Interleukin-23 and Interleukin-27 Exert Quite Different Antitumor and Vaccine Effects on Poorly Immunogenic Melanoma

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Abstract

Recent studies revealed that two novel interleukin (IL)-12-related cytokines, IL-23 and IL-27, have potent antitumor activities. However, the antitumor effects were mainly evaluated in relatively highly immunogenic tumors and have not been fully evaluated against nonimmunogenic or poorly immunogenic tumors. In this study, we investigated the antitumor efficacies of IL-23 and IL-27 on poorly immunogenic B16F10 melanoma and found that the antitumor responses mediated by IL-23 and IL-27 were clearly different. In syngeneic mice, mouse single-chain (sc) IL-23-transfected B16F10 (B16/IL-23) tumors exhibited almost the same growth curve as B16F10 parental tumor about until day 20 after tumor injection and then showed growth inhibition or even regression. In contrast, scIL-27-transfected B16F10 (B16/IL-27) tumors exhibited significant retardation of tumor growth from the early stage. In vivo depletion assay revealed that the antitumor effect of B16/IL-23 was mainly mediated by CD8+ T cells and IFN-γ whereas that of B16/IL-27 mainly involved natural killer cells and was independent of IFN-γ. We also found that antitumor effects of B16/IL-23 and B16/IL-27 were synergistically enhanced by treatment with IL-18 and IL-12, respectively. Furthermore, B16/IL-23-vaccinated mice developed protective immunity against parental B16F10 tumors but B16/IL-27-vaccinated mice did not. When combined with prior in vivo depletion of CD8+ T cells, 80% of B16/IL-23-vaccinated mice completely rejected subsequent tumor challenge. Finally, we showed that the systemic administration of neither IL-23 nor IL-27 induced such intense toxicity as IL-12. Our data support that IL-23 and IL-27 might play a role in future cytokine-based immunotherapy against poorly immunogenic tumors. (Cancer Res 2006; 66(12): 6395-404)

Introduction

Cytokines play a critical role in the developmental regulation of naïve CD4+ T cells into either T helper 1 (Th1) or Th2 cells. Interleukin (IL)-12, a heterodimeric cytokine composed of two subunits designated p40 and p35, has been widely accepted as the main cytokine regulating Th1 differentiation. IL-12 induces interferon (IFN)-γ production by natural killer (NK) cells, T cells, dendritic cells, and macrophages. The ability of IL-12 to regulate Th1 differentiation and facilitate cell-mediated immune responses, including the enhancement of NK cell cytotoxicity and the generation of CTLs, is favorable for the antitumor response (1–3). Indeed, several comparative studies have shown that IL-12 is the most effective cytokine for eradication of experimental tumors, prevention of metastasis development, and attainment of long-term antitumor immunity (4–6).

Recently, two novel IL-12-related cytokines, IL-23 and IL-27, were identified (7, 8). IL-23 is composed of the p19 subunit, a molecule related to the p35 subunit of IL-12, and the p40 subunit of IL-12. IL-23 is mainly produced by monocytes/macrophages and the dendritic cell population. IL-23 acts on target cell populations via binding to a heterodimeric receptor consisting of a β1 subunit that is also a component of IL-12R and a unique IL-23R chain (9). IL-23 has similar biological activities to IL-12 in vitro (7) but IL-23 is not as efficient as IL-12 in the induction of IFN-γ production and in the polarization of T cells to the Th1 pattern. In contrast, IL-23 is more effective than IL-12 in the induction of memory T-cell proliferation. On the other hand, IL-27 is the newest member of the IL-12-related cytokine family. Like other members, IL-27 is a heterodimeric cytokine composed of EBV-induced gene 3 (EBI3) and p28 subunits that are structurally related to the p40 and p35 subunits of IL-12, respectively (8). IL-27 is mainly produced by activated antigen-presenting cells including lipopolysaccharide-stimulated monocytes and monocyte-derived dendritic cells (8). IL-27 binds target cells via a heterodimeric receptor consisting of WSX-1/TCCR and gp130 subunits (10). IL-27 preferentially induces the proliferation of naïve but not memory T cells in combination with T-cell receptor cross-linking. Furthermore, IL-27 synergizes with IL-12 to potentiate IFN-γ production by activated naïve T- and NK-cell populations (8). Thus, IL-27 is thought to promote Th1 polarization. These functional characteristics of IL-23 and IL-27 suggest that they could be useful candidates in the cytokine-based treatment of solid tumors. Indeed, recent studies in experimental animals revealed that both cytokines have potent antitumor activities (11–15). However, the antitumor effects were mainly evaluated in relatively highly immunogenic tumors. Considering clinical applications, it is very important to evaluate the immunotherapeutic effects against poorly immunogenic tumors.

B16 melanoma cells are poorly immunogenic tumor cells which originally developed in the C57BL/6 mouse spontaneously, and B16F10 cells, a subline of B16 melanoma cells, are thought to reflect the poor immunogenicity of metastatic tumors in humans (16). In this study, therefore, we investigated the antitumor efficacies of IL-23 and IL-27 on B16F10 melanoma cells. It had been shown that effective tumor-protective immunity could be achieved in a poorly immunogenic, syngeneic tumor model of murine neuroblastoma by the transduction of a fusion gene encoding a linearized single-chain (sc) IL-12 into tumor cells (17). Thus, we constructed mouse...
scIL-23 and scIL-27, cloned them into the same expression vector, and then transfected the plasmids into B16F10 cells, which enabled us to compare both antitumor efficacies. We found that IL-23 and IL-27 exert antitumor effects on poorly immunogenic melanoma through quite different mechanisms. To the best of our knowledge, this is the first report showing comparative antitumor effects of IL-23 and IL-27 on poorly immunogenic tumors under the same experimental conditions.

Materials and Methods

Tumor cell lines and mice. B16F10 melanoma and Lewis lung carcinoma cells were maintained in Eagle’s modified essential medium supplemented with 5% and 10% heat-inactivated fetal bovine serum (FBS), respectively, at 37°C in a humidified atmosphere of 5% CO2/air. Specific pathogen-free C57BL/6N mice (6- to 8-week-old females) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine.

Preparation of B16F10 transfectants. The cDNAs encoding scIL-23 composed of the p40 chain, (Gly4Ser)3 linker, and the p19 chain were amplified from an scIL-23-immunoglobulin fusion protein composed of the p40 chain, (Gly4Ser)3 linker, and the p19 chain were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine.

Preparation of purified recombinant IL-23 and IL-27 proteins. Purified mouse recombinant IL-27 (rIL-27) was prepared as a single-chain protein by flexibly linking EB3 to p28 using HEK293T cells as previously reported, with yeast artificial chromosome-1 cells as the target and spleen cells as effector cells at effector-to-target ratios of 100:1, 50:1, and 25:1. Depletion completely abrogated the detectable NK cell activity. To neutralize IFN-γ activity in vivo, 2.8 mg of B4-624 (ATCC) rat mAb against mouse IFN-γ were injected i.p. on day −1 or day 20 and every 7 days thereafter. Rat IgG (Wako Fine Chemicals) and rabbit serum (Sigma Chemical) were used as the control antibody.

Systemic treatment with rIL-12 or rIL-18. Mouse rIL-12 and rIL-18 were gifts from Hayashibara Biochemical Laboratories (Okayama, Japan). Mice were injected s.c. with 106 cells of B16/IL-23, B16/IL-27, or B16/control on day 0 and injected i.p. with rIL-12 (200 ng per mouse) or rIL-18 (1 mg per mouse) from day −1 and thrice a week thereafter.

Prophylactic vaccine treatment models. B16/IL-12, B16/IL-27, and B16/control were preincubated with mitomycin C (50 μg/mL; Sigma Chemical) at 37°C for 30 minutes and then washed with PBS thrice. On days −14 and −7, 105 cells of these transfectants were injected s.c. in the left flank as vaccine treatment. Mice were then s.c. challenged with 106 cells of parental B16F10 cells on day 0 into the right flank. The prophylactic efficacy of those transfectants was also examined in combination with anti-CD25 mAb (PC61). Cells were washed and incubated in the following mAbs for 30 minutes at 4°C in 1% bovine serum albumin-containing PBS: phycoerythrin-conjugated anti-LT4 (CT4) mAb (GK1.5; BD Pharmingen) and FITC-conjugated anti-CD25 (IL-2Rα) mAb (7D4; BD Pharmingen). After incubation, the cells were washed, suspended in PBS, and analyzed using FACSscan (Becton Dickinson Co., Mountain View, CA).

Flow cytometry. Spleen cells and lymph node cells were prepared from C57BL/6 mice that were untreated or treated with 11 μg of anti-CD25 mAb (PC61). Cells were washed and incubated with the following mAbs for 30 minutes at 4°C in 1% bovine serum albumin-containing PBS: phycoerythrin-conjugated anti-LT4 (CT4) mAb (GK1.5; BD Pharmingen) and FITC-conjugated anti-CD25 (IL-2Rα) mAb (7D4; BD Pharmingen). After incubation, the cells were washed, suspended in PBS, and analyzed using FACSscan (Becton Dickinson Co., Mountain View, CA).

In vitro cytotoxic assay. Spleen cells were collected on day 0 from mice that had been vaccinated on days −14 and −7 or from naive mice, resuspended in 10% FBS-RPMI 1640 (105 cells per 150-μm2 flask) after erythrocyte depletion, and then restimulated in vitro with mitomycin C–treated parental B16F10 cells (5 × 104) for 5 days. Stimulated splenocytes were recloned and cocultured with untreated parental B16F10 cells or Lewis lung carcinoma cells (control target) in a 96-well round-bottomed plate (1 × 104/well/100 μL) by an appropriate effector cell count/target cell count ratio for 4 hours. Released lactate dehydrogenase (LDH) levels in the culture supernatants were measured and the percent cytotoxicity of each well was calculated according to the manufacturer (CytotoX 96 Non-Radioactive Cytotoxicity Assay Kit, Promega).
Assessment of toxicity by IL-12, IL-23, or IL-27. Adverse effects by systemic treatments with IL-12, IL-23, or IL-27 were evaluated according to the protocol previously described (21). In brief, mice were injected i.p. daily for 4 days with vehicle or with 1 μg of murine rIL-12, rIL-23, or rIL-27. Mice were sacrificed the day after the final injection and their organs (liver and spleen) and sera were collected. Liver tissues were fixed in 10% buffered formalin for histology, sectioned, stained with H&E, and evaluated microscopically. Serum alanine transaminase (ALT) activities were determined using the Bettman-Frankel method (S. TA test Wako, Wako Pure Chemical Industries, Ltd.). The serum concentration of IFN-γ was measured with mouse IFN-γ ELISA kit (BioSource International).

Data analysis. Each experiment was done at least twice. The statistical significance of differences in means among groups was determined using Dunnett’s test or Turkey-Kramer post hoc test. Survival curves were computed with the Kaplan-Meier method and differences in survival were validated by log-rank test. The differences were considered statistically significant at P < 0.05. All data were tabulated and analyzed using StatView 5.0 software (SAS Institute, Inc., Cary, NC).

Results

B16F10 melanoma transfected with scIL-12, scIL-23, or scIL-27 secreted biologically active heterodimeric cytokines. It is crucial whether heterodimeric forms of IL-12, IL-23, or IL-27 are secreted from each transfectant because those monomers or homodimers have no original biological functions. Therefore, we developed sandwich ELISA systems to detect only heterodimeric forms of IL-23 and IL-27, as described in Materials and Methods, and measured the cytokine levels in each culture supernatant. Several clones of scIL-23-transfected B16F10 cells (B16/IL-23) showed remarkable secretion of the heterodimeric form of IL-23, and two clones (no. 7 and no. 10) were selected as high-level producers. Similarly, two clones (no. 17 and no. 7) of scIL-27-transfected B16F10 cells (B16/IL-27) and two clones (no. 6 and no. 9) of scIL-12-transfected B16F10 cells (B16/IL-12) were selected as high-level producers. The selected clones (B16/IL-23 no. 7, B16/IL-23 no. 10, B16/IL-27 no. 17, B16/IL-27 no. 7, B16/IL-12 no. 6, and B16/IL-12 no. 9) produced 145 ng, 87 ng, 87 ng, 53 ng, 1,455 pg, and 780 pg of each cytokine per 10^6 cells during 24 hours, respectively. Control vector–transfected B16F10 cells (B16/control) did not secrete detectable amount of IL-23, IL-27, or IL-12. ELISA systems for IL-23 and IL-27 did not detect IL-27 secreted by B16/IL-27 and IL-23 secreted by B16/IL-23, showing that the specificity of this system is quite high (Fig. 1A and B).

Next, we analyzed the biological activity of IL-23, IL-27, and IL-12 in the supernatants from the selected transfectants by measuring the proliferation of CD4+ T cells. All the supernatants of B16/IL-23, B16/IL-27, and B16/IL-12 could proliferate CD4+ T cells. The result of the T-cell proliferation assay is summarized in Fig. 1C. The biological activity of IL-27 in the supernatants of B16/IL-27 no. 7 and no. 17 was also confirmed by measuring the proliferation of naïve CD4+ T cells purified from the syngeneic murine spleen (data not shown).

Table 1. Production and bioactivity of IL-23 and IL-27 in 24-hour culture supernatants collected from each transfectant clone in vitro.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>ELISA for IL-23 (ng/ml/10^6 cells/24 hr)</th>
<th>ELISA for IL-27 (ng/ml/10^6 cells/24 hr)</th>
<th>T-cell proliferation assay (% Bioactivity)</th>
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<tr>
<td>B16/IL-23 no.7</td>
<td>50</td>
<td>30</td>
<td>180</td>
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<tr>
<td>B16/IL-27 no.7</td>
<td>180</td>
<td>120</td>
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<td>B16/IL-12 no.6</td>
<td>120</td>
<td>90</td>
<td>180</td>
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<tr>
<td>B16/IL-12 no.9</td>
<td>90</td>
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Figure 1. Production and bioactivity of IL-23 and IL-27 in 24-hour culture supernatants collected from each transfectant clone in vitro. To estimate the cytokine production levels, 24-hour culture supernatants (3 mL) from 10^6 cells of each transfectants were collected. A. sandwich ELISA for mouse IL-23 was established by using mouse rIL-23 R/Fc chimera on the capture side and ANTI-FLAG biotinylated M2 monoclonal antibody as the primary detection antibody. B16/IL-23 no. 7 and no. 10 showed marked secretion of IL-23 in vitro. B. sandwich ELISA for mouse IL-27 was established by using ANTI-FLAG M2-coated 96-well plate on the capture side, anti-mouse IL-27 p28 antibody (primary antibody), and biotinylated anti-goat IgG (H+L) (secondary antibody). B16/IL-27 no. 17 and no. 7 exhibited marked secretion of IL-27 in vitro. C. bioactivity was examined by stimulating purified mouse CD4+ spleen cells with the represented dilution of each culture supernatant under the condition of a 96-well plate coated with anti-CD3 for 4 days (2 × 10^4 per well), and the proliferation activity of each well was measured by adding MTS assay reagent in the last 4 hours of culture and detected using an ELISA reader. Values are the percentage compared with the experiment with stimulation by culture medium only. Supernatant from B16/IL-23 no. 7 was capable of proliferating CD4+ spleen cells in a dose-dependent manner, and those of B16/IL-27 no. 17 and B16/IL-12 no. 6 were also capable of proliferating CD4+ spleen cells. Columns, mean; bars, SD.
IL-23 or IL-27 on tumor growth, $10^5$ cells of B16/IL-23, B16/IL-27, B16/control, or parental B16F10 were injected s.c. into the right flank of syngeneic mice. The tumor growth exhibited by parental B16F10 and B16/control was almost identical (Fig. 2A, top). Compared with them, to our surprise, both B16/IL-23 no. 7 and no. 10 showed quite unique kinetics of tumor growth; i.e., as shown in Fig. 2A (second rung), most of B16/IL-23 tumors exhibited almost same growth curve as the parental B16F10 or B16/control until about day 20 (referred to as the progression phase) and then showed growth inhibition or even regression (referred to as the regression phase). Consequently, the survival times of mice challenged with B16/IL-23 tumors were significantly elongated compared with those of parental B16F10 (Fig. 2B, top; $P < 0.01$ and $P < 0.05$). On the other hand, B16/IL-27 no. 7 and no. 17 exhibited significant retardation of tumor growth from the early stage (Fig. 2A, third rung). The survival times of mice challenged with B16/IL-27 tumors were also elongated compared with those of parental B16F10 and B16/control (Fig. 2B, middle; $P < 0.05$). B16/IL-12 no. 6 and no. 9 also exhibited significant retardation of tumor growth from the early stage (Fig. 2A, bottom) and challenge with B16/IL-12 tumors resulted in prolonged survival (Fig. 2B, bottom; $P < 0.05$).

**In vivo depletion assay reveals distinct antitumor effectors between B16/IL-23 and B16/IL-27.** To characterize the cells or cytokines responsible for the inhibition of tumor growth of B16/IL-23 and B16/IL-27, tumor-bearing hosts were depleted in vivo of selected effector cell populations (CD8+ T cells and NK cells) or cytokine (IFN-γ) from 1 day before tumor challenge. As a result, the unique growth inhibition in the regression phase of B16/IL-23 tumor was completely canceled by depleting CD8+ T cells, NK cells, or IFN-γ (Fig. 3A). Next, we tried to clarify the effectors directly involved in the growth inhibition in the regression phase of B16/IL-23 tumors. As shown in Fig. 3C, treatment with anti-CD8 mAb or anti-IFN-γ mAb from day 20 clearly canceled growth inhibition whereas anti-asialo GM1 sera treatment from day 20 did not affect inhibition. This result suggests that CD8+ T cells as well as IFN-γ,
but not NK cells, are the key factors of this unique regression of B16/IL-23 tumors. On the other hand, only the depletion of NK cells by anti–asialo GM1 sera remarkably accelerated the tumor growth of B16/IL-27 to almost the same rate as the tumor growth of B16/control (Fig. 3B). However, neither the depletion of CD8\(^+\) T cells nor the neutralization of IFN-\(\gamma\) affected B16/IL-27 tumor growth.

Antitumor effects of B16/IL-23 and B16/IL-27 are synergistically enhanced by systemic treatment with IL-18 and IL-12, respectively. To investigate the synergistic antitumor effects of IL-12 or IL-18 on B16/IL-23 or B16/IL-27 in vivo, mouse rIL-12 (200 ng per mouse) or rIL-18 (1 \(\mu\)g per mouse) was administered i.p. from day \(-1\), thrice a week, and \(10^5\) cells of B16/control, B16/IL-23, or B16/IL-27 were inoculated s.c. on day 0. Whereas the tumor growth of B16/control was not affected by treatment with rIL-12 or rIL-18 under this experimental condition (Fig. 4A), that of B16/IL-23 showed significant retardation when treated with rIL-18 but not with rIL-12 (Fig. 4B; \(P < 0.05\)). On the other hand, B16/IL-27 showed a synergistic antitumor effect in groups treated with rIL-12 but not with rIL-18 (Fig. 4C; \(P < 0.01\) and \(P < 0.05\)).

B16/IL-23-vaccinated mice developed protective immunity but B16/IL-27-vaccinated mice did not. Next, we investigated the potent tumor-vaccine effect of IL-23 and IL-27. B16/IL-23-vaccinated mice showed significant protective immunity against B16F10 parental tumor cells compared with B16/control-vaccinated mice (Fig. 5A; \(P < 0.01\) and \(P < 0.05\)). On the other hand, B16/IL-27-vaccinated mice did not exhibit any significant protective response. To confirm the prophylactic ability of vaccination with these transfectants, splenocytes from mice vaccinated with B16/IL-23, B16/IL-27, or B16/control were cultured in vitro and their cytotoxic ability was assessed in a functional assay. As shown in Fig. 5B (left), splenocytes from B16/IL-23-vaccinated mice exerted a significant cytotoxic effect against B16F10 melanoma cells at ratios of 200:1 (\(P < 0.01\) and 100:1 (\(P < 0.05\)) compared with the control group. On the other hand, splenocytes from B16/IL-27-vaccinated mice did not show any significant cytotoxicity. Significant cytotoxic activities against Lewis lung carcinoma cells were not observed in those splenocytes (Fig. 5B, right), indicating that specific cytotoxic activity was induced in B16/IL-23-vaccinated mice.

![Figure 3](image-url)

**Figure 3.** In vivo depletion assay. A and B, C57BL/6 mice were i.p. administered with anti-CD8 mAb, anti–asialo GM1 sera, anti-IFN-\(\gamma\) mAb, or control antibodies from day \(-1\) and s.c. challenged with \(10^5\) of B16/IL-23 no. 7 (A) or B16/IL-27 no. 17 (B) on day 0. Points, mean tumor size of five mice per group; bars, SE. C, mice were injected s.c. with \(1 	imes 10^5\) of B16/IL-23 no. 7 in the right flank on day 0 and were i.p. administered with anti-CD8 mAb, anti–asialo GM1 sera, or anti-IFN-\(\gamma\) mAb from day 20. The tumor size of individual mice is represented (\(n = 5-10\)).
It was shown that the removal of CD25+CD4+ regulatory T cells as well as injections of anti-CD25 mAb could induce antitumor response (22, 23). Recently, Sutmuller et al. (24) showed that in a nonimmunogenic B16 melanoma model, vaccination with granulocyte macrophage colony-stimulating factor–transfected tumor cells, together with anti-CD25 treatment, improved the survival of mice. Therefore, we set the following prophylactic treatment protocol: mice were injected i.p. with a low dose of anti-CD25 mAb (PC61) on day −15, followed by immunization with the transfectants on day −14, and challenged with B16F10 parental tumor cells on day 0. First, we confirmed that the populations of CD25+CD4+ cells were clearly reduced by anti-CD25 mAb in the spleen (Fig. 5C) as well as in the lymph nodes (data not shown) using flow cytometry. The reduced population mostly composed of CD25-highly positive CD4+ cells. The efficacy of anti-CD25 mAb lasted for 9 days but not for 11 days (data not shown). Next, we investigated the prophylactic vaccine effects combined with anti-CD25 mAb. In the setting described above, the protective immunity against B16F10 parental tumor of B16/IL-23-transfected mice was remarkably enhanced and resulted in 80% complete tumor rejection (Fig. 5D; P < 0.01). In contrast, there was no difference in the enhanced effects on protective immunity by anti-CD25 mAb treatment between B16/IL-27-vaccinated and B16/control-vaccinated mice.

**IL-23 and IL-27 have much fewer systemic side effects than IL-12.** Finally, we evaluated the adverse effects by systemic treatments with IL-23 or IL-27 because IL-12 is well known to have considerable toxicity. Mice were injected i.p. daily for 4 days with 1 μg of murine rIL-12, rIL-23, or rIL-27. As shown in Fig. 6A, marked splenomegaly was observed in IL-12-treated mice, but not in IL-23- or IL-27-treated mice. Spleen weight was significantly increased in only IL-12-treated mice (P < 0.01). Histologic examination showed that IL-12 induced hepatic perivascular cellular infiltrates but IL-23 and IL-27 scarcely did (Fig. 6B). Correlated with the liver histology, significant elevation of ALT was documented in mice treated with IL-12 (Fig. 6C; P < 0.01). ALT levels in the sera of IL-23- and IL-27-treated mice showed no significant elevation compared with the control. IFN-γ concentrations were remarkably elevated in the sera of IL-12-treated mice, but not in those of IL-23- or IL-27-treated mice (Fig. 6D; P < 0.01).

**Discussion**

We here showed that both IL-23 and IL-27 exerted antitumor effects even on poorly immunogenic B16F10 melanoma cells. To our interest, however, the antitumor responses mediated by IL-23 and IL-27 were clearly different. Whereas the antitumor response of IL-27 was observed from an early stage, that of IL-23 was only evident in the late phase (after about day 20 of tumor injection). To our surprise, some B16/IL-23 tumors regressed after they grew to be large masses (Figs. 2A, second rung). Such phenomenon of delayed regression following maximum growth has never been observed in IL-12-transfected melanoma cells by us (25) and other investigators (26, 27). Thus, IL-23-mediated tumor regression seems to be quite unique. A similar antitumor response had been shown in studies using other tumor cells. Lo et al. (11) showed that IL-23-transduced CT26 colon adenocarcinoma cells grew progressively until day 26, then the tumors started to regress in most mice, resulting in a final 70% rate of complete tumor rejection. They also showed that antitumor activity was mediated through CD8+ T cells but not through CD4+ T cells or NK cells, and that mice rejecting IL-23-transduced tumors developed a memory response against subsequent wild-type tumor challenge. In addition, Wang et al. (12) showed that IL-23 overexpression in Colon 26 tumors produced T cell–dependent antitumor effects and induced systemic immunity. Also in their study, IL-23-transduced Colon 26 cells exhibited transient tumor growth, although it was significantly retarded, and disappeared thereafter. In our study, however, there was no mouse that completely rejected IL-23-transduced B16F10 melanoma...
We suspect that this distinction may be attributed to differences in the immunogenicity of tumor cells and the microenvironment surrounding the tumor. In vivo depletion assay revealed that the antitumor effects of B16/IL-23 were dependent on IFN-γ and mediated through CD8+ T cells and NK cells. Further depletion studies from 20 days after inoculation revealed that CD8+ T cells, but not NK cells, play an essential role in the late regression phase (Fig. 3A and C). It has been shown that IL-23 induces stronger sustained CTL than IL-12 in hepatitis C virus envelope protein immunization (28). In conjunction with the finding that vaccination with B16/IL-23 induced a significant CTL activity, we strongly believe that IL-23 production from tumor cells is effective for priming CD8+ CTL, thereby showing a unique phenomenon of delayed tumor regression. On the other hand, our observation that depletion of NK cells from the beginning of B16/IL-23 inoculation abrogated the tumor regression indicates that NK cells are other effectors indispensable for the antitumor effect during early phase when CTLs are possibly primed. Recent accumulative evidence has revealed that NK cells cooperate with dendritic cells and play a key role in the induction of CTL (29-33). NK cells and dendritic cells bidirectionally influence the process of CTL development (29, 33). Indeed, it was shown that NK cell depletion suppressed the induction of antitumor CTL in the experiments using CD40 knockout mice (30).

On the other hand, IL-27 exhibited antitumor effects on B16F10 cells from an early stage in a similar fashion to IL-12 (Fig. 2B, third rung and bottom). The most characteristic finding was that the antitumor efficacy of IL-27 on B16F10 cells was not dependent on

**Figure 5.** Tumor vaccine effects of B16/IL-23 and B16/IL-27. A, C57BL/6 mice (n = 5-8) were immunized in the left flank with 10⁶ of mitomycin C–treated B16/IL-23, B16/IL-27, or B16/control cells on days -14 and -7. B16F10 parental cells (1 × 10⁵) were challenged s.c. in the shaved right flank on day 0. Left, tumor sizes of parental B16F10 tumor in mice vaccinated with each transfectant. Points, mean; bars, SE (**, P < 0.05; ***, P < 0.01). Right, survival rate of the mice (**, P < 0.01).

B, cytotoxic assay. Splenocytes from mice immunized with B16/IL-23, B16/IL-27, or B16/control cells were collected, stimulated with mitomycin C–treated B16F10 parental cells or Lewis lung carcinoma cells and cocultured as target cells in appropriate effector cell/target cell ratios (E/T ratio) for 4 hours. Released LDH levels in the culture supernatants were measured by CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit and specific percent cytotoxicity was calculated. Points, mean; bars, SD. C, flow cytometry analysis for detection of CD4+CD25+ regulatory T cells. C57/BL6 mice were injected with 11 μg of anti-CD25 mAb (PC61) or rat IgG on day -3, and then spleen cells and lymph node cells (data not included) were analyzed on day 0.

D, survival rate of mice challenged with parental B16F10 cells, when combined with prior in vivo depletion of CD25+ T cells and vaccination with B16/IL-23, B16/IL-27, or B16/control cells. Mice (n = 10) were injected i.p. with anti-CD25 mAb (PC61) or rat IgG on day -15, followed by vaccination with the transfectants on day -14, and challenged with B16F10 parental tumor cells on day 0. In this setting, 80% of B16/IL-23-vaccinated mice completely rejected subsequent tumor challenge (**, P < 0.01).
IFN-γ (Fig. 3B), in contrast with those of IL-12 and IL-23 that depended on IFN-γ. The independence from IFN-γ and involvement of NK cells, but not of CD8+ T cells, in the antitumor effect of B16/IL-27 are different from the results of studies using other tumor cells. We recently reported that the antitumor effects of IL-27-producing C26 murine colon carcinoma cells were mediated through CD8+ T cells and IFN-γ (13). Just recently, Chiyo et al. (15) also showed that the expression of IL-27 in Colon 26 murine colon carcinoma cells produced antitumor effects which were partially mediated through T cells and NK cells. Furthermore, Salcedo et al. (14) showed that IL-27 overexpression in TBJ neuroblastoma cells markedly delayed tumor growth and led to complete tumor regression in >90% of mice and that CD8+ T cells, but not CD4+ T cells or NK cells, were critical for tumor suppression. They also showed that IL-27 overexpression induced the up-regulation of local IFN-γ gene expression and cell-surface MHC class I expression within TBJ tumors, which might contribute to effective tumor destruction by cytotoxic CD8+ T cells. Indeed, we recently confirmed that IL-27 directly acts on naïve CD8+ T cells and augments the generation of CD8+ CTL with enhanced granzyme B expression (34). All these results seem to be contradictory to the results of this study. The reason why the effector CD8+ T cells play a lesser role in antitumor effects of IL-27 on B16F10 melanoma is unknown, but one reason might be the difference of IFN-γ expression in tumor tissue. IFN-γ independence in the antitumor effect of B16/IL-27 suggested that IFN-γ expression might not be fully up-regulated in the tumor tissue. Therefore, it is expected that the class I molecule on B16F10 cells was hardly expressed, which might contribute to the susceptibility to cytotoxic reaction by NK cells rather than that of specific effector cells such as CD8+ T cells. On the other hand, the antitumor effects of IL-27 on C26 and TBJ tumors involved IFN-γ, suggesting that class I molecules of those cells might be fully up-regulated in tumor
tissues. Thus, cytotoxic CD8+ T cells could be the main effector cells against those tumors.

The difference between IL-23 and IL-27 in antitumor response against B16F10 cells was also observed in synergism with other cytokines. IL-23 significantly exhibited a synergistic antitumor effect with IL-18, but not with IL-12 (Fig. 4B). Wang et al. (35) recently showed that combinatorial gene-gun therapy using IL-23 and IL-18 cDNA elicited a synergistic antitumor effect on B16 melanoma tumors. Although the mechanism is not fully clarified, the combination of IL-23 with IL-18 should be noted as a novel combination therapy against tumors. In contrast, IL-27 exhibited significant synergism with IL-12, but not with IL-18 (Fig. 4C). IL-27 induces T-bet and subsequent IL-12Rβ2 expression and suppresses GATA-3 expression, and therefore synergistically enhances IFN-γ production with IL-12 in naïve CD4+ T cells (36, 37). Thus, IL-27 plays an important role in the early steps of Th1 commitment by regulating IL-12 responsiveness. The combined use of these cytokines should be one strategy to minimize adverse effects and maximize the therapeutic efficacy of cytokine-based immunotherapy against tumors.

Furthermore, a quite different result was also obtained in the vaccine effects between B16/IL-23 and B16/IL-27. As shown in Fig. 5, vaccination with B16/IL-23, but not with B16/IL-27, enabled mice to develop significant protective immunity. A cytotoxic assay also clearly showed that CTLs were significantly induced only with B16/IL-23 vaccination. The difference became remarkable when mice were pretreated with anti-CD25 mAb for depletion of CD4+CD25+ regulatory T cells, and as a consequence, 80% of B16/IL-23-vaccinated mice survived the tumor challenge. These results suggest that IL-23 has more potent efficacy than IL-27 in cytokine-based tumor vaccines and that anti-CD25 treatment can be a candidate as an efficient adjuvant in IL-23-based tumor vaccines.

Finally, we evaluated the adverse effects by systemic treatment with rIL-23 or rIL-27 because the clinical use of IL-12 is limited by its considerable toxicity (38–40). The toxicity associated with the systemic administration of IL-12 has been documented in preclinical studies using experimental animals (21). Therefore, we think that the evaluation of toxicity by IL-23 or IL-27 using experimental animals would be significant for predicting the future clinical usefulness of both cytokines. As shown in Fig. 6A to D, marked toxicity was observed in rIL-12-treated mice but not in rIL-23- or rIL-27-treated mice. In particular, systemic treatment with rIL-12, but not with rIL-23 and rIL-27, induced highly elevated levels of serum IFN-γ (Fig. 6D), which plays a major role in IL-12-associated toxicity, suggesting that the therapeutic use of IL-23 and IL-27 might be more tolerated than that of IL-12, although further examinations using other protocols are required.

In conclusion, we here showed that IL-23 and IL-27 exerted significant antitumor effects even on poorly immunogenic B16F10 melanoma cells. Although both cytokines belong to the IL-12 family, they contrastingly differ in their antitumor response and mechanism, the synergistic antitumor effects with other cytokines (IL-12 and IL-18), and the tumor vaccine effects. Although additional preclinical studies will be required, the results in this study support that IL-23 and IL-27 might play a role in future cytokine-based approaches for the treatment of poorly immunogenic tumors.

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References

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