment (100 μg/mouse) became evident at the later time points when compared with that of IL-12 treatment (100 μg/mouse) ($p < 0.05$). Administration of the same amount of EGFP- plasmid had no significant effect on tumor growth. In addition, IL-23 treatment resulted in prolongation of the animal survival, whereas all mice in control group receiving EGFP treatment had died by day 36 after tumor inoculation (data not shown). Interestingly, the elevation of IFN-γ levels in the serum, splenomegaly, and lymphadenopacy, which were observed in mice treated with IL-12 as previously reported (32), was not observed in mice treated with IL-23 (Fig. 2*B*). We detected no significant levels of IL-17 in the serum of the animals treated with IL-23-IVE or IL-12-IVE at any time point (data not shown). Similar antitumor effects of IL-23 treatment were also observed with MC38 tumor cell line (data not shown). These experiments were repeated five times with similar results.

To examine the potency of the CTLs in the animals treated with IL-23, the cells in draining lymph nodes were harvested from the mice treated with EGFP, IL-12, or IL-23 and tested for specific

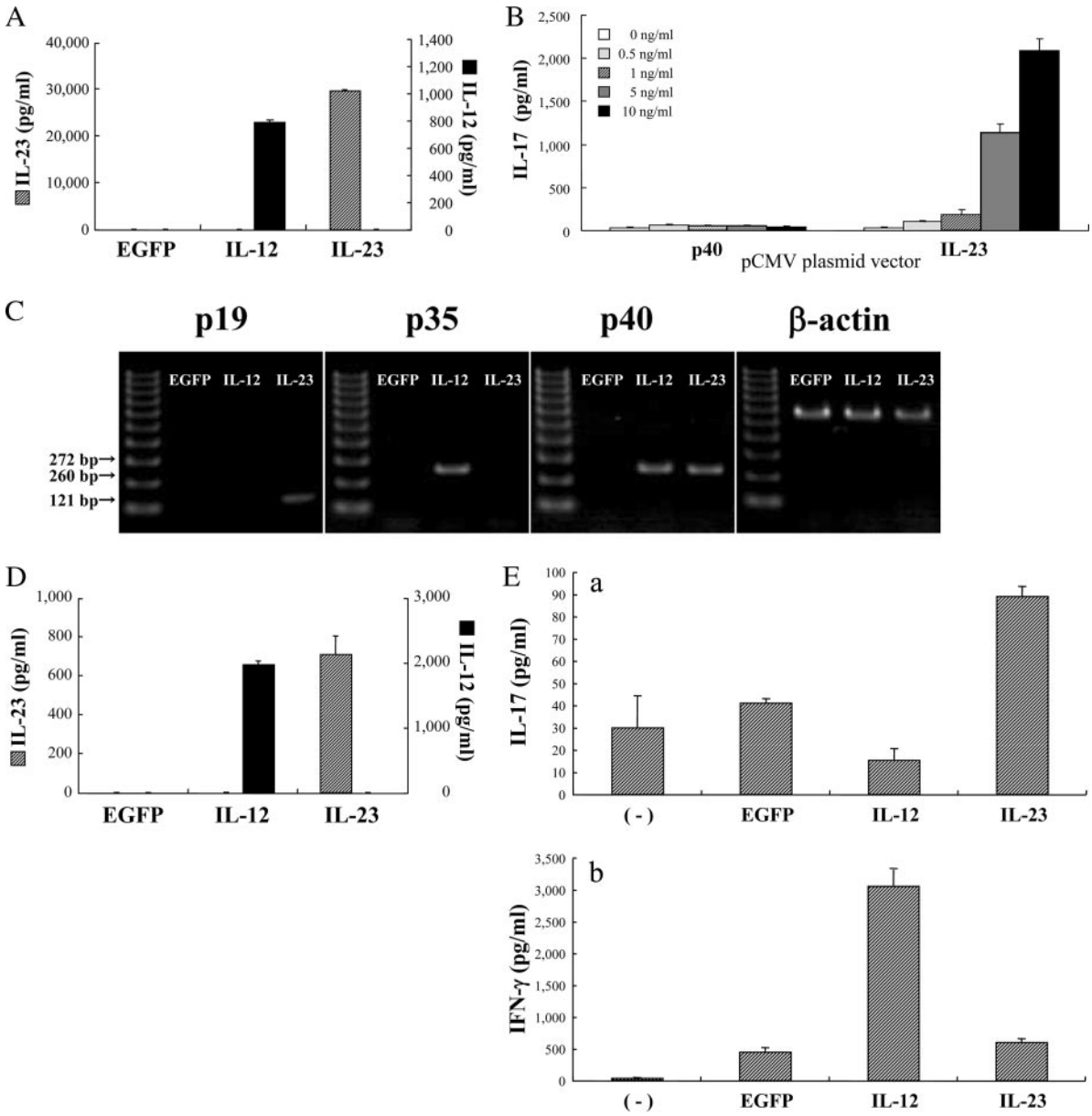

FIGURE 1. Biologically active IL-23 was expressed in vitro and in vivo with transfection using pCMV-A-IL-23. *A*, Transfection of NIH3T3 cells with pCMV-A-IL-23 was associated with IL-23 protein expression in vitro without IL-12 expression. To examine IL-23 and IL-12 expression of pCMV-A-IL-23, the NIH3T3 cells were transfected using Effecten with 2 μg of pCMV-A-IL-23 or control vectors such as pCMV-A-EGFP and pCMV-A-IL-12, and the culture supernatant was harvested 48 h after the transfection and tested for concentration of IL-23 and IL-12 using the ELISA specific for respective cytokines. The 30 ng/ml immunoreactive IL-23 was detected only in the supernatant of the cells transfected with pCMV-A-IL-23, and 800 pg/ml immunoreactive IL-12 was detected only in the supernatant of the cells transfected with pCMV-A-IL-12. *B*, Biological activity of the expressed protein was confirmed. The biological activity of the protein expressed by the pCMV-A-IL-23 was confirmed. The culture supernatant of NIH3T3 cells transfected with pCMV-A-IL-23 was tested for its ability to induce IL-17 secretion from Con A-stimulated blast T cells (Con A-blast T cells). Significant levels of IL-17 were detected in the culture supernatant of Con A-blast T cells in a dose-dependent manner when the supernatant of NIH3T3 cells transfected with pCMV-A-IL-23 was added. In contrast, IL-17 was not detected on either of the culture supernatants of Con A-blast T cells stimulated with the supernatant of NIH3T3 cells transfected with p40-IRES-DsRed2 as a negative control. These results indicate that biologically active IL-23 can be expressed by the pCMV-A-IL-23. *C*, The RNA expression of IVE in vivo was confirmed. To study in vivo effects associated with systemic administration of IL-23, we used IVE into the bilateral pretibial muscles, which allows the prolonged production and release to systemic blood circulation of large amount of proteins (18). To confirm the cytokine secretion, expression plasmids for EGFP, IL-12, and IL-23 were transfected into the pretibial muscles of mice, and the expression of each subunit was examined using RT-PCR. In IL-23-transfected mice, p19 and p40 were detected, but p35 was not detected. In contrast, only the p35 and p40 were detected in IL-12-transfected mice. *D*, Significantly high IL-23-protein expression was confirmed in the serum of the animals treated with IVE using pCMV-A-IL-23. We also examined the in vivo expression of IL-23 and IL-12 protein in the serum after IVE using pCMV-A-IL-23 or control vectors such as pCMV-A-EGFP and pCMV-A-IL-12. Significantly high IL-23-protein expression was also confirmed in the serum of the animals treated with IVE using pCMV-A-IL-23 4 days prior. However, significantly high IL-12-protein expression was detected only in the serum of the animals treated with IVE using pCMV-A-IL-12. *E*, Biological activity of immunoreactive IL-23 in the serum was confirmed. The mice were treated with IVE using 100 μg of IL-23 and sacrificed 4 days after the treatment to harvest the serum. Con A-blast T cells (1×10^6) were cultured for 72 h in the presence of the serum containing 0.5 ng/ml total p40 defined by ELISA. Significant amount of IL-17 was detected only in the supernatant of the culture stimulated with the serum harvested from the mice treated with IL-23-IVE (*a*), while IFN-γ was not detected in the same serum (*b*). Therefore, administration of pCMV-A-IL-23 using IVE results in the expression of biological active IL-23 in vivo at high levels. This system was used through out the following studies.

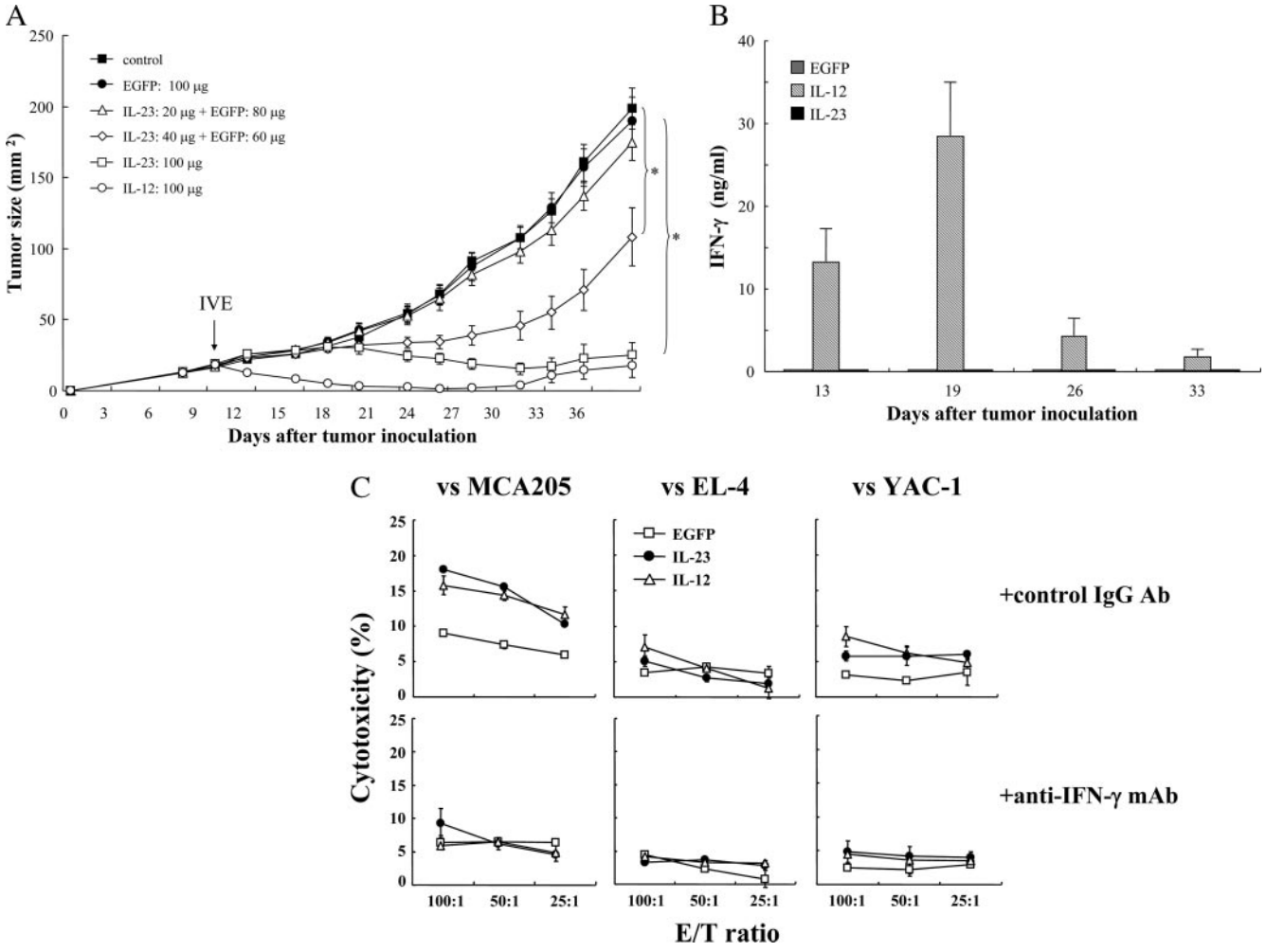

FIGURE 2. IL-23 treatment resulted in significant suppression of the growth of MCA205 tumor cells with characteristic immune responses, including the low serum IFN-γ concentration. *A*, Treatment with IL-23 was associated with significant antitumor effects. Mice were inoculated with 1×10^5 cells of MCA205 tumor cells i.d. on day 0 and treated the mice with increasing doses of IL-23 (20, 40, and 100 μg of plasmid DNA) on day 9. *B*, The significant elevation of IFN-γ levels in the serum was not observed in the animals treated with IL-23. IFN-γ levels in the serum were determined in EGFP, IL-12, and IL-23 treatment mice on day 13, 19, 26, and 33 after tumor inoculation. *C*, Treatment with IL-12 and IL-23 was associated with the induction of tumor-specific CTL. The lymphoid cells from lymph nodes of these three mice on day 30 after tumor inoculation and stimulated 1×10^6 of these cells in vitro with irradiated 1×10^5 of MCA205 fibrosarcoma or EL-4 tumor cells in the presence of IL-2 (20 U/ml) with anti-IFN-γ mAb or control IgG Ab. After 72 h of coculture, CTL activities were detected. Tumor sizes of the animals in the respective groups were compared with two-way repeated-measures ANOVA by Fisher's PLSD test. The statistical deference of the in vitro data depict mean ± SE of three mice are representative of three comparable experiments. *, $p < 0.0001$ and **, $p < 0.05$ vs respective controls.

cytotoxic activities against inoculated tumor cells using a 4-h ^{51}Cr release assay. We isolated lymphoid cells from lymph nodes of these three mice on day 30 after tumor inoculation and stimulated 1×10^6 of these cells in vitro with irradiated 1×10^5 of MCA205 fibrosarcoma or MC38 tumor cells in the presence of IL-2 (20 U/ml) for 72 h with anti-IFN-γ mAb or control IgG Ab. As shown in Fig. 2*C*, the cytotoxic activities of the lymph node cells from IL-23-treated mice and IL-12-treated ones were significantly increased only against MCA205 tumor cells when compared with those of EGFP-treated mice. The positive activities were abrogated when the anti-IFN-γ mAb was added. These results indicated that IFN-γ response is involved in the induction of cytotoxic activities.

In vivo depletion of CD4$^+$ T cells, CD8$^+$ T cells, or NK cells significantly impaired the antitumor effects of IL-23 treatment

We examined the involvement of CD4$^+$ T cells, CD8$^+$ T cells, or NK cells in antitumor effects associated with IL-23 treatment. Each cell type was depleted using specific Ab as described in *Materials and Methods*, and the flow cytometric analysis confirmed that the treatment successfully depleted the appropriate cell population at least by 95%. Depletion of either CD4$^+$ T cells, CD8$^+$ T cells, or CD4$^+$ T cells or CD8$^+$ T cells resulted in complete abrogation of antitumor activity of IL-23 treatment (Fig. 3*A*). In contrast, depletion either with anti-asialo GM1 antiserum or anti-NK1.1 Ab significantly but not completely inhibited IL-23 antitumor effects (Fig. 3*B*). These results demonstrated the absolute requirements for CD4$^+$ T cells or CD8$^+$ T cells and the significant but partial involvement of NK cells in the antitumor responses associated with IL-23 treatment in vivo.

CD4$^+$ T cells were activated to produce IFN-γ and IL-17 during the antitumor responses induced with IL-23 treatment

To examine the nature of the antitumor immune responses of the mice treated with IL-23 in detail, we harvested lymph nodes of treated mice on day 30 when the transgene expression of IL-23 is

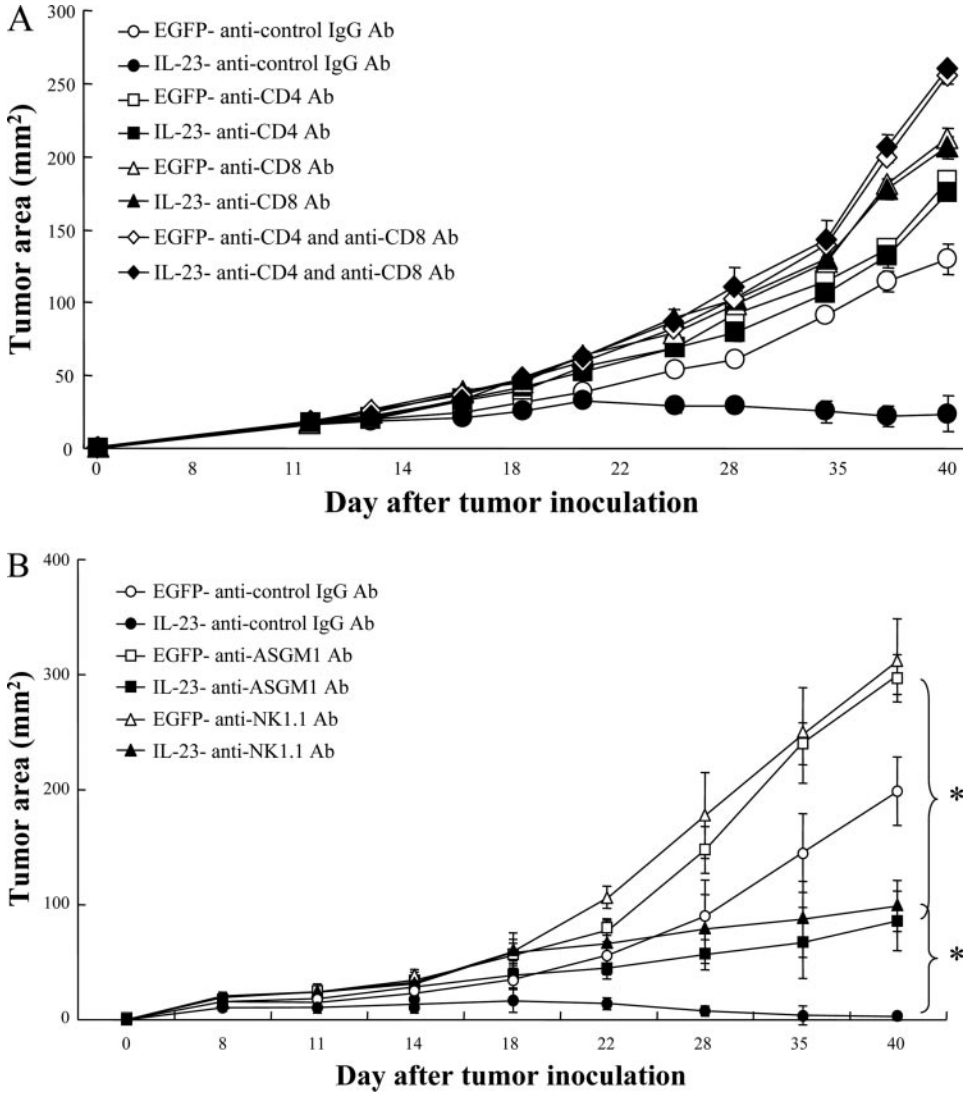

FIGURE 3. In vivo depletion of $CD4^+$ T cells, $CD8^+$ T cells (*A*), or NK cells (*B*) significantly impaired the antitumor effects of IL-23 treatment. The specific cells were depleted in vivo as described in *Materials and Methods*, and the mice were inoculated with 1×10^5 cells of MCA205 tumor cells i.d. on day 0 and treated with IL-23. The data depict mean ± SE of three mice are representative of three comparable experiments.

no longer observed but the suppression of the tumor growth was significant. Lymphoid cells were prepared from these lymph nodes immediately after the harvest, stimulated with immobilized anti-CD3 mAb (10 μg/ml) in vitro for 6 h in the presence of BFA, and analyzed for the intracellular IFN-γ production by flow cytometry (Fig. 4*Aa*). The percentage of positive IFN-γ response in $CD4^+$ T cells (15.7%) of the IL-23-treated mice was significantly high when compared with that of the EGFP-treated ones. The staining for both IFN-γ and IL-17 was performed on T cells gated for CD4-positive staining as well. As shown in Fig. 4*Ab*, there was no significant population showing simultaneously positive for both IFN-γ and IL-17 expression. The lymphoid cells separated from the lymph nodes were also tested for cytokine responses upon CD3 stimulation (Fig. 4*B*). When they were stimulated with soluble anti-CD3 mAb (0.1 μg/ml) for 36 h, they produced significantly higher amount of IFN-γ when compared with that of EGFP-treated mice, and this IFN-γ secretion was almost completely blocked by the addition of anti-CD4 mAb in the assay (Fig. 4*Ba*). This type of IFN-γ response can be induced with the IL-23 treatment even in the non-tumor-bearing mice as well (data not shown). Furthermore, they also produced significantly more IL-17 when compared with that of EGFP-treated mice, and this IL-17 secretion was completely blocked by the addition of anti-CD4 mAb in the assay as well (Fig. 4*Bb*). The $CD4^+$ T cells were activated to produce IFN-γ and IL-17 during the antitumor responses induced with IL-23 treatment. To see the levels of IFN-γ and IL-17 induced by a tumor Ag, we measured the concentration of these cytokines during MLTR using lymphoid cells isolated from the lymph nodes of the treated mice on day 30 after tumor inoculation. As shown in Fig. 4*C*, *a* and *b*, significantly high levels of IFN-γ and IL-17 were detected in the supernatant of MLTR. These responses were almost completely blocked with the addition of the anti-CD4 mAb in the culture. Furthermore, the IFN-γ response appears to be MCA205 specific as shown in Fig. 4*Cc*.

The levels of TNF-α were also measured in the supernatant of the T cells stimulated with anti-CD3 mAb or irradiated MCA205 tumor cells (Fig. 4*D*). Although its expression was detected upon stimulation with CD3 (Fig. 4*Da*) or tumor cells (Fig. 4*Db*), it was 100-fold lower when compared with the ones for IFN-γ or IL-17, and the expression was significantly reduced to the basal level with the addition of anti-IL-17 blocking Ab (Fig. 4*Da*).

IL-23 antitumor effects were absent in IFN-γ KO mice

To determine the role of IFN-γ in the development of antitumor effects with IL-23, we used IFN-γ KO mice. Before performing in vivo experiments, we examined the characteristics of cytokine responses related to our questions in IFN-γ KO mice because this

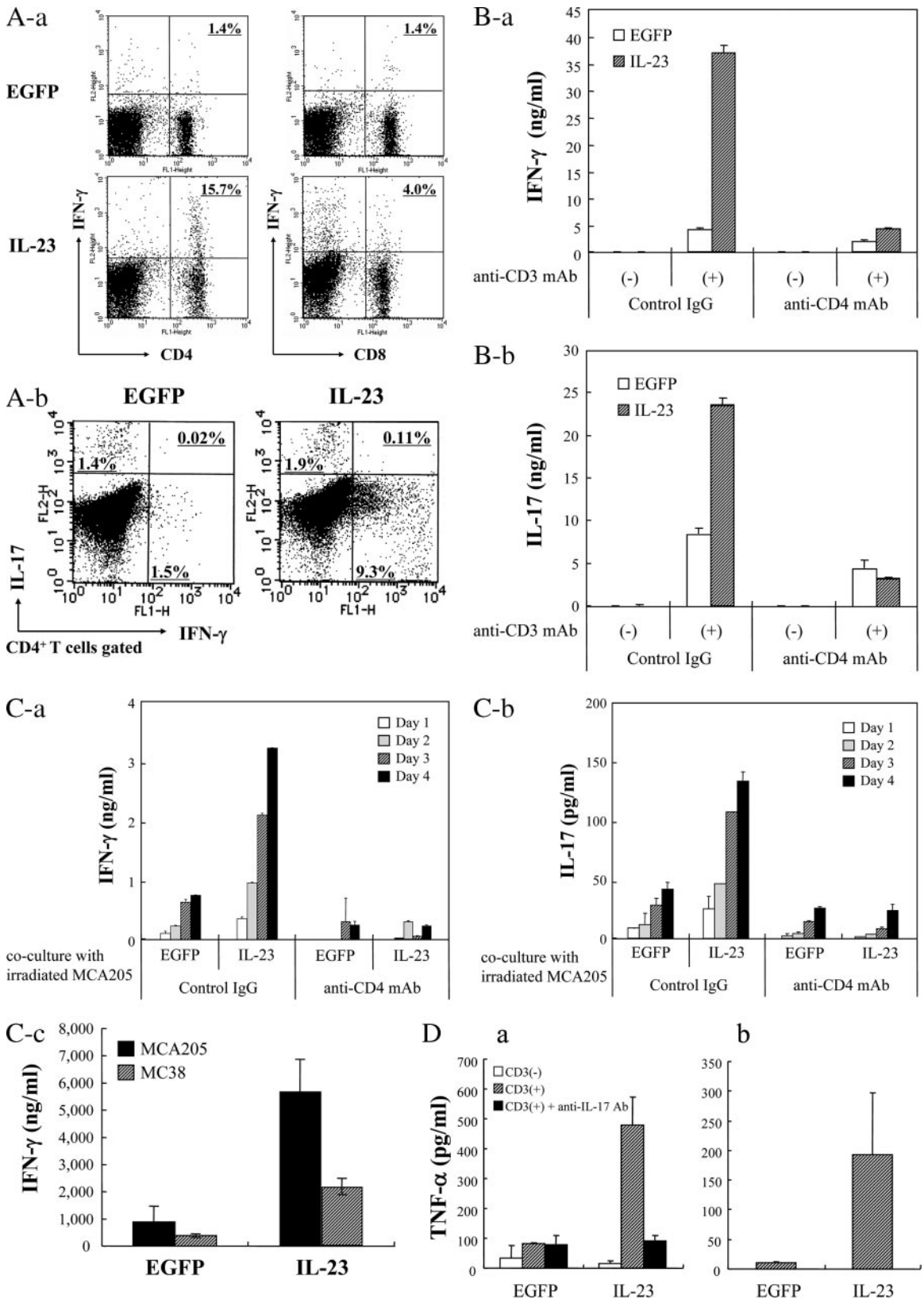

FIGURE 4. CD4$^+$ T cells were activated to produce IFN-γ and IL-17 during the antitumor responses induced with IL-23 treatment. *A*, The percentage of CD4$^+$ T cells expressing only IFN-γ but not IL-17 was significantly higher in the IL-23-treated mice when compared with that of the EGFP-treated ones. The lymph nodes cells were stimulated with immobilized anti-CD3 mAb (10 μg/ml) for 6 h in the presence of BFA and then analyzed for the intracellular IFN-γ or IL-17 production by flow cytometry to examine the T cell responses with the in vitro stimulation. The percentage of CD4$^+$ T cells expressing IFN-γ was significantly high in the IL-23-treated mice (*a*). The staining for both IFN-γ and IL-17 was performed on T cells gated for CD4-positive staining as well, and there was no significant population showing simultaneously positive for both IFN-γ and IL-17 expression (*b*). *B*, The T cells harvested from the mice treated with IL-23 were activated to produce IFN-γ and IL-17 upon CD3 stimulation. IFN-γ (*a*) and IL-17 (*b*) production were measured using ELISA kit with the stimulation using soluble anti-CD3 mAb (0.1 μg/ml) for 36 h with or without anti-CD4-blocking Ab. Representative results of three

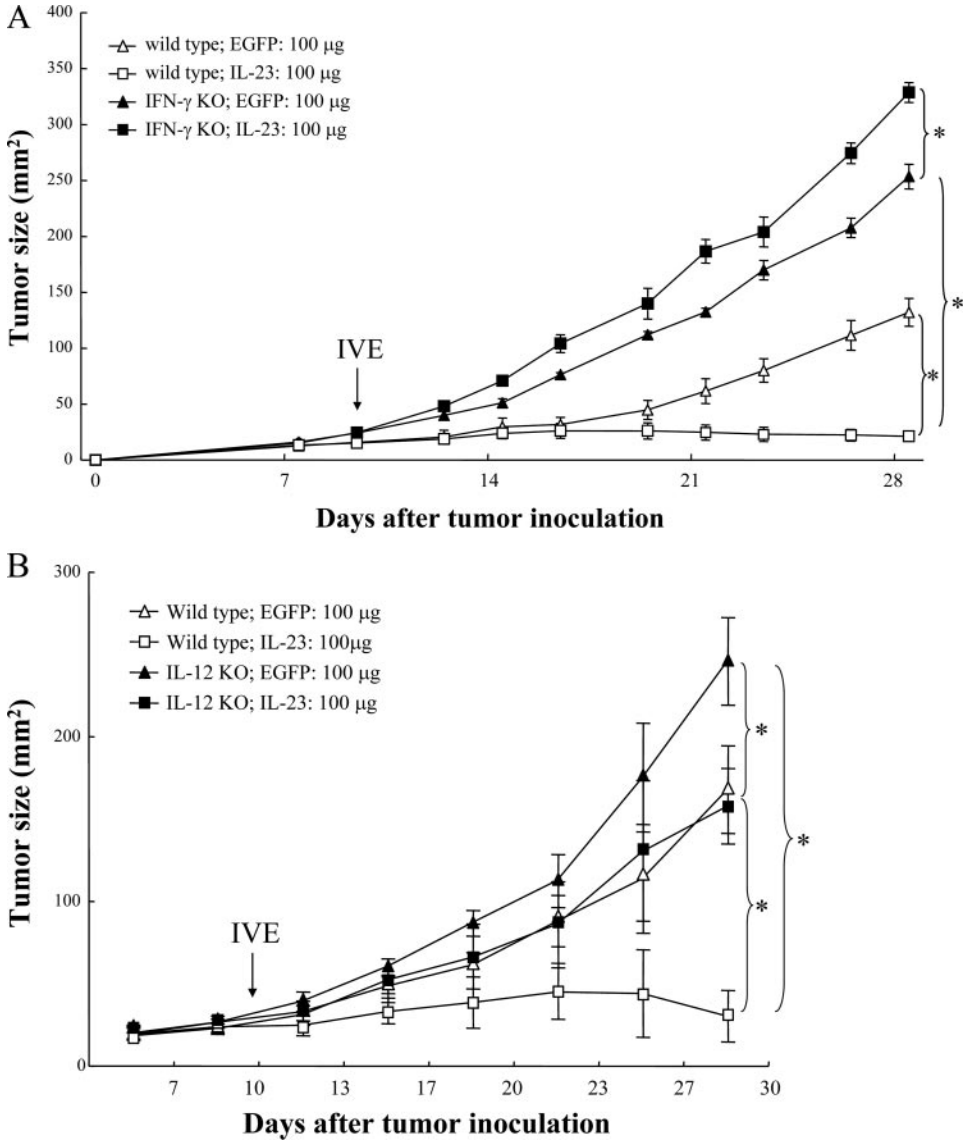

FIGURE 5. IL-23 antitumor effects were completely abrogated in IFN-γ KO mice and significantly reduced in IL-12 KO mice. *A*, IL-23 antitumor effects were completely abrogated in IFN-γ KO mice. *B*, IL-23 antitumor effects were reduced significantly in IL-12 KO mice. The IFN-γ KO mice or IL-12 KO mice were inoculated i.d. with 1×10^5 cells of MCA205 tumor cells on day 0, and they were treated with IVE of IL-23 using 100 μg of plasmid DNA when the tumor mass became palpable (6–8 mm) in 9–11 days. Tumor sizes of the animals in each group were measured and compared with two-way repeated-measures ANOVA by Fisher's PLSD test. Representative results of three experiments with consistent results are shown. The mean ± SE were calculated from the results of three mice in such representative experiment. *, $p < 0.05$ vs respective controls.

genetic manipulation could cause immune dysfunction other than IFN-γ secretion in these animals. We prepared Con A-blast T cells from the splenocytes of wild-type and IFN-γ KO mice and stimulated these splenocytes in vitro to see whether IFN-γ KO maintains the immune responses to IL-23 in inducing IL-17. Although the magnitude of the response was reduced, significant IL-17 response was observed with the stimulation using recombinant human IL-23 protein on the Con A-blast T cells of IFN-γ KO mice (data not shown).

The IFN-γ KO mice were inoculated with 1×10^5 of MCA205 tumor cells, and IVE-EGFP or IVE-IL-23 was performed on these mice when the tumor mass became palpable (6–8 mm) in 9–11 days (Fig. 5*A*). Although the biological activities of IL-23, including IL-17 induction, other than IFN-γ responses, were maintained in IFN-γ KO mice, antitumor effects of IL-23 treatment were completely abrogated in IFN-γ KO mice. These results suggest that IFN-γ is primarily involved for the development of antitumor activities induced with IL-23 treatment.

Furthermore, we used IL-12 KO mice as well to determine the role of endogenously expressed IL-12 in the development of antitumor effects with IL-23. The IL-12 KO mice were inoculated with 1×10^5 of MCA205 tumor cells, and IVE-EGFP or IVE-IL-23 was performed on these mice when the tumor mass became palpable (6–8 mm) in 9–11 days (Fig. 5*B*). In this experiment, antitumor effects associated with IL-23 treatment were reduced significantly in IL-12 KO mice. These results might indicate that

experiments with consistent results are shown. The mean ± SE were calculated from the results of three mice in such representative experiment. *C*, The T cells harvested from the mice treated with IL-23 were activated to produce IFN-γ and IL-17 upon stimulation using tumor cells. To see the levels of IFN-γ and IL-17 induced by a tumor Ag, we measured the concentration of these cytokines during MLTR using lymphoid cells isolated from the lymph nodes of the treated mice on day 30 after tumor inoculation. Significantly high levels of IFN-γ (*a*) and IL-17 (*b*) were detected in the supernatant of MLTR. These responses were almost completely blocked with the addition of the anti-CD4 mAb in the culture. Furthermore, the IFN-γ response appears to be MCA205 specific as shown in *c*. *D*, The T cells harvested from the mice treated with IL-23 produced TNF-α at low levels upon stimulation using CD3 mAb or tumor cells. Although its expression was detected upon stimulation with CD3 (*a*) or tumor cells (*b*), it was 100-fold lower when compared with the ones for IFN-γ or IL-17, and the expression was reduced significantly to the basal level with the addition of anti-IL-17-blocking Ab (*a*).

endogenous IL-12 in the host has significant contribution to the antitumor effects of IL-23 treatment.

Discussion

In this study, we first established the experimental system that could examine in vivo effects associated with systemic administration of IL-23 in mice. We injected IL-23 expression plasmid into the pretibial muscles of the mouse and performed IVE, which could have the gene to be transduced efficiently and expressed continuously at the high level in vivo. In the mice treated with this method, the assay with ELISA showed that a significant amount of IL-23 was present in the serum of the treated animals for at least 7 days. We also confirmed that the immunoreactive IL-23 in the serum could induce the secretion of IL-17 from Con A-blast T cells (33). These results indicate that we have established the method to systemically administer biologically active IL-23 protein into the mice.

Using this IL-23-IVE system, we treated the established MCA205 fibrosarcoma inoculated i.d. Although the endogenous IL-23 expression has been reported to promote tumor incidence and growth (24), significant suppression of the tumor growth and prolongation of the survival of the mice were observed in the mice treated with IL-23-IVE when compared with those of the control mice treated with EGFP. This type of discordant function has been reported on multiple cytokines. For instance, we have previously reported that IL-10, which is recognized to have immune regulatory roles with physiological doses, is associated with potent antitumor effects when IL-10 is administered at excessive amount for prolonged period (34, 35). The results of the IL-23 studies reported by us and the others may suggest that administration of excessive amount of IL-23 could induce antitumor immune responses, but endogenously provided IL-23 at a physiological level would act contrary.

The potency of the antitumor effects was dose dependent and equivalent to that of IL-12. Furthermore, the cytotoxic activities of the lymph node cells harvested from IL-23-treated mice were increased significantly against MCA205 tumor cells when compared with those of EGFP-treated mice. These results indicate that the systemic administration of IL-23 is associated with significant antitumor immune responses in this mouse tumor model. Because systemic administration of IL-12, a cytokine sharing some important structures and functions with IL-23, has potent antitumor effects with significant toxicities related to highly elevated IFN-γ level in the serum (36), we examined the possible side effects with IL-23 administration. In the mice treated with IL-23-IVE, we found no obvious toxicities, including treatment-related death, splenomegaly, or lymphadenopathy. Furthermore, IFN-γ was not detected in the serum of the mice treated with IL-23-IVE, whereas the serum IFN-γ was elevated significantly in the ones treated with IL-12-IVE in the same manner. Because systemic administration of IL-23 exerts potent antitumor effects without obvious side effects related to IFN-γ in our tumor model, our results suggest that IL-23 could be a candidate for clinical development as an antitumor agent for systemic administration. Overwijk et al. (24) recently reported that systemic IL-23 administration showed immunological and antitumor effects only as a cancer vaccine adjuvant. The difference between their results and ours appears to be caused by the difference of the tumor models. The translation to the human clinical trials would require further and careful study on this approach.

The absence of the serum IFN-γ elevation in the animals treated with IL-23 strongly suggest that IL-23 administration induces immune responses significantly different from those of IL-12. We examined this hypothesis using multiple means. We first examined which immune cell types are involved in IL-23-induced antitumor immunity. Antitumor effects of IL-23 treatment were completely abrogated when the blocking Abs specific to $CD4^+$ T cells or $CD8^+$ T cells was administered. In contrast, administration of anti-asialo-1 or NK1.1 Ab, which could deplete NK cells, significantly but partially abrogate the antitumor effects of IL-23 treatment. These results suggest that IL-23 can induce tumor-specific antitumor immune responses, which appear to be exerted by the $CD8^+$ CTLs, solely through the activation of $CD4^+$ T cells. Because only the administration of both anti-CD4- and anti-CD8-blocking Abs could abrogate IL-12 antitumor effects as shown in our previous study (36), the mechanism of the antitumor effects associated with IL-23 might be significantly different from the ones with IL-12. Furthermore, although the previous studies elsewhere using IL-23 local production system (25–27) showed no evidence for the involvement of NK cells, our results in the depletion study using anti-asialo-1 Ab or NK1.1 Ab suggest that NK cells might be significantly involved in the antitumor responses associated with IL-23 treatment in vivo. Because it has not been clearly demonstrated whether NK cells express receptors for IL-23, NK cells might be involved in IL-23-mediated antitumor effects directly or through the activation of $CD4^+$ T cells. This interpretation on indirect activation of NK cells could also explain why the suppression of the tumor growth associated with IL-23 treatment becomes significant not immediately after the administration but ~2 wk after the initiation of the treatment, whereas the significant antitumor effect can be observed immediately after the IL-12 treatment. All these results might suggest that IL-23 treatment could directly induce only the activation of $CD4^+$ T cells and then indirectly activate multiple effector cell populations, including $CD8^+$ T cells and NK cells through the functions of the activated $CD4^+$ T cells.

We also examined the immune responses of the treated mice further to learn how $CD4^+$ T cells are involved in IL-23-induced antitumor immunity. The $CD4^+$ T cells in lymph nodes of mice treated with IVE-IL-23 showed significant expression of both IFN-γ and IL-17 upon stimulation with anti-CD3 mAb or the tumor Ag. Because the IL-17 has recently shown to be involved in inducing characteristic immune reaction termed Th17-type reaction (18, 19), these results suggest that IL-23 treatment might promote Th17-type immune responses as well as Th1-type ones in exerting antitumor effects. However, we have shown in the next set of the experiments that antitumor effects of IL-23 treatment were completely abrogated in IFN-γ KO mice. Because the capability of IL-23 for IL-17 induction are lower but significantly maintained in the IFN-γ KO mice (data not shown), the findings in IFN-γ KO mice might suggest that Th1-type immune responses induced with IL-23 treatment play the primary role in inducing potent antitumor effects. Furthermore, the results using IFN-γ KO mice (Fig. 5*A*) and IL-12 KO mice (Fig. 5*B*) might collectively suggest that potent antitumor effects associated with IL-23 treatment could be achieved when the Th1-type response is fully promoted in the presence of endogenously expressed IL-12. Because IL-6 and TGF-β together, but not IL-23, have been reported to induce the differentiation of pathogenic Th17 cells from naive T cells (37), the role of Th17 in IL-23 treatment is still unclear. In addition, the roles of the TNF-α, which was found to be modestly expressed by T cells in the animals treated with IVE-IL-23 (Fig. 4*D*), is also unclear. Further studies appear to be necessary to solve these questions.

In conclusion, we demonstrated using in vivo mouse tumor systems that the administration of IL-23 is associated with significant antitumor effects primarily mediated through Th1-type response

without showing significant elevation of serum IFN-γ levels. Although the clinical application of IL-23 treatment as a cancer treatment appears to be promising, further examination would be required on the mechanism of its antitumor immune responses.

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Disclosures

The authors have no financial conflict of interest.

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