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*J Immunol* 2007; 178:5086-5098; doi: 10.4049/jimmunol.178.8.5086

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IL-28 Elicits Antitumor Responses against Murine Fibrosarcoma

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IL-28 is a recently described antiviral cytokine. In this study, we investigated the biological effects of IL-28 on tumor growth to evaluate its antitumor activity. IL-28 or retroviral transduction of the IL-28 gene into MCA205 cells did not affect in vitro growth, whereas in vivo growth of MCA205IL-28 was markedly suppressed along with survival advantages when compared with that of controls. When the metastatic ability of IL-28-secreting MCA205 cells was compared with that of controls, the expression of IL-28 resulted in a potent inhibition of metastases formation in the lungs. IL-28-mediated suppression of tumor growth was mostly abolished in irradiated mice, indicating that irradiation-sensitive cells, presumably immune cells, are primarily involved in the IL-28-induced suppression of tumor growth. In vivo cell depletion experiments displayed that polymorphonuclear neutrophils, NK cells, and CD8 T cells, but not CD4 T cells, play an equal role in the IL-28-mediated inhibition of in vivo tumor growth. Consistent with these findings, inoculation of MCA205IL-28 into mice evoked enhanced IFN-γ production and cytotoxic T cell activity in spleen cells. Antitumor action of IL-28 is partially dependent on IFN-γ and is independent of IL-12, IL-17, and IL-23. IL-28 increased the total number of splenic NK cells in SCID mice and enhanced IL-12-induced IFN-γ production in vivo and expanded spleen cells in C57BL/6 mice. Moreover, IL-12 augmented IL-28-mediated antitumor activity in the presence or absence of IFN-γ. These findings indicate that IL-28 has bioactivities that induce innate and adaptive immune responses against tumors. The Journal of Immunology, 2007, 178: 5086–5098.

Interleukin-28 (IFN-α) is a newly identified class II cytokine receptor ligand that is distantly related to members of the IL-10 family and to type I IFNs (1–3). Like the type I IFN family, the expression of IL-28 is induced by virus infection or dsRNA (1–3). IL-28 signals through the heterodimeric receptor complex that is composed of IL-10Rβ and a novel IL-28R (1–3). The downstream IL-28 signaling is indistinguishable from type I IFN signaling through the IFN-α/βR. IL-28 also elicits STAT3 and STAT5 activation, which is more characteristically associated with signaling by IL-10 and IL-10-related cytokines. IL-28 signaling induces two cellular genes, myxovirus resistance A, and 2’-5’-oligoadenylate synthetase 1, that are known to play a role in the IFN-mediated protection from virus infection (1–3).

IFNs form an important group of cytokines that have a wide range of immunomodulatory activities including activation of NK cells and T cell cytotoxicity, promotion of Th1 responses, and up-regulation of MHC class I molecules (4). Especially, IFN-α and IFN-β have been reported to have potent antitumor activity and are applied to clinical treatments of several malignancies, including renal cell carcinoma, melanoma, and hairy cell leukemia. IL-28 exerts bioactivities that overlap those of type I IFNs such as antiviral activity and up-regulation of MHC class I expression (1–3). However, in sharp contrast to type I IFNs, the antitumor effect of IL-28 has not been fully elucidated.

There are multiple means to evaluate the biological actions of cytokines and chemokines. One way to define the biological activities of a cytokine is to express it under a strong constitutive or inducible promoter in cell lines or in mice and observe its local and systemic effects. Especially, the transfer of a cytokine gene into tumor cells is regarded as a valuable approach for investigating antitumor activities in experimental models. We constructed a retroviral vector-expressing mouse IL-28 and transduced tumor cells to evaluate the antitumor action of IL-28. IL-28 secreted by transduced tumor cells markedly inhibited in vivo tumor growth, depending on the polymorphonuclear neutrophils, NK cells, CD8 T cells, and IFN-γ. These findings provide the first evidence that IL-28 has a unique function in sequentially activating both innate and adaptive immune responses against tumors, leading to the suppression of in vivo tumor growth.

Materials and Methods

Mice and reagents

Female C57BL/6Ncl mice, female BALB/c mice, and male BALB/c SCID mice, 8 wk of age, were obtained from CLEA Japan. IFN-γ gene-deficient (IFN-γ knockout) (KO) mice with a C57BL/6 background were purchased from The Jackson Laboratory. IL-17 gene-deficient (IL-17 KO) mice with a C57BL/6 background were generated as reported previously.

Received for publication February 9, 2006. Accepted for publication December 8, 2006.

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Abreviations used in this paper: KO, knockout; CM, complete medium.

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(5). Animals were housed under specific pathogen-free conditions in accordance with the guidelines of the institutional Animal Care and Use Committee. Human IL-2 protein, mouse IFN-α, IL-12 and IL-28 proteins, and mouse IL-28 DuoSet Immunoassay were purchased from R&D Systems. Retroviral vector DFG-mIFN-α-ires-Neo was supplied by Dr. M. T. Lotze (University of Pittsburgh, Pittsburgh, PA).

Cells and cell cultures

MCA205 is a fibrosarcoma cell line. B16 is a melanoma cell line. Yac-1 is a lymphoma cell line. A primary culture of fibroblasts was established from the lung of a 7-wk-old female C57BL/6 mouse as described previously (6). These lines were maintained in RPMI 1640 (Sigma-Aldrich) with 10% FCS (MP Biomedicals), 0.1 mM nonessential amino acids (Sigma-Aldrich), 100 IU/ml penicillin, and 100 μg/ml streptomycin (both from Sigma-Aldrich), which is designated as complete medium (CM). Spleen cells were harvested from naive C57BL/6 mice and cultured in CM with or without 100 μg/ml poly(I:C) or 5 μg/ml Con A.

Flow cytometric analysis

MCA205 cells or primary lung fibroblasts were cultured with or without 10, 100, or 1000 ng/ml IL-28 for 24 h. MCA205 cells or lung fibroblasts were also cultured with or without 1000 IU/ml IFN-α for 24 h. Cells were harvested and washed with PBS containing 0.1% NaN₃. The surface expression of MHC class I (H-2Kb), IFN-γ, IFN-α/βR, and IFN-γR1 was examined by a FACScan (BD Biosciences) using PE-conjugated mouse IgG2a κ (clone G12A-2), PE-conjugated mouse IgG2a κ (clone G1178-157; both from BD Biosciences), PE-conjugated anti-mouse IFN-γR alpha (clone 2E2), and PE-conjugated anti-mouse IFN-γR beta mAb (clone MOB-47) (Santa Cruz Biotechnology). The surface expression of IL-28R on MCA205 cells was also examined using goat polyclonal anti-IL-28R Ab (Abcam).

Immunoblotting

Immunoblottings for STAT1 and STAT2 were performed as follows. After starvation for 17 h, cells were treated with 1000 IU/ml IFN-α or 50 ng/ml IL-28 for 0, 10, 30, or 60 min and then lysed with lysis buffer (20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 1% Triton X-100, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM PMSF, 0.1 mM leupeptin, and 10 μM ml aprotinin). Cell lysates were centrifuged at 14,000 rpm for 5 min at 4°C. Aliquots of supernatants were suspended in SDS sample buffer and boiled for 5 min. Proteins were separated by 4–20% acrylamide gel and equilibrated electrophoretically to nitrocellulose membranes. The membranes were incubated with 5% BSA in TBST containing 0.1% Tween 20 for 1 h and then incubated with anti-STAT1 Ab (Cell signaling Technology), anti-phospho-STAT1 Ab (Cell Signaling Technology), anti-STAT2 Ab (Cell Signaling Technology), or anti-phospho-STAT2 Ab (Medical Biological Laboratories) overnight. The total and phosphorylated STAT1 and STAT2 were visualized using a Phospho-HP Western Blot Detection System (Cell Signaling Technology).

MTT assay

Cells (1 × 10⁶/50 μl) were seeded into 96-well plates (BD Biosciences Labware). After 24 h, 50 μl of RPMI 1640 containing 5% FCS with or without IL-28 at the indicated final concentrations were added to each well (on day 0). On day 5, 10 μl of MTT (Dojindo) solution (5 mg/ml in RPMI 1640 with 10% FCS) was added to each well. Plates were incubated for 4 h. Next, the MTT solution was removed and 100 μl of DMSO (Sigma-Aldrich) was added to each well. The absorbance was read at a wavelength of 590 nm on an ELISA plate reader (Molecular Devices).

Isolation of mouse IL-28 cDNA, construction of recombinant retroviral vector carrying mouse IL-28 and retroviral infection

Mouse IL-28 cDNA was cloned by RT-PCR with mRNA extracted from 100 μg/ml poly(I)/poly(C)-stimulated spleen cells from BALB/c mice. Synthesized first-strand cDNAs were amplified with two primers: sense, 5'-CAAGAACAAGACTGAGGGGCCACATGCTCCTC-3' and antisense, 5'-GTACCCTGAGCAGACACAGTGCTCCAC-3'. Amplification was performed according to the manufacturer’s recommendation (PerkinElmer Cetus), and it consisted of 30 cycles under the following conditions: 30 s at 92°C for denaturation, 30 s at 64°C for primer annealing, and 60 s at 72°C for primer extension. The sequence of the product was confirmed to be identical to the published sequence. The retrovirus vector LXSXN (provided by D. W. Pear, MIT, and D. Baltimore, Rockefeller University, New York, NY) was used to generate retroviral supernatant. The supernatant of the retroviral supernatant was used to infect 1 × 10⁶ cells of parental MCA205 cells. MCA205 cells were infected with 5 ml of DFG-mIFN-α-ires-Neo retroviral supernatant in the presence of 8 μg/ml polybrene and selected in CM with 800 μg/ml G418. As a control, a retroviral vector carrying only the neo-mycin phosphotransferase gene (Neo) was used.

To establish IFN-α-producing MCA205 cells, the DFG-mIFN-α-ires-Neo proviral construct was transfected into the ecotropic packaging cell line BOSC 23 (provided by Drs. W. Pear and D. Baltimore, Rockefeller University, New York, NY) to generate retroviral supernatant. The supernatant of the retroviral supernatant was used to infect 1 × 10⁶ cells of DFG-mIFN-α-ires-Neo retroviral supernatant in the presence of 8 μg/ml polybrene and selected with 800 μg/ml G418.

ELISA for IL-28

ELISA was performed to measure the concentration of IL-28 protein. Microtiter plates with flat-bottoms were coated with 1 μg/ml rat anti-IL-28 mAb overnight at room temperature. After washing with 0.05% Tween 20 in PBS, the plates were incubated with 30 μg/ml polyclonal rat anti-IL-28 Ab (Abcam). After washing, 100 μl of recombinant mouse IL-28 protein or samples per well were added and incubated for 2 h. After washing, 250 ng/ml biotinylated rat anti-IL-28 Ab was added and incubated for 2 h. Then, the plate was incubated with 100 μl of streptavidin-HRP solution for 20 min. After washing, the reaction was revealed by the addition of the peroxidase substrate. After incubation for 20 min, 50 μl of 2 N H₂SO₄ was added. The OD was read at a wavelength of 450 nm.

In vitro cell growth

To examine the in vitro growth rate, parental MCA205 (MCA205WT), MCA205Neo, MCA205IL-28, or MCA205IFN-α cells were seeded at 8 × 10⁵ cells in 75-cm² culture flasks on day 0, and the cell number was counted on days 2, 3, 4, and 5.

In vivo evaluation of tumor growth

Wild-type mice were inoculated s.c. into the right flank with either 1 × 10⁵ or 5 × 10⁵ cells of MCA205WT, MCA205Neo, MCA205IL-28, or MCA205IFN-α cells. In some experiments, mice were subjected to 550 rad of whole-body irradiation and then inoculated s.c. with 1 × 10⁵ cells of MCA205Neo or MCA205IL-28. To evaluate the cooperative antitumor effects of IL-12 and IL-28, animals were inoculated s.c. with 1 × 10⁵ cells of MCA205Neo or MCA205IL-28 and received daily i.p. injections of PBS or 0.2 or 0.4 μg of IL-12 from days 7 to 14. Mice that had rejected the tumors were rechallenged with 2.5 × 10⁵ cells of parental MCA205 or irrelevant B16 in the other flank on day 60. Tumor size was determined by periodically measuring perpendicular tumor diameters with a Vernier caliper. Tumor volume (cubic millimeter) was calculated using the formula $a \times b^2/2$, where $a$ = largest diameter and $b$ = smallest diameter.

Lung metastases

Experimental lung metastases were obtained after i.v. injection of 250 μl of a suspension of tumor cells (3 × 10⁵) in PBS into C57BL/6 mice via the tail vein. Experimental metastases were evaluated 21 days after tumor cell inoculation. The tumor metastatic nodules were contrasted using black ink solution before counting under a dissecting microscope. The number of lung tumor metastases was counted in a blinded fashion.

IFN-γ production assay

The spleen was removed from each mouse, and spleen cells (2 × 10⁶/ml) were resuspended in vitro for 60 h by coculture with parental MCA205 cells (2 × 10⁷/ml) irradiated with 8000 rad. These culture supernatants were collected and assayed for IFN-γ production using a commercially available ELISA kit (R&D Systems). To examine the biological ability of IL-28 to induce IFN-γ production by NK cells in vitro, NK cells were obtained from spleen cells of SCID mice and cultured with or without a wide range of doses of IL-28 or 10 ng/ml IL-12 for 48 h. These culture supernatants were collected and assayed for IFN-γ production using a commercially available ELISA kit (R&D Systems). To examine the biological ability of IL-28 to induce IFN-γ production by CD8 T cells in vitro, CD8 T cells were isolated from spleen cells of C57BL/6 mice using a CD8 T
Cell Enrichment Column (R&D Systems) and cultured for 36 or 48 h with or without a wide range of doses of IL-28 or 10 ng/ml IL-12 in the presence or absence of 3 μg/ml anti-mouse CD3 mAb (eBioscience). These culture supernatants were collected and assayed for IFN-γ production using a commercially available ELISA kit (R&D Systems). To examine the biological ability of IL-28 to induce IFN-γ production in vivo, mice were administered i.p. either with 0.2 or 1.0 μg of IL-28 or 0.4 μg of IL-12 alone or with 0.2 or 1.0 μg of IL-28 in combination with 0.4 μg of IL-12 once a day for 3 consecutive days. Twenty-four hours after the final injection, blood samples were collected from four mice per group and stored at 4°C overnight. Serum was separated by centrifugation and stored at −80°C until use.

Cytotoxic T cell assay

Spleen cells were harvested and pooled from three mice per group 12 days after tumor cell inoculation. These cells (4 × 10^6) were restimulated in vitro with 4 × 10^5 irradiated (8000 rad) MCA205 cells in the presence of 10 IU/ml human IL-2. Five days later, restimulated cells were used as effectors for the standard 31Cr release assay against either MCA205 cells or B16 cells. In some experiments, restimulated cells were used as effectors for the standard 31Cr release assay against either MCA205Neo or MCA205IL-2 target cells. In brief, 10^6 of respective target cells were labeled with 100 μCi of Na_2^11003^11002CrO_4 for 1 h. After washing twice, effector and target cells were plated at an appropriate E/T ratio in 96-well round-bottom microtiter plates. Supernatants were collected after 4 h of incubation, and the radioactivity of each supernatant was counted in a gamma counter. The percentage of specific cytolytic activity was calculated as follows: percentage of specific release = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

In vivo cell depletion study

To deplete polymorphonuclear neutrophils, NK cells, CD4 T cells, or CD8 T cells in vivo, C57BL/6 mice received the i.p. injections of 400 μg of anti-mouse Gr-1 mAb (RB6-8C5; e Bioscience), 20 μl of anti-asialo GM1 antiserum (Wako Pure Chemical Industries), 500 μg of anti-mouse CD4 mAb (GK1.5; hybridoma, L3T4), 50 μg of anti-mouse CD8 α mAb (M5/105 hybridoma, Ly 2), normal rat IgG (Sigma-Aldrich), and/or normal rabbit serum (Biowest) 1 day before tumor inoculation and subsequently once every 3 days afterward for an additional 4 days (six times in total).

Immunohistochemical staining

Tumor tissues were harvested on days 9, 13, and 18, immediately embedded in OCT compound, and frozen in liquid nitrogen and stored at −80°C until use. For immunohistochemical stainings for CD4 T cells and CD8 T cells, cryostat sections were fixed in cold acetone. After washing twice, sections were incubated with rat anti-mouse CD4 mAb (clone H129.19), rat anti-mouse CD8α mAb (clone 53-6.7), or isotype-matched rat IgG (all from BD Biosciences) for 30 min at room temperature. After washing twice, endogenous peroxidase activity was blocked with 1% hydrogen peroxide. To block the biological activities of both IL-12 and IL-23, mice received s.c. injections of 0.4 g of IL-28 once a day for 3 consecutive days (three times in total). After 7 days of treatment with IL-28, tumors were harvested and assayed for IFN-γ activity of IL-28 to induce IFN-5088.

Lymphocyte chemotaxis assays

In vitro chemotaxis assays were performed as previously described (8). Cell migration was assessed by using a 48-well microchemotaxis chamber separated by a nitrocellulose membrane with a 5-μm pore size (Neuro Probe). NK cells, CD4 T cells, or CD8 T cells (5 × 10^5/ml) were stimulated with various concentrations of IL-28 or medium alone. The number of cells that migrated was counted according to the following formula: percentage of specific release = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Inhibition of IL-12 and IL-23 bioactivities

To block the biological activities of both IL-12 and IL-23, mice received i.p. injections of 700 μg of rat anti-mouse neutralizing IL-12/IL-23 p40 mAb (clone C17.8) 1 day before tumor cell inoculation and subsequently once every 3 days afterward for an additional 24 days (nine times in total).

RT-PCR

The primer sequences of the oligonucleotides used for PCR were as follows: IL-10R, sense: 5′-TCGATCTCAGGGTCTCAGAT-3′, antisense: 5′-TTGGGAGGTTGAAGGTACATT-3′; IL-28, sense: 5′-TGTGAAGCCTAAGGAAGATT-3′, antisense: 5′-GGCTTGTGCTACATT-3′; IL-28R, sense: 5′-CTTCTCAATAGCTGCACTGATT-3′, antisense: 5′-GCCACATTGGAGCTCC-3′; β-actin, sense: 5′-TGGTAGGTGGGAATGCGGTC-3′, antisense: 5′-TTGGATGTACGCCAGCATTC-3′. Total cellular RNA was extracted from the cells using RNAzolTM (Tel-Test) according to the manufacturer’s instructions. In addition, to confirm the expression of IL-28 mRNA in tumor tissues, total cellular RNA was extracted from either MCA205Neo tumors or MCA205IL-28 tumors on day 14. Four micrograms of total RNA was applied for the synthesis of cDNA with SuperScript RNAaseH-Reverse Transcriptase (Invitrogen Life Technologies). PCR was performed in a DNA Thermal Cycler (PerkinElmer Cetus) using Taq polymerase (Boehringer Mannheim).

Statistical analyses

Statistical analysis was performed using an unpaired two-tailed Student’s t test with a comparison by parametric and F tests. Differences were considered to be statistically significant when the p value was <0.05.

Results

IL-28 does not signal in MCA205 cells

We first examined the mRNA expression of the receptor subunits for IL-28 in MCA205 cells by RT-PCR and found that the transcripts for IL-10Rβ and IL-28R, which compose the functional heterodimeric receptor complex for IL-28, were expressed in MCA205 cells (Fig. 1A). We next examined the IL-28R expression in MCA205 cells by flow cytometry and found that IL-28R is expressed on the cell surface of MCA205 (Fig. 1B). Because IL-28R is expressed and the transcript for IL-10Rβ is detected, we further examined whether IL-28 can activate the intracellular signaling pathway in MCA205 cells. IL-28 has been reported to signal through, at least, STAT1, STAT2, and STAT3 (2, 9). We therefore determined whether treatment with IL-28 activates STAT1.
**FIGURE 1.** IL-28 has no direct biological effects on MCA205 cells. A, IL-10Rβ and IL-28R mRNA expression in MCA205 cells. The cDNAs generated from the total cellular mRNAs extracted from MCA205 cells were amplified with the primers specific for IL-10Rβ, IL-28R, or β-actin. Amplified PCR products were then loaded on a 1% agarose gel and stained with ethidium bromide for UV visualization. B, Flow cytometric analysis of IL-28R expression. After washing with PBS containing 0.1% NaN₃, MCA205 cells were incubated with goat IgG or goat anti-mouse IL-28R polyclonal Ab. After washing with PBS three times, cells were incubated with porcine anti-goat IgG mAb. The expression of IL-28R in MCA205 cells was analyzed by flow cytometry. Goat IgG (thin solid line) or anti-mouse IL-28R Ab (thick solid line). C, IL-28 does not activate STAT1 in MCA205 cells. Activation and expression of STAT1 was assessed by immunoblotting. STAT1 is activated in primary lung fibroblasts after stimulation with either 1000 IU/ml IFN-α or 50 ng/ml IL-28. STAT1 is also activated in MCA205 cells after stimulation with 1000 IU/ml IFN-α, but not with 50 ng/ml IL-28. Lanes 1 and 5, 0 min; lanes 2 and 6, 10 min; lanes 3 and 7, 30 min; lanes 4 and 8, 60 min. D, Flow cytometric analysis of MHC class I Ag expression. Lung fibroblasts or MCA205 cells were cultured with or without 100 ng/ml IL-28 for 24 h. After washing with PBS containing 0.1% NaN₃, cells were incubated with PE-conjugated mouse IgG or anti-H-2Kᵇ mAb for 15 min. The MHC class I molecule expression in either lung fibroblasts or MCA205 cells was analyzed by flow cytometry. Nontreated cells stained with PE-conjugated mouse IgG (dotted line) or PE-conjugated anti-H-2Kᵇ mAb (gray solid line). IL-28-treated cells stained with PE-conjugated mouse IgG (dotted line) or PE-conjugated anti-H-2Kᵇ mAb (black solid line). E, Flow cytometric analysis of MHC class I Ag expression. Lung fibroblasts or MCA205 cells were cultured with or without 1000 IU/ml IFN-α for 24 h. After washing with PBS containing 0.1% NaN₃, cells were incubated with PE-conjugated mouse IgG or anti-H-2Kᵇ mAb for 15 min. The MHC class I molecule expression in either MCA205 cells or lung fibroblasts was analyzed by flow cytometry. Nontreated cells stained with PE-conjugated mouse IgG (dotted line) or PE-conjugated anti-H-2Kᵇ mAb (thin solid line). IFN-α-treated cells stained with PE-conjugated mouse IgG (dotted line) or PE-conjugated anti-H-2Kᵇ mAb (black solid line). F, IL-28 has no direct effects on in vitro growth of MCA205 cells. MCA205 cells (1 × 10⁴) were seeded into 96-well plates and cultured in RPMI 1640 containing 5% FCS with or without 0.5–500 ng/ml IL-28. On day 5, cells were washed with RPMI 1640 medium, and 10 μl of MTT solution was added to each well. Each value represents mean ± SD (n = 10). The result is representative of two independent experiments.
FIGURE 2. Local secretion of IL-28 reduces tumorigenicity and metastatic ability of MCA205 cells. A, IL-28 mRNA expression in parental MCA205, MCA205Neo, or MCA205IL-28 cells assessed by RT-PCR. Total cellular RNA was isolated from the respective tumors. The cDNA generated from the extracted RNA was amplified with the primers for IL-28 mRNA or control actin mRNA. PCR products were resolved in 1% agarose gel and stained with ethidium bromide. Lane 1, MCA205WT; lane 2, MCA205Neo; and lane 3, MCA205IL-28. B, Transduction with the IL-28 gene has no direct effects on in vitro growth of MCA205 cells. Each value represents mean ± SD (n = 4). The result is representative of two independent experiments. C, IL-28 mRNA expression in MCA205IL-28 tumors on day 14 assessed by RT-PCR. Fourteen days after inoculation with MCA205Neo or MCA205IL-28 cells, total cellular RNA was isolated from the respective tumors. The cDNA generated from the extracted RNA was amplified with the primers for IL-28 mRNA or control actin mRNA. PCR products were resolved in 1% agarose gel and stained with ethidium bromide. Lane 1, MCA205Neo; lane 2, 1 × 10^5 MCA205IL-28; and lane 3, 5 × 10^5 MCA205IL-28. D, The time course of in vivo growth for 1 × 10^5 cells of MCA205WT, MCA205Neo, MCA205IL-28, or MCA205IFN-α in C57BL/6 wild-type mice. Each value represents the mean tumor volume ± SD for 10 mice/group. The result is representative of two independent experiments. E, The survival of mice inoculated with 1 × 10^5 cells of MCA205WT, MCA205Neo, MCA205IL-28 or MCA205IFN-α. F, The time course of in vivo growth for 5 × 10^5 cells of MCA205WT, MCA205Neo, MCA205IL-28, or MCA205IFN-α in C57BL/6 wild-type mice. Each value represents the mean tumor volume ± SD for 10 mice/group. The result is representative of two independent experiments. G, The survival of mice inoculated with 5 × 10^5 cells of MCA205WT, MCA205Neo, MCA205IL-28, or MCA205IFN-α. H, Lung metastases after i.v. injection of tumor cells. Mice were injected with 3 × 10^5 cells of MCA205WT, MCA205Neo, or MCA205IL-28 via tail vein. On day 21, the tumor metastatic nodules in the lungs were counted. Data are presented as mean ± SD for 10 mice/group. The result is representative of two independent experiments. (MCA205IL-28 vs MCA205WT or MCA205Neo; *, p < 0.0001). I, The time course of the in vivo growth for 1 × 10^5 cells of MCA205Neo or MCA205IL-28 in irradiated C57BL/6 wild-type mice. Each value represents the mean tumor volume ± SD for five mice per group. The result is representative of two independent experiments.
and STAT2. Although 1000 IU/ml IFN-α activated STAT1 and STAT2 in both primary lung fibroblasts and MCA205 cells, 50 ng/ml IL-28 slightly phosphorylated STAT1 and STAT2 in lung fibroblasts, but not in MCA205 cells (Fig. 1C and data not shown). To confirm that IL-28 does not signal in MCA205 cells, we evaluated whether IL-28 enhances MHC class I Ag expression in MCA205 cells because IL-28 has been reported to up-regulate MHC class I Ag expression (2). One thousand International Units per milliliter IFN-α enhanced MHC class I molecule expression in both lung fibroblasts and MCA205 cells, and 100 ng/ml IL-28 also slightly up-regulated the expression in lung fibroblasts, whereas the expression levels of MHC class I molecules in MCA205 cells treated with 10, 100, or 1000 ng/ml IL-28 for 24 h were not influenced when compared with those of nontreated cells (Fig. 1D and data not shown). In addition, we found that neither IFN-α nor IL-28 enhance expression of IFN-γRα and IFN-γRβ in lung

FIGURE 3. Involvement of polymorphonuclear neutrophils, NK cells, and CD8 T cells in the induction of antitumor activity mediated by IL-28. C57BL/6 wild-type mice were inoculated with 1 × 10^5 cells of either MCA205Neo or MCA205IL-28 and injected i.p. with anti-Gr-1 mAb (A), anti-asialo GM1 antiserum (B), anti-CD4 mAb (C), anti-CD8a mAb (D), or anti-asialo GM1 antiserum plus anti-CD8a mAb (E) as described in Materials and Methods. Each value represents the mean tumor volume ± SD for six mice per group. The result is representative of two independent experiments.
fibroblasts and MCA205 cells (data not shown). We also found that a wide range of doses of IL-28 had no direct effects on in vitro growth of MCA205 cells (Fig. 1E). Taken collectively, we concluded that IL-28 is not capable of signaling effectively in MCA205 cells.

IL-28 significantly inhibits the in vivo tumor growth and lung metastasis and prolongs survival of tumor-bearing mice

We then generated a retroviral vector PA317IL-28 as described in Materials and Methods and used it to transduce MCA205 cells. The G418-resistant MCA205 bulk culture was established and designated as MCA205IL-28. The expression of IL-28 mRNA could be weakly detected in MCA205 cells before transduction. After G418 selection, the expression of IL-28 mRNA in MCA205IL-28 cells markedly increased (Fig. 2A). The amount of IL-28 released by MCA205IL-28 cells was determined with an ELISA for mouse IL-28. MCA205IL-28 produced 27 ng of IL-28/1 x 10^6 cells per 48 h. In addition, the G418-resistant MCA205 cells generated by infection with retroviral supernatant produced by DFG-mIFN-α-IRES-Neo were designated as MCA205IFN-α. MCA205IFN-α produced 90 ng of IFN-α/1 x 10^6 cells per 48 h. On the contrary, MCA205WT or MCA205Neo cells did not secrete detectable amounts of IL-28 or IFN-α protein. Significant changes were not observed in in vitro growth of IL-28 transfectant when compared with that of parental cells or Neo transfectant (Fig. 2B). IFN-α transduction was previously reported to have no direct effects on in vitro growth of MCA205 cells (10), and we confirmed this finding (data not shown).

To evaluate the antitumor response mediated by local secretion of IL-28, groups of 10 mice were inoculated s.c. with either 1 x 10^5 or 5 x 10^5 cells of MCA205IL-28 and were monitored for tumor development and survival. We confirmed in vivo expression of IL-28 in MCA205IL-28 tumors on day 14 by RT-PCR (Fig. 2C). Mice receiving MCA205IFN-α cells served as positive controls, and those inoculated with MCA205Neo or parental MCA205WT cells were negative controls. When MCA205WT, MCA205Neo, or MCA205IL-28 cells were implanted into syngeneic mice, they all formed solid tumors. The tumors in the MCA205Neo, or MCA205IL-28 groups grew progressively, whereas MCA205WT cells at both doses developed tumors with a markedly reduced growth rate (Fig. 2, A and B). Animals with MCA205IL-28 tumors survived significantly longer than those with MCA205WT or MCA205Neo tumors (Fig. 2, C and D). However, transduction of IL-28 into MCA205 cells resulted in no long-term tumor-free animals. In comparison, implantation of MCA205IFN-α cells developed palpable tumors, and 9 of 10 these tumors were subsequently rejected (Fig. 2, D–G). All mice inoculated with MCA205IFN-α cells survived for the whole observation period of 120 days (data not shown).

To evaluate the antitumoral effect of IL-28, groups of mice (n = 10) were administrated MCA205WT, MCA205Neo, or MCA205IL-28 cells by tail vein injection, and the number of nodules of pulmonary metastases in the different groups was counted on day 21. All animals receiving MCA205WT or MCA205Neo cells displayed a large number of metastatic foci in their lungs. Transduction of IL-28 significantly reduced the number of lung foci. These findings demonstrate that IL-28 has effective antimetastatic activity (Fig. 2H).

We next examined whether immunological mechanisms were involved in the decreased in vivo growth of the IL-28 transfectant. To address this issue, groups of mice (n = 5) received sublethal total-body irradiation and were challenged with MCA205Neo or MCA205IL-28 cells. In irradiated mice, the growth rate of MCA205IL-28 tumors was mostly identical to that of MCA205Neo tumors (Fig. 2J). Therefore, irradiation-sensitive cells, presumably immune cells, primarily mediate the decreased in vivo tumor growth induced by locally produced IL-28.

Cell populations involved in the growth suppression of IL-28-transduced cells

To assess the phenotype of effector cells in the IL-28-mediated antitumoral activity, mice were depleted of polymorphonuclear neutrophils, NK cells, CD4 T cells, and/or CD8 T cells by injection of anti-Gr-1 mAb, anti-asialo GM1 antiserum, anti-CD4 mAb, and/or anti-CD8a mAb. Fig. 3 shows the results of a representative experiment, in which the effects of various immunosuppressive treatments on the growth of MCA205Neo and MCA205IL-28 tumors...
Intratumoral infiltration of CD8 T cells by IL-28

We further examined the phenotype of antitumor effector cells by immunohistochemical analysis of tumor tissues at various time points. Moderate infiltration of CD8 T cells in MCA205IL-28 tumor tissues was observed on day 9 (Fig. 4D). On day 13, intratumoral infiltration of CD8 T cells in MCA205IL-28 tumor tissues was more pronounced (Fig. 4H). In addition, moderate numbers of infiltrating CD4 T cells were also present in MCA205IL-28 tumor tissues on day 13 (Fig. 4G). On day 18, the number of infiltrating CD4 T cells and CD8 T cells in MCA205IL-28 tumors slightly decreased (data not shown). On the contrary, the minimum numbers of infiltrating CD4 T cells and CD8 T cells were found in MCA205Neo tumors on days 9, 13, and 18 (Fig. 4, A, B, E and F and data not shown).

Enhanced IFN-γ production and tumor-specific CTL responses by IL-28

To further examine the mechanism underlying the antitumor activity of IL-28, we then analyzed IFN-γ production of spleen cells, which were obtained from mice with either MCA205Neo or MCA205IL-28 tumors and restimulated in vitro for 60 h. Markedly enhanced IFN-γ production was observed in the culture supernatants of spleen cells from mice inoculated with MCA205IL-28 cells as compared with those inoculated with MCA205Neo cells (Fig. 5A). To further assess the ability of IL-28 produced by MCA205IL-28 cells to induce tumor-specific CTLs, CTL activity of spleen cells was evaluated. A significantly higher cytolytic activity of IL-28, which was reproducibly observed (Fig. 5B). These in vitro results reflected the enhanced tumor-specific cytolytic activity of primed CD8 T cells present in the spleen of wild-type mice with
of the IL-12-induced in vivo IFN-γ/H9253 injected i.p. either with PBS, 0.4 μg of IL-12, or 0.2 or 1.0 μg of IL-28 increases the total number of splenic NK cells in SCID mice and enhances IL-12-induced IFN-γ production by NK cells and CD8 T cells, whereas unstimulated spleen cells are unresponsive. We next tested the biological effects of IL-28 on the in vitro growth of NK cells and CD8 T cells and found that while 200 IU/ml IL-2 displayed no biological abilities to stimulate the in vitro growth of NK cells and CD8 T cells (data not shown). We also tested whether IL-28 directly stimulates the chemotaxis of NK cells, CD4 T cells, and CD8 T cells and found that a wide range of doses of IL-28 did not significantly induce the chemotaxis of NK cells, CD4 T cells, and CD8 T cells (data not shown). We then evaluated the effect of IL-28 on IFN-γ production by NK cells and CD8 T cells in vitro. Ten nanograms per milliliter IL-28 markedly enhanced IFN-γ secretion by NK cells and CD8 T cells, whereas a wide range of doses of IL-28 showed no direct effects on the release of IFN-γ (data not shown). We further evaluated the biological effects of IL-28 on CD8 T cells stimulated with anti-mouse CD3 mAb and found that IL-28 has no biological abilities to enhance the in vitro growth and IFN-γ production by CD8 T cells stimulated with anti-mouse CD3 mAb (data not shown). Because NK cells were involved in the IL-28-mediated antitumor activity, we further examined the effect of IL-28 on NK cell cytotoxic activity. SCID mice were injected i.p. with PBS, 0.4 μg of IL-12, or 0.2 or 1.0 μg of IL-28 once a day for 3 consecutive days. The cytolytic activity of spleen cells, harvested from three mice per group and tested for cytotoxic activity against Yac-1 cells. All results are shown as mean ± SD (n = 4 per data point). The result is representative of two independent experiments. 

**Effects of IL-28 on NK cells and CD8 T cells**

Although antitumor action of IL-28 is, at least in part, mediated by NK cells and CD8 T cells, the biological effects of IL-28 on immune cell populations such as NK cells and CD8 T cells still remain largely unknown. We first examined the expression of mRNA specific for IL-28R in unstimulated and poly(I:C)- or Con A-stimulated spleen cells and found that the IL-28R mRNA expression is clearly detected in poly(I:C)- or Con A-stimulated spleen cells, but not in unstimulated naive spleen cells by RT-PCR (data not shown). These results indicated that activated spleen cells may be responsive to IL-28, whereas unstimulated spleen cells are unresponsive. We next tested the biological effects of IL-28 on the total number of spleen cells, harvested from four mice per group, to determine whether IL-28 directly stimulates the chemotaxis of NK cells, CD4 T cells, and CD8 T cells (data not shown). We then evaluated the effect of IL-28 on IFN-γ production by NK cells and CD8 T cells in vitro. Ten nanograms per milliliter IL-28 markedly enhanced IFN-γ secretion by NK cells and CD8 T cells, whereas a wide range of doses of IL-28 showed no direct effects on the release of IFN-γ (data not shown). We further evaluated the biological effects of IL-28 on CD8 T cells stimulated with anti-mouse CD3 mAb and found that IL-28 has no biological abilities to enhance the in vitro growth and IFN-γ production by CD8 T cells stimulated with anti-mouse CD3 mAb (data not shown). Because NK cells were involved in the IL-28-mediated antitumor activity, we further examined the effect of IL-28 on NK cell cytotoxic activity. SCID mice were injected i.p. with PBS, 0.4 μg of IL-12, or 0.2 or 1.0 μg of IL-28 once a day for 3 consecutive days. The cytolytic activity of splenic NK cells from SCID mice treated with PBS, IL-12, or IL-28 was tested by a standard 51Cr release assay. We found that IL-28 did not enhance NK cell cytotoxicity against Yac-1 cells (Fig. 6A). In contrast, IL-12, a positive control, significantly augmented the cytolytic activity of splenic NK cells, as was consistent with a previous report (11) (Fig. 6A). We also examined the effect of IL-28 on the total number of splenic NK cells and found that administration of 0.2 μg of IL-28 significantly increased the number of splenic NK cells. IL-12 also markedly expanded the splenic NK cells when compared with PBS (Fig. 6B). On the contrary, daily injections of 1.0 μg of IL-28 into mice for 3 consecutive days did not affect the total collected from four mice per group. The concentration of IFN-γ in each sample was measured using a commercially available ELISA kit. All results are shown as mean ± SD (n = 4 per data point). The result is representative of two independent experiments. 

**FIGURE 6.** IL-28 increases the total number of splenic NK cells in SCID mice and enhances IL-12-induced IFN-γ production and expansion of spleen cells in C57BL/6 mice. A, NK cell cytotoxicity against Yac-1 cells. SCID mice were injected i.p. with PBS, 0.4 μg of IL-12, or 0.2 or 1.0 μg of IL-28 once a day for 3 consecutive days. Twelve hours after the final injection, spleen cells were harvested from three mice per group and tested for cytotoxic activity against Yac-1 cells. All results are shown as mean ± SD (n = 4 per data point). The result is representative of two independent experiments. B, Total number of splenic NK cells in SCID mice. SCID mice were injected i.p. with PBS, 0.4 μg of IL-12, or 0.2 or 1.0 μg of IL-28 once a day for 3 consecutive days. Twenty-four hours after the final injection, the total number of spleen cells, harvested from four mice per group, was counted. All results are shown as mean ± SD (n = 4 per data point). The result is representative of two independent experiments. PBS vs 0.2 μg of IL-28, *, p < 0.007; PBS vs 1.0 μg of IL-28, **, p > 0.05; PBS vs IL-12, ***, p < 0.0001. C, Augmentation of the IL-12-induced in vivo IFN-γ production by IL-28. Mice were injected i.p. either with PBS, 0.4 μg of IL-12, or 0.2 or 1.0 μg of IL-28 or with 0.2 or 1.0 μg of IL-28 + 0.4 μg of IL-12 once a day for 3 consecutive days. Twenty-four hours after the final injection, blood was collected from four mice per group. The concentration of IFN-γ in each sample was measured using a commercially available ELISA kit. All results are shown as mean ± SD (n = 4 per data point). The result is representative of two independent experiments. PBS vs IL-12, *, p < 0.006; IL-12 vs IL-12 + 0.2 or 1.0 μg of IL-28, **, p < 0.02.
number of splenic NK cells (Fig. 6B). To determine whether IL-28 induces IFN-γ production in vivo, we analyzed the serum levels of IFN-γ in mice treated with IL-28. Although daily injections of 0.2 or 1.0 μg of IL-28 for 3 consecutive days did not induce measurable serum IFN-γ levels, treatment with 0.2 μg of IL-28 combined with 0.4 μg of IL-12 raised serum IFN-γ levels ~1.5 times higher than those with 0.4 μg of IL-12 alone (Fig. 6C). Serum IFN-γ levels in the animals receiving 1.0 μg of IL-28 combined with 0.4 μg of IL-12 were 1.3 times higher than those of IL-12 alone (Fig. 6C). We then examined the effects of IL-28 on the total number of spleen cells and found that daily injections of 0.2 or 1.0 μg of IL-28 into mice for 3 consecutive days did not significantly affect the total number of spleen cells, whereas IL-12 markedly expanded the spleen cells when compared with PBS (Fig. 6D). Moreover, treatment with 0.2 or 1.0 μg of IL-28 in combination with 0.4 μg of IL-12 for 3 consecutive days significantly increased the total number of spleen cells when compared with IL-12 alone (Fig. 6D).

We further examined the cytotoxic activity of MCA205 cellspecific CTLs against either MCA205Neo or MCA205IL-28 target cells. CTL activity was measured against 51Cr-labeled MCA205Neo or MCA205IL-28 target cells in a standard 51Cr-labeling assay. We found that the cytotoxic activity of MCA205 cell-specific CTLs against MCA205IL-28 target cells is almost equal to that against MCA205Neo target cells (data not shown), suggesting that IL-28 has no direct biological actions to enhance the cytotoxic activity of CTLs.

**IFN-γ is involved in the IL-28-mediated antitumor effect**

The IL-12/IFN-γ Th1 pathway has been considered to be critically involved in activating immune cells against tumors, both in murine models and in humans (12, 13). Recently, a novel IL-23/IL-17 immune pathway, which is distinct from the Th1 and Th2 pathways, has been found (14, 15). Both IL-17 and IL-23 have been also reported to elicit effective antitumor immune responses (16, 17). To definitively determine whether these cytokines actively participated in the IL-28-mediated antitumor action, we performed studies using a neutralizing mAb for IL-12/IL-23 p40, IFN-γ gene KO mice, and IL-17 gene KO mice. MCA205Neo tumors in IFN-γ KO mice displayed very rapid growth kinetics when compared to those in wild-type mice. Consistent with the increased production of IFN-γ by IL-28 as shown in Fig. 3A, MCA205IL-28 tumors in IFN-γ KO mice also grew more rapidly when compared with those in wild-type mice (Fig. 7A). These results point to a relevant role of IFN-γ as a mediator of the antitumor immune responses triggered by IL-28, although the effect of IL-28 was not completely abrogated in the absence of IFN-γ (Fig. 7A). In addition, MCA205Neo tumors in wild-type mice treated with a neutralizing anti-IL-12/IL-23 p40 mAb, which blocks the biological activities of both IL-12 and IL-23, grew more rapidly when compared to those in wild-type mice treated with an isotype-matched control mAb (Fig. 7B) (anti-p40 mAb vs control IgG; p = 0.02, on day 32). On the contrary, the difference of MCA205IL-28 tumor growth between groups treated with a neutralizing anti-IL-12/IL-23 p40 mAb and treated with an isotype-matched control mAb was not statistically significant (Fig. 7B) (anti-p40 mAb vs control IgG; p > 0.1, on day 35). Furthermore, both MCA205Neo and MCA205IL-28 tumors showed almost the same growth kinetics in wild-type mice and IL-17 KO mice (data not shown). These findings demonstrate that IL-12, IL-17, and IL-23 are not involved in the antitumor activity of IL-28.

**IL-12 enhances the antitumor action of IL-28 in the presence or absence of IFN-γ**

IL-12 was not involved in the IL-28-mediated antitumor responses. Based on this evidence, we evaluated whether concomitant systemic administration of IL-12 (0.2 μg/day per mouse) would augment the efficacy of the IL-28-elicited antitumor effect. Animals were inoculated with either MCA205Neo or MCA205IL-28 cells, and daily i.p. injections of either PBS or IL-12 was begun on day 7 for 8 consecutive days. Systemic administration of IL-12 moderately suppressed the MCA205Neo tumor growth in vivo, whereas IL-12 treatment with MCA205IL-28 tumors was associated with a more potent antitumor effect, and 4 of 10 mice completely rejected the MCA205IL-28 tumors (Fig. 8, A and B). These mice were rechallenged with parental MCA205 and B16 cells on day 60. All mice were protected from MCA205, but not from B16. To assess the phenotype of effector cells in the IL-12-mediated enhanced antitumor action, mice were depleted of NK cells, CD4 T cells, or CD8 T cells. Fig. 8C displays the result of a representative experiment, in which the effects of various immunosuppressive treatments on the IL-12-mediated enhanced antitumor activity of IL-28 were compared. Depletion of NK cells or CD8 T cells, but not of CD4 T cells, completely attenuated the enhanced antitumor action of IL-28 (Fig. 8C). These findings indicate that both NK cells and CD8 T cells are involved in the IL-12-induced enhanced antitumor immune responses of IL-28. We further evaluated whether concomitant systemic administration of IL-12 (0.4 μg/day per mouse) would augment the efficacy of the IL-28-elicited antitumor effect in IFN-γ KO mice. Mice were inoculated with MCA205Neo or MCA205IL-28 cells, and daily i.p. injections of
PBS or IL-12 was begun on day 7 for 8 consecutive days. IL-12 did not suppress the growth of MCA205Neo tumors, whereas IL-12 significantly inhibited the growth of MCA205IL-28 tumors (Fig. 8D). On day 31, the mean tumor volume of MCA205IL-28 tumors in the IL-12-treated group was $3002 \pm 11006$ mm$^3$ compared with $5550 \pm 1048$ mm$^3$ in the PBS-treated group. In addition, on day 28, the mean tumor volume of MCA205Neo tumors in the IL-12-treated group was $6647 \pm 1559$ mm$^3$ compared with $7699 \pm 2129$ mm$^3$ in the PBS-treated group. Each value represents the mean tumor volume ± SD for five mice per group. The result is representative of two independent experiments.

Discussion

The cytokine gene therapy approach as a form of molecular pharmacology applied to tumor models has contributed greatly to identifying immune responses mediated by cytokines that were previously either unknown or not fully evaluated (18). In the current study, to investigate the in vivo biology of IL-28, we used the cytokine gene-transfer technology using a retroviral vector harboring mouse IL-28 gene in a MCA205 fibrosarcoma tumor model. We found that IL-28 produced by tumor cells augmented the antitumor cellular immunity that led to the marked suppression of in vivo tumor growth and experimental lung metastases and consequently a prolonged survival of mice. The antitumor action of IL-28 was mediated mainly through NK cells, CD8 T cells, and IFN-γ, and polymorphonuclear neutrophils were also involved in the elicitation of antitumor activity. In contrast, the IL-28-induced suppression of tumor growth in vivo was independent of IL-12, IL-17, and IL-23, all of which were reported to enhance antitumor immune responses (16, 17, 19). Moreover, cooperative antitumor effects of the combination with IL-12 and IL-28 were also noted.

MCA205 cells were found to express IL-28R on the cell surface and mRNA for IL-10Rβ. However, the activation of STAT1 and STAT2 was slightly detected in primary lung fibroblasts, but not in MCA205 cells, in response to treatment with IL-28. Furthermore, we found that a wide range of doses of IL-28 has no direct effects on the growth of MCA205 cells in vitro, although IL-28 has been reported to have cytostatic activity (20, 21). In addition, IL-28 slightly up-regulated MHC class I Ag expression in lung fibroblasts, as was consistent with a previous report (2), whereas IL-28 did not enhance the MHC class I molecule expression in MCA205 cells. In contrast, IFN-α apparently activated STAT1 and STAT2 and up-regulated MHC class I molecule expression in both lung fibroblasts and MCA205 cells, indicating that the Jak-STAT signaling pathway and the MHC class I molecule were functional in MCA205 cells. Therefore, these findings indicate that MCA205

FIGURE 8. IL-12 enhances IL-28-elicited antitumor activity in the presence or absence of IFN-γ. Mice were inoculated with $1 \times 10^6$ cells of either MCA205Neo or MCA205IL-28 and received i.p. injections of PBS or 0.2 μg of IL-12 from days 7 to 14 for 8 consecutive days. A, The time course of in vivo growth for MCA205Neo or MCA205IL-28 tumors in mice treated with either PBS or IL-12. Each value represents the mean tumor volume ± SD for five mice per group. B, Percentage of tumor-bearing mice in each group. The result is representative of two independent experiments. C, Mice were inoculated with $1 \times 10^6$ cells of either MCA205Neo or MCA205IL-28 and received i.p. injections of PBS or 0.2 μg of IL-12 from days 7 to 14 for 8 consecutive days. Animals were also injected i.p. with anti-asialo GM1 antiserum, anti-CD4 mAb, or anti-CD8 mAb as described in Materials and Methods. The result is representative of two independent experiments. D, IFN-γ KO mice were inoculated with $1 \times 10^6$ cells of either MCA205Neo or MCA205IL-28 and received i.p. injections of PBS or 0.4 μg of IL-12 from days 7 to 14 for 8 consecutive days. Each value represents the mean tumor volume ± SD for five mice per group. The result is representative of two independent experiments.
cells are unresponsive to IL-28, although MCA205 cells express IL-28R on the cell surface and mRNA for IL-10RB. Nevertheless, immunocompetent animals inoculated with MCA205IL-28 cells had much smaller tumor nodules and survived significantly longer when compared with mice injected with either MCA205WT or MCA205Neo cells. In addition, the suppression of in vivo growth of MCA205IL-28 tumors was completely abrogated in irradiated mice. Taken collectively, these findings demonstrate that irradiation-sensitive host-defense mechanisms, presumably immune system, play a critical role in mediating antitumor activities of IL-28 in vivo.

It seems that the potency of the antitumor activity of IL-28 was slightly lower than that of IFN-α, although MCA205IL-28 cells secreted ~3-fold less cytokine than did MCA205IFN-α cells. Actually, almost all mice inoculated with MCA205IFN-α cells developed palpable tumors that were quickly rejected and survived significantly longer, whereas MCA205IL-28 tumors were not rejected at all. IFN-α showed direct biological actions including enhancement of the MHC class I Ag expression in MCA205 cells, whereas IL-28 failed, suggesting that IFN-α, but not IL-28, has direct effects on immunogenicity of MCA205 cells. Therefore, IFN-α might be able to mediate the antitumor activity more effectively than IL-28. These results suggest that the underlying mechanisms of antitumor activities of IFN-α and IL-28 are not quite identical.

In regard to the cellular antitumor mechanisms of IL-28, our results in mice selectively depleted of various immune cell populations strongly suggest that CD8 T cells play an important role in the IL-28-mediated antitumor action because the protective effect was partially abolished in CD8 T cell-depleted animals. Moreover, local secretion of IL-28 induced more powerful tumor-specific CTLs against MCA205 cells. Immunohistochemical analyses confirmed the infiltration of CD8 T cells into MCA205IL-28 tumor tissues on day 9, and the number of infiltrating CD8 T cells was further increased on day 13. We also observed the moderate infiltration of CD4 T cells on day 13, but these cells appeared to be not necessary for the antitumor action of IL-28. In fact, we consistently observed a slower tumor growth rate of MCA205IL-28 in CD4 T cell-depleted mice than in control mice, indicating that CD4 T cells might rather inhibit IL-28-induced antitumor activity. Both CD4 T cells and CD8 T cells have been described to be important for the efficient induction of antitumor cellular immunity (22, 23). Our finding that CD4 T cells are not required for the antitumor activity of IL-28 is not in agreement with the notion that CD4 T cell help is necessary for the full activation of naive CD8 T cells (24). However, a similar inhibitory effect of CD4 T cells has been reported in the IL-12- or IL-23-transduced CT26 tumor model (17, 25). This is accomplished by either skewing the cytokine milieu to the Th1 phenotype or removing CD4+CD25+ T regulatory cells (26). Because of technical difficulties, we were unable to evaluate the infiltration of NK cells into tumor tissues. However, our finding that NK cell depletion partially abrogated IL-28-elicited antitumor response strongly suggests that NK cells also play an important role in the antitumor activity of IL-28 in this MCA205 tumor model. Furthermore, we observed that depletion of both NK cells and CD8 T cells at once almost completely impaired the antitumor effect of IL-28.

Our finding that treatment of animals with anti-Gr-1 mAb resulted in partial impairment of IL-28-mediated antitumor effect might indicate that polymorphonuclear neutrophils contribute, in some way, to the generation of a CD8 T cell-mediated antitumor response. Notably, the evidence that polymorphonuclear neutrophils may be important for the induction of an antitumor immunity has already been suggested (27), and a specific role for polymorphonuclear neutrophils in the development of CD8 T cell-mediated antitumor responses was also demonstrated (28).

Our finding indicated that NK cells are partially involved in the IL-28-mediated antitumor activity. IL-28 was unable to enhance NK cell cytolytic activity, whereas a relatively lower dose of IL-28 significantly expanded the splenic NK cells. Thus, IL-28 appeared to augment the NK cell-mediated antitumor activity in vivo via increasing the total number of NK cells. Moreover, although IL-28 alone did not induce IFN-γ production, IL-28 significantly enhanced the IL-12-induced IFN-γ production in vivo and the IL-12-induced expansion of spleen cells. Therefore, another possible explanation of underlying mechanisms is that IL-28, like IL-21 (29), could enhance the cytolytic activity of NK cells previously activated by stimulators such as other cytokines and chemokines in vivo, but could not induce cytoxic activity in resting NK cells. Further analyses are needed to elucidate the detailed mechanisms by which NK cells contribute to the antitumor activity of IL-28.

IL-28 inhibited the in vivo growth of MCA205 cells via enhancement of antitumor immune responses mediated by polymorphonuclear neutrophils, NK cells, and CD8 T cells. However, we found that the mRNA specific for IL-28R is expressed in poly(I: C)- or Con A-stimulated spleen cells, but not in unstimulated naive spleen cells. Therefore, it seemed that IL-28 has direct biological effects on only activated immune cell populations. In addition, IL-28 has the biological function to induce chemokine secretion by fibroblasts (our unpublished data). Taken together, IL-28 is likely to primarily elicit antitumor immune responses through indirect mechanisms such as induction of other cytokines and chemokines, which subsequently stimulate the immune cells.

We found that IFN-γ plays a partial role in the IL-28-mediated antitumor responses because MCA205IL-28 cells were significantly more tumorigenic in IFN-γ KO mice than in immunocompetent mice. IFN-γ is a pleiotropic cytokine that can act on both tumor cells and host immunity (30, 31). IFN-γ directly inhibits proliferation of some tumor cells and indirectly suppresses tumor growth in vivo by activating NK cells and macrophages and inducing angiostatic chemokines such as monokine induced by IFN-γ and IFN-γ-inducible protein 10 with consequent inhibition of tumor angiogenesis (32–34). Nevertheless, depletion of IFN-γ could not completely abolish the antitumor action of IL-28. This finding indicates that IFN-γ-independent pathways also play a role in the IL-28-mediated antitumor activity. In addition, IL-12 and other cytokines (IL-17 and IL-23) are not required for the IL-28-induced antitumor activity.

IL-12 enhances the cytolytic activities and antitumor functions of both NK cells and CD8 T cells (9, 35, 36). NK cells and CTLs are also important effector cells mediating the antitumor activity of IL-28, as shown in our experiments. Furthermore, IL-28 and IL-12 in combination significantly enhanced IFN-γ secretion in vivo and expanded spleen cells. Thus, it is of interest to determine whether the effectiveness of IL-28 in suppressing tumor growth in vivo can be further enhanced in combination with IL-12. We found that the combination therapy with local production of IL-28 by genetically modified tumor cells and systemic administration of IL-12 had an apparent additive antitumor effect, and did not have the apparent deleterious side effects. The presence of protective antitumor immunity in the surviving mice indicates that the effectiveness of this combination strategy extends beyond initial rejection of MCA205IL-28 cells to the development of protective immunity, which is specific for the initial MCA205 tumor. Our in vivo depletion experiments displayed that NK cells and CD8 T cells play an important role in the IL-28-elicited enhanced antitumor effect of IL-28.
The antitumor effect of IL-12 was not observed in IFN-γ KO mice because daily injections of 0.4 μg of IL-12 for 8 consecutive days did not significantly suppress the growth of MCA205Neo tumors in IFN-γ KO mice. Thus, it is of particular interest that IL-12 enhanced the antitumor effect of IL-28 even in IFN-γ KO mice. A possible explanation of the underlying mechanism is that, in IFN-γ KO mice, IL-28 could enhance IL-12-induced production of other mediators such as GM-CSF and IL-15 instead of IFN-γ.

Further analyses are needed to elucidate the detailed mechanisms through which IL-12 enhances the antitumor activity of IL-28 in IFN-γ KO mice.

In conclusion, IL-28 has a biological action to significantly inhibit in vivo tumor growth and lung metastases. IL-28-mediated antitumor action is mediated by polymorphonuclear neutrophils, NK cells, and CD8 T cells, but not CD4 T cells. In addition, antitumor activity of IL-28 is partially dependent on IFN-γ and independent of IL-12, IL-17, and IL-23. Furthermore, an additive antitumor effect of IL-28 with IL-12 in vivo was also observed. These results demonstrate that IL-28 has the bioactivities to induce both innate and adaptive immune responses against tumors. Our findings also indicate a possibility for the application of IL-28 in cancer immunotherapy.

Acknowledgments

We thank Toshie Suzuki and Yumiko Ito for their excellent technical assistance in carrying out this study. We also thank Dr. Naoto Ishii for useful discussions.

Disclosures

The authors have no financial conflict of interest.

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