The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 1998

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J Immunol (1998) 160 (4): 1742–1749.
https://doi.org/10.4049/jimmunol.160.4.1742

IFN-γ-Inducing Factor/IL-18 Administration Mediates IFN-γ- and IL-12-Independent Antitumor Effects[1]

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We evaluated the mechanism of the antitumor effects of mouse rIFN-γ-inducing factor/IL-18 protein on the growth of mouse tumor cell lines in vivo. Mice received IL-18 before or after challenge with CL8-1, a mouse melanoma cell line. Both regimens significantly suppressed tumor growth and reduced the number of mice with growth of tumor from 60% (3/5) to 20% (1/5). Furthermore, IL-18 administered before and after tumor inoculation completely abrogated the establishment of CL8-1 in all animals. IL-18 administration also significantly suppressed the growth of MCA205, a sarcoma cell line, even when treatment was delayed to 7 days following tumor inoculation. Although IL-18/IL-12 combination therapy had the most significant and immediate antitumor effects, many mice so treated succumbed with markedly elevated serum IFN-γ levels. The antitumor effects of IL-18 were abrogated almost completely when NK cells were eliminated using anti-asialo GM1 Ab administration, but only marginally impaired in IFN-γ or IL-12 gene-disrupted mice. Immunohistochemical staining revealed that the number of the $CD8^+$ T cells, but not $CD4^+$ T cells, found at the tumor site was reduced in animals treated with IL-18. These results indicate that IL-18 has potent antitumor effects mediated by $CD4^+$ T cells and NK cells, but in IFN-γ- and IL-12-independent pathways. ***The Journal of Immunology,*** **1998, 160: 1742–1749.**

Antitumor effects mediated by the administration of recombinant cytokines, including IL-1 (1), IL-2 (2), IL-4 (3), IFN-α (4, 5), IFN-γ (6), and TNF-α (7), have been investigated in mice bearing transplantable tumors, as well as in patients with cancer. These cytokines have had limited antitumor effects with significant side effects. Recently, IL-12 has been shown to be effective in the treatment of murine tumors with minimal side effects (8–10). In these studies, the antitumor effects of IL-12 on cancer are mediated by NK cells, $CD4^+$, $CD8^+$ T cells, as well as IFN-γ elaboration (9, 11, 12). IL-12 antitumor effects have also been attributed to its potent anti-angiogenic function that is mediated in part by IP-10,[3] in turn induced by IFN-γ (13–15). Phase I clinical trials of rIL-12 administration have been completed recently, based on the promising preclinical animal studies (16) and phase II studies initiated.

IL-18 is a recently discovered cytokine cloned from mice with fluminant hepatitis induced by challenge with *Propionibacterium acnes* and subsequent administration of LPS (17). This cytokine was initially termed IFN-γ-inducing factor based on high level IFN-γ secretion from NK and T cells at levels substantially greater than that observed with IL-12, another potent IFN-γ inducer (17). IL-18 has many similarities with IL-1 (12% homology with IL-1α, and 19% with IL-1β, respectively), demonstrated in its structure and requirement for processing to an active molecule. It, like IL-1, lacks a signal sequence (17) and is processed into the mature form by an IL-1β-converting enzyme (18). Although IL-18 shares these characteristics with IL-1, IL-18 appears to have its own receptor (19). IL-18 is produced by cells of monocyte lineage, augments NK cytolytic activity, and enhances proliferation of T cells (17). IL-18 also promotes NK and T cells to secrete IFN-γ as well as granulocyte-macrophage CSF (20). Although IL-18 and IL-12 synergize in IFN-γ production, their receptors or signal-transduction pathways appear to be different (21). These findings strongly suggest that IL-18 has significant immunoregulatory functions that could be modulated further in combination with IL-12.

To examine the role of IL-18 in tumor immunity, we evaluated the effect of administration of murine rIL-18 to tumor-bearing mice. IL-18 significantly suppressed the growth of both CL8-1, a melanoma cell line, and MCA205, a fibrosarcoma cell line. Pretreatment of mice with IL-18 before tumor transplantation also significantly suppressed the subsequent establishment of tumor. Furthermore, the antitumor effects of IL-18 were augmented by combination with systemic IL-12 administration.

Materials and Methods

Recombinant cytokines

Murine rIL-18 was supplied by Hayashibara Biochemical Laboratories (Okayama, Japan). Murine rIL-12 was a gift from Hoffman-La Roche (Nutley, NJ).

Tumor cell lines and animals

MCA205, a methylcholanthrene-induced murine fibrosarcoma cell line, was a generous gift from National Cancer Institute (Dr. S. A. Rosenberg, Bethesda, MD). CL8-1, a BL6 melanoma cell line, was provided by Dr. E. Gorelik (University of Pittsburgh, Pittsburgh, PA). These cell lines were maintained as described previously (22) and were demonstrably free of mycoplasma contamination with the Mycoplasma PCR ELISA kit (Boehringer Mannheim Corp., Mannheim, Germany) periodically.

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Received for publication August 22, 1997. Accepted for publication October 30, 1997.

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[1] This work was supported in part by Career Development Award of American Association of Clinical Oncology '94 (H.T.) and a National Institutes of Health Grant 1PO1 CA 48047-01 (M.T.L.).

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[3] Abbreviations used in this paper: IP-10, IFN-γ-inducible 10-kDa protein; ASGM1, asialo GM1; i.d., intradermal; ko, knockout.

0022-1767/98/$02.00

C57/BL6 mice were purchased from Taconic Farms (Germantown, NY) and were used for experiments when they were 7 to 10 wk old. The IFN-γ gene knockout (ko) mice were kindly provided by Dr. J. Flynn (University of Pittsburgh, Pittsburgh, PA), and the IL-12 p40 gene ko mice were generated and supplied by Dr. J. Magram (Hoffman-La Roche) (23). These animals were bred in our animal facility at Center for Biotechnology and Bioengineering, University of Pittsburgh.

Animal experiments and statistical evaluation

A quantity amounting to 3×10^4 to 1×10^6 cells of CL8-1 or MCA205 was inoculated intradermally (i.d.) in the flank of C57BL/6 mice on day 0. Each group consisted of five to six animals. Mice received once daily i.p. injections of IL-18, IL-12, or HBSS (Life Technologies, Grand Island, NY) as a control. Treatment schedule including the dosage of IL-18 and IL-12 injected into mice is specified in each experiment. The tumor size was measured every 2 to 4 days and expressed as the product of the perpendicular diameters of individual tumors. Each animal experiment was repeated at least twice. Blood samples from three to four mice per group were obtained by tail-vein phlebotomy 1 day before and 5 days after cytokine injections. Serum was separated by centrifugation and stored at -80°C until assayed. Nonparametric Wilcoxon rank test was used in the statistical analysis of the size of tumor in individual groups. The difference was considered statistically significant when $p < 0.05$.

Ab depletion of NK cells

To deplete NK cells in mice, 20 μl of anti-asialo GM1 antiserum (ASGM1; Wako Bioproducts, Richmond, VA) was administered i.p. 1 day prior to the tumor inoculation, and subsequently once every 5 days afterward for an additional 20 days (five times in total). This dose was confirmed to be effective in suppressing more than 95% of the lytic activity against YAC-1 cells mediated by spleen cells harvested from the animals receiving only two injections.

ELISA for cytokines

Concentration of IFN-γ and IL-10 in the serum of the mice treated with IL-18 and/or IL-12 was measured by ELISA, respectively (PharMingen, San Diego, CA). The lower limits of the detection of both cytokines were 15.6 pg/ml.

Immunohistologic analysis

Mice received 7 days of treatment with 1 μg of IL-18 either before (pretreatment) or after (post-treatment) the i.d. inoculation of CL8-1 cells into the flank. Tumors were harvested 6 days after tumor challenge. One-half of the tumors were immediately frozen and embedded in OCT compound. Serial 5-μm sections were made from these tumors using cryostat, and underwent immunochemical staining using Abs to CD4, CD8a, and CD11b (all from PharMingen, San Diego, CA) with the Vectastain ABC kit (Vector, Burlingame, CA). The stained cells were counted in five fields at a magnification of $\times$400 in each sample. Evaluation of the results was performed in a blinded fashion. The rest of the samples were immediately frozen, stored at -80°C, and used for extracting RNA.

Analysis of IFN-γ and IP-10 expressions in tumors by reverse-transcriptase-mediated PCR

CL8-1 tumors were inoculated, treated with 1 μg of IL-18 for 6 days, and harvested. Total cellular RNA was extracted from whole tumor tissue using RNAzol B (TEL-TEST, Friendswood, TX). Four micrograms of total RNA were reverse transcribed using an RNase H^- reverse transcriptase (Superscript; Life Technologies). Two hundred nanograms of RNA-equivalent cDNA were amplified by PCR. The numbers of amplification cycles were chosen for each primer pair that shows maximum signal intensity on the linear portion of an amplified product (24). The sense and antisense primer sequences used, respectively (followed in parenthesis by annealing temperature and amplification cycles), were as follows: IP-10, 5′-ACCATG AACCAAGTGCTGCCGTC-3′ and 5′-GCTTCACTCCAGTTAAGGAGC CCT-3′ (22, 66°C); IFN-γ, 5′-TGCGGCCTAGCTGAGACAATGA-3′ and 5′-TGAATGCTTGGCGCTGGACCTGTG-3′ (31, 66°C); and β-actin, 5′-TTCTACAATGAGCTGCGTGTG-3′ and 5′-CACTGTGTTGGCATA GAGGTC-3′ (21, 58°C). The amplified products were electrophoresed on 1.5% agarose gel, and the intensity of the bands was quantified by densitometry (Molecular Dynamics, Sunnyvale, CA).

Results

Systemic administration of IL-18 abrogates the establishment of murine tumors

To evaluate the effects of IL-18 on preventing the establishment of tumor in vivo, 1×10^5 of CL8-1 cells were inoculated i.d. into the right flank on day 0. Mice received daily i.p. injections of 1 μg of IL-18 for 7 days either from day -7 (pretreatment) or from day 0 (post-treatment) (Fig. 1*A*). Suppression of tumor growth was first observed on day 6 in all IL-18-treated groups. The mean tumor area was significantly smaller in all IL-18-treated groups (groups II, III, IV vs I, $p < 0.05$ on day 8; groups IV vs I, $p < 0.05$ on day 11). This growth suppression was attained by day 13 in pretreated mice (group II) and by day 17 in post-treated mice (group III). Furthermore, pretreatment combined with post-treatment with IL-18 (group IV) completely eradicated tumor in all animals (Fig. 1, *A* and *B*).

Although CL8-1 is an immunogenic tumor (22), when 1×10^5 cells were injected, only 40% of the animals rejected tumor. However, the majority (80%) of the animals rejected the CL8-1 tumors when they received either pretreatment or post-treatment with IL-18. Similarly, pretreatment combined with post-treatment with IL-18 (group IV) was also associated with complete tumor eradication in all animals (100%) (Fig. 1*B*). In animals rejecting the tumor, specific immunity against CL8-1 was evaluated by rechallenge of 2×10^6 of CL8-1 cells applied to the opposite flank. Thirteen of fifteen animals rejected the rechallenge. Of the two mice that failed to reject the subsequent challenge, one was in group II (pretreatment) and the other was in group IV (pre- and post-treatment).

IL-12 enhances the antitumor effects of IL-18 on established tumor in vivo

To further evaluate the antitumor effects of IL-18 in vivo, 1×10^5 of MCA205 cells were inoculated into the right flank of mice on day 0, and treatment was begun on day 7 with daily i.p. injections of either HBSS, IL-18 or IL-12 alone, or IL-18 combined with IL-12 for 7 days (Fig. 2). Each group consisted of six mice. IL-18 treatment (group II) (1 μg/day) suppressed tumor growth through day 18 to 24 (groups II vs I, $p < 0.05$ on days 18, 20, and 24). IL-12 treatment (group III) (0.1 μg/day) was associated with more potent antitumor effects (groups II vs III, $p < 0.05$ on day 16 to 29; groups III vs I, $p < 0.05$ on day 12, and $p < 0.01$ on day 14 to 29). Although combination treatment with IL-18 and IL-12 (group IV) was associated with the most marked suppression of tumor growth (groups IV vs I, $p < 0.05$ on day 8, and $p < 0.01$ on days 12 and 14), all mice in this group died before completion of treatment.

To avoid the lethal side effects of combination treatment with IL-18 and IL-12, we examined the effectiveness of reduced doses of IL-18 and IL-12 on the MCA205 tumor (Fig. 3). Groups of six mice received i.d. injections with 5×10^4 of MCA205 cells in the right flanks on day 0 and were treated with daily administration on day 7 to 13 with HBSS, IL-18, or IL-12 i.p. Treatment with IL-18 alone (groups III and IV) or IL-18 combined with IL-12 (groups V and VI) suppressed the growth of the tumor during treatment at both doses (0.2 and 1 μg/day) of IL-18 (groups III vs I and groups IV vs I, $p < 0.05$ on day 13, and $p < 0.01$ on day 18 to 30; groups V vs I and groups VI vs I, $p < 0.01$ on day 9 to 30). IL-12 (group II) also mediated antitumor effects at the lower dose (groups II vs I, $p < 0.05$ on day 9, and $p < 0.01$ on day 13 to 30). Tumor growth suppression was also observed in mice treated with IL-18 combined with IL-12 (groups V and VI), and somewhat better than what was observed with IL-18 alone (groups III and IV) (groups VI vs III and groups VI vs IV, $p < 0.05$ on day 11; groups V vs

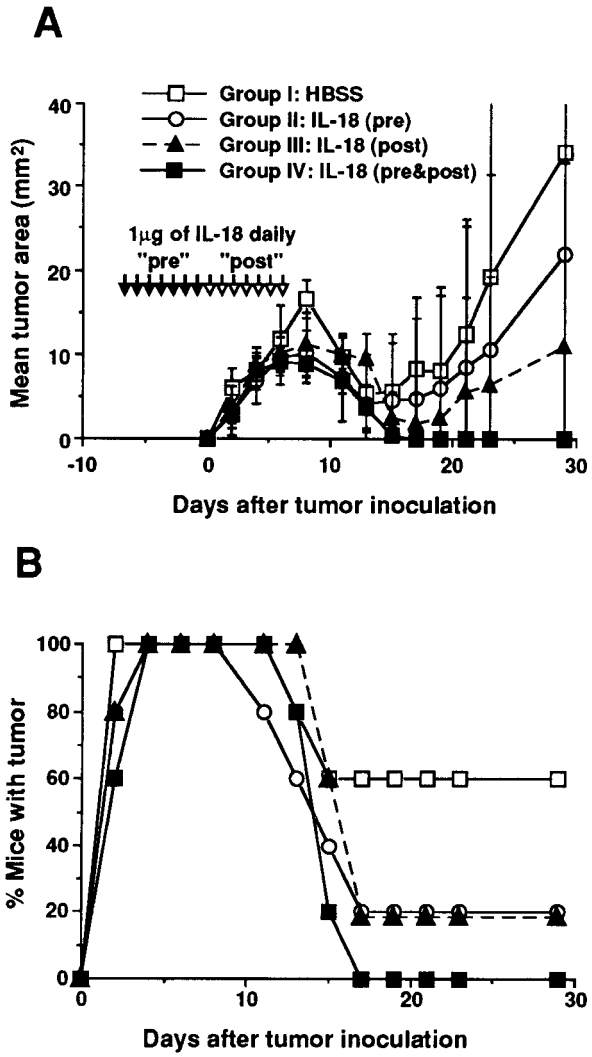

FIGURE 1. Inhibition of CL8-1 tumor growth following IL-18 administration. Groups of five animals were inoculated i.d. with 1×10^5 of murine melanoma CL8-1 cells in the right flank on day 0. Mice subsequently received daily injections of either HBSS or 1 μg of IL-18. Group I, control, HBSS on day -7 to 6; group II, pretreatment, IL-18 on day -7 to -1, and HBSS on day 0 to 6; group III, post-treatment, HBSS on day -7 to -1, and IL-18 on day 0 to 6; group IV, pre- and post-treatment, IL-18 on day -7 to 6. *A*, Treatment with IL-18 suppressed CL8-1 tumor growth. Tumor area was calculated by the products of the perpendicular diameters of each tumor. Bars represent mean ± SD. Treatment with IL-18 was associated with significant antitumor effects (groups II, III, and IV). *B*, Treatment with IL-18 enhanced rejection of CL8-1 in most animals. Either pretreatment (group II) or post-treatment (group III) with IL-18 inhibited establishment of CL8-1 cells in most animals (80% for both groups). Furthermore, pretreatment combined with post-treatment with IL-18 (group IV) abrogated tumors completely. On day 34, 2×10^6 of CL8-1 cells were rechallenged i.d. in the left flank of mice free of palpable tumor. Rechallenged CL8-1 cells were rejected in all but two of these mice (1/15 in group II, and 1/15 in group IV).

III, $p < 0.05$ on days 21 and 24). Complete abrogation of the tumor was observed in one animal treated with the combination of 1 μg of IL-18 and 0.01 μg of IL-12 (group VI; 1/6, 17%). However, three of the mice in this treatment group became ill with this treatment, and it was discontinued after the fourth injection. The animals in other groups completed the treatment cycle without apparent deleterious effects.

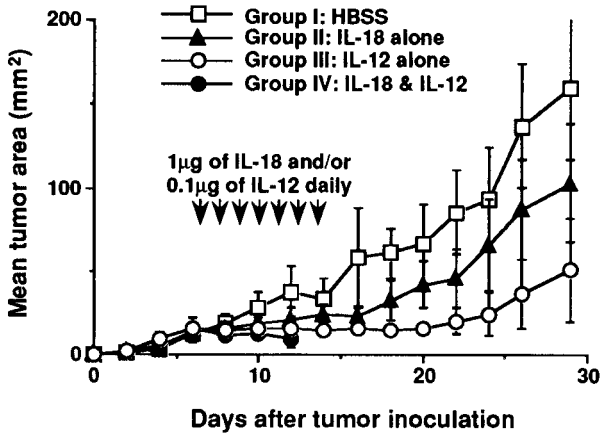

FIGURE 2. Suppression of s.c. MCA205 tumor growth following treatment with IL-18. Six mice in each group were inoculated in the flank with 1×10^5 of MCA205 murine fibrosarcoma cells on day 0. Mice received daily i.p. injections of either HBSS (group I), IL-18 (group II, 1 μg IL-18 alone), IL-12 (group III, 0.1 μg IL-12 alone), or IL-18 combined with IL-12 (group IV, 1 μg IL-18 and 0.1 μg IL-12) on day 7 to 13. Tumor area is presented as the mean ± SD of the product of the perpendicular diameters of each tumor. IL-18 treatment was associated with transient, but significant tumor growth suppression (group II). IL-12 treatment had more potent antitumor effects alone (group III) and in combination treatment with IL-18 (group IV), but all mice in the latter group died before completion of treatment.

Systemic delivery of IL-18 elevates the serum IFN-γ level, but not IL-10

IL-18 and IL-12 are reported to induce the production of IFN-γ in vitro (17), and administration of IL-12 induces both IFN-γ and

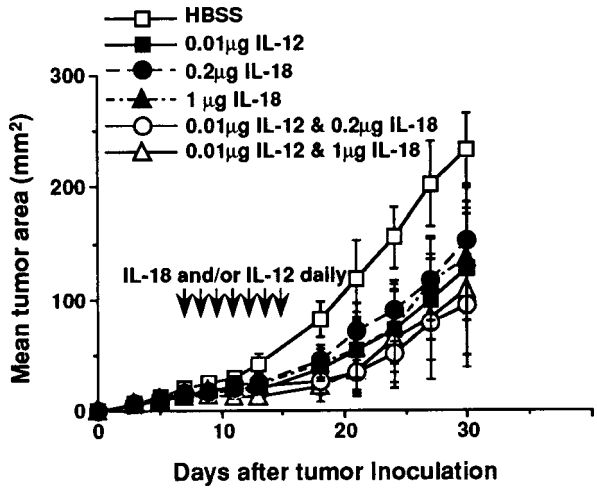

FIGURE 3. Suppression of established MCA205 i.d. tumor growth with combined treatment with IL-18 and IL-12 in vivo. Groups of six mice were injected i.d. with 3×10^4 of murine fibrosarcoma MCA205 cells in the right flank on day 0. Each group of mice was administered cytokines i.p. daily on day 7 to 13 with HBSS, IL-18, or IL-12. Group I, HBSS; group II, 0.01 μg IL-12; group III, 0.2 μg IL-18; group IV, 1 μg IL-18; group V, 0.2 μg IL-18 and 0.01 μg IL-12; group VI, 1 μg IL-18 and 0.01 μg IL-12. Tumor area is shown by the mean ± SD of the products of the perpendicular diameters of each tumor. Treatment with IL-18 comparably suppressed the growth of tumor during treatment at both doses. Complete abrogation of the tumor was observed only in animals treated with a combination of 1 μg IL-18 and 0.01 μg IL-12 (1/6: 17%). However, one-half of the mice in this treatment group became morbid and the treatment was discontinued after the fourth injection. The animals in other groups completed the treatment cycle without apparent side effects.

Table I. *Elevation of serum IFN-γ levels following IL-18 administration*

Treatment[a]	IFN-γ ± SD (pg/ml)[b,c] Before	5th Day
Group I: HBSS	<15.6	<15.6
Group II: IL-18	<15.6	57.12 ± 64.9
Group III: IL-12	<15.6	400.0 ± 577.5
Group IV: IL-18 and IL-12	<15.6	17671.5 ± 107.1

[a] Each group of C57BL/6 mice received 1.0 μg of IL-18 and/or 0.1 μg of IL-12 i.p. once daily on days 7–13 after the i.d. inoculation of MCA205 tumor cells.
[b] Results represent mean ± SD of serum IFN-γ from three mice. Blood samples were collected 1 day before (Before) and on the 5th day (5th Day) following protein injection. Serum IFN-γ was assayed by ELISA.
[c] IL-10 levels were only measured in HBSS and IL-18-treated animals and were <15.6 pg/ml, which was not changed by IL-18 treatment.

IL-10 production in vivo. To determine whether IL-18 induces productions of IFN-γ and IL-10 in vivo, we analyzed the serum levels of IFN-γ and IL-10 of the mice treated with IL-18 by ELISA (Table I). While five daily injections of 1 μg of IL-18 induced measurable serum IFN-γ levels to 57.1 ± 64.9 pg/ml, the treatment with 0.2 μg of IL-18 did not (data not shown). Treatment with 1 μg of IL-18 combined with 0.1 μg of IL-12 raised serum IFN-γ level about 300 times higher than that observed with IL-18 alone (17,671.5 ± 107.1 pg/ml). Serum IFN-γ levels in the animals receiving 1 μg of IL-18 combined with 0.01 μg of IL-12 were only nine times higher than IL-18 alone (data not shown). Five daily injections of 1 μg of IL-18 did not cause significant elevation of IL-10 above the detection level of the ELISA.

Depletion of NK cell activity by anti-ASGM1 Ab abrogates the antitumor effects mediated by IL-18

Treatment with IL-18 inhibited the establishment of CL8-1 cells with administration before tumor inoculation (Fig. 1). This result suggests the involvement of NK cells in the rejection of this tumor. To investigate the role of NK cells in the antitumor effects of IL-18, NK cells were depleted using anti-ASGM1 administered before and during treatment with IL-18 (Fig. 4). Mice received 1 μg of IL-18 daily for 7 days after the inoculation of 1×10^6 of CL8-1 cells as a treatment (group III, IL-18, and group IV, IL-18 + anti-ASGM1). The treatment with IL-18 significantly suppressed the growth of tumor (groups III vs I, $p < 0.05$ on day 15 to 29, and $p < 0.01$ on days 22 and 33) (Fig. 4*A*) and eradicated the tumor in all mice without NK depletion (Fig. 4*B*). However, depletion of NK cells with anti-ASGM1 administration completely abrogated the growth-inhibitory effects of IL-18 (groups III vs IV, $p < 0.01$ on day 12 to 33) (Fig. 4, *A* and *B*).

Antitumor effects of IL-18 were still noted in IFN-γ gene or in IL-12 gene-disrupted mice

Studies using an Ab for IFN-γ or alternatively, using IFN-γ gene ko mice, demonstrated that the antitumor effects of IL-12 require IFN-γ elaboration (8, 9, 12), presumed essential for the activation of macrophages and for enhancing the cytolytic activity of NK and T cells (25). Since IL-18 induces IFN-γ from NK cells and T cells and augments NK cell activities in a synergistic fashion with IL-12, the IL-12 itself and/or induced IFN-γ expression might play an important role in the antitumor effects of IL-18 on tumor in vivo. To test this hypothesis, we examined the antitumor effects of IL-18 in IL-12 gene or IFN-γ gene ko mice. Groups of five wild-type, IL-12, or IFN-γ gene ko mice received i.p. treatment with 1 μg of IL-18 daily for 7 days immediately after inoculation of 1×10^6 of CL8-1 cells (Fig. 5). The tumors inoculated in the IL-12 gene ko

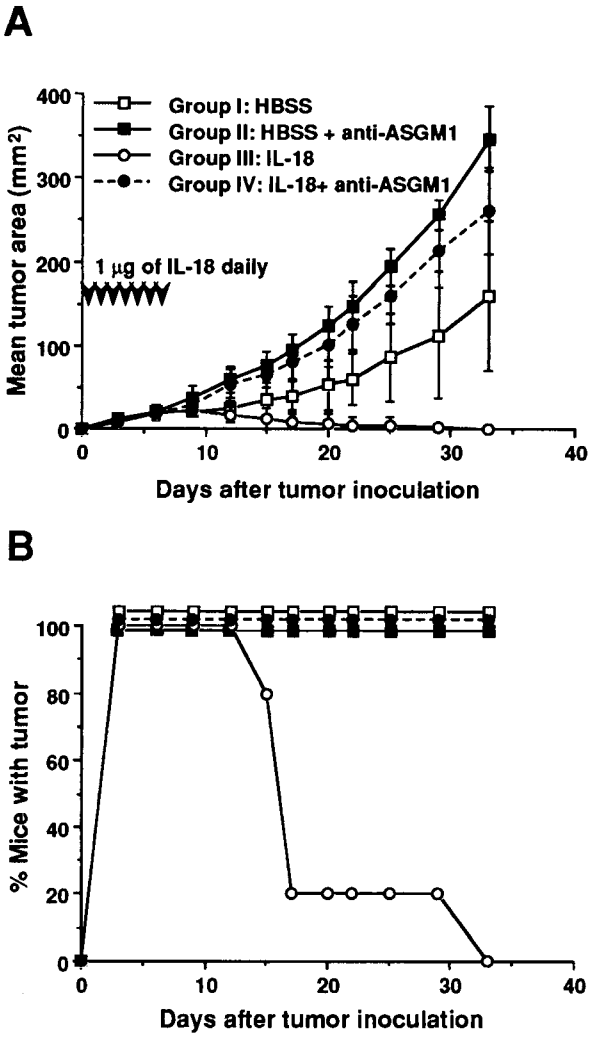

FIGURE 4. Antitumor effects of IL-18 were abrogated completely by administration of anti-GM1. Animals were pretreated with anti-ASGM1 to eradicate NK cells before tumor inoculation of 1×10^6 of murine melanoma CL8-1 cells in the right flank on day 0. These mice received daily administration of either HBSS or 1 μg of IL-18 beginning on day 0 for 7 days. Twenty microliters of anti-ASGM1 were administered i.p. 1 day before tumor inoculation and thereafter every 5 days for an additional 20 days (five times in total). Group I, HBSS, treated with HBSS; group II, HBSS + anti-ASGM1, treated with HBSS and anti-ASGM1; group III, IL-18, treated with IL-18; group VI, IL-18 + ASGM1, treated with IL-18 and anti-ASGM1. Tumor area was calculated as the product of perpendicular diameters of individual tumors. Data represent the mean ± SD of five mice per group. *A*, Treatment with anti-ASGM1 abrogated the growth-inhibitory effects of IL-18 on CL8-1. Growth suppression of CL8-1 was observed in the IL-18-treated groups (group III), and treatment with IL-18 was associated with complete inhibition of tumor establishment (group III) in all animals. Depletion of NK cell activity almost completely abrogated the antitumor effects of IL-18 in this tumor system (group IV). All mice rejected a subsequent rechallenge with CL8-1. *B*, Treatment with IL-18 inhibited establishment of tumor. Treatment with IL-18 led to complete inhibition of tumor growth (group III). However, depletion of NK cells following anti-ASGM1 treatment abrogated the effects of IL-18 completely (group IV).

(Fig. 5, *B* and *E*, HBSS) or the IFN-γ gene ko mice (Fig. 5, *C* and *F*, HBSS) with HBSS treatment (control) grew more rapidly when compared with tumors inoculated in the wild-type, immunocompetent mice (Fig. 5, *A* and *D*, HBSS) (average tumor area of Fig. 5*A*, HBSS vs that of Fig. 5*B*, HBSS; $p < 0.05$ on day 12 to 31,

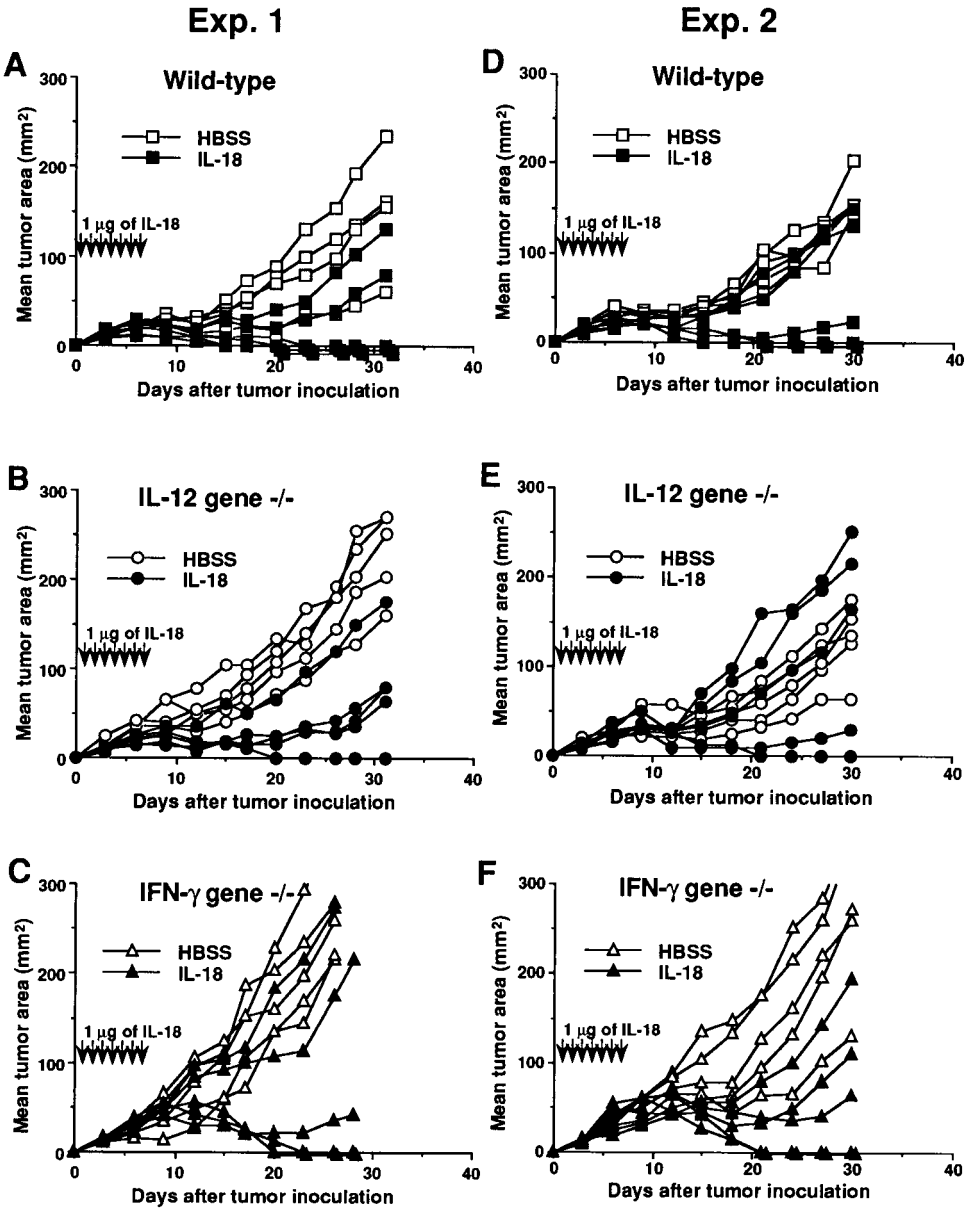

FIGURE 5. Antitumor effects of IL-18 were abrogated only marginally in IFN-γ gene ko mice and IL-12 gene ko mice. Groups of five wild-type, IL-12 gene ko, or IFN-γ gene ko mice, respectively, were inoculated i.d. with 1×10^6 of CL8-1 cells in the right flank on day 0 and received i.p. injections of 1 μg of IL-18 from day 0 for 7 days. Tumor area is shown by the products of the perpendicular diameters of the tumor of individual animals. The untreated tumors in IFN-γ (*C* and *F*, HBSS) or in IL-12 gene ko mice (*B* and *E*, HBSS) grew more quickly when compared with untreated tumors in wild-type mice (*A* and *D*, HBSS). The untreated tumor in IFN-γ gene ko mice grew most rapidly. Treatment with IL-18 significantly suppressed tumor growth in IL-12 gene ko mice (*B* and *E*, IL-18) to the same extent as wild-type mice (*A* and *D*, IL-18). The significant antitumor effects of IL-18 were also observed in the IFN-γ gene ko mice (*C* and *F*, IL-18). Two experiments are shown.

average tumor area of Fig. 5*A*, HBSS vs that of Fig. 5*C*, HBSS; $p < 0.01$ on day 12 to 31). Treatment with IL-18 in the IL-12 gene ko mice (Fig. 5, *B* and *E*, IL-18) effectively suppressed tumor growth at the same order of magnitude as that in the wild-type mice (Fig. 5, *A* and *D*, IL-18) (average tumor area of Fig. 5*B*, HBSS vs that of Fig. 5*B*, IL-18; $p < 0.05$ on day 9, and $p < 0.01$ on day 12 to 31, average tumor area of Fig. 5*A*, IL-18 vs that of Fig. 5*B*, IL-18; $p = 0.114-0.837$ on day 0 to 31). In the IFN-γ gene ko mice, the growth-suppressive effects of IL-18 were impaired at earlier time points, but became significant later (Fig. 5, *C* and *F*) (average tumor area of Fig. 5*C*, HBSS vs that of Fig. 5*C*, IL-18; $p < 0.05$ on day 17 to 24).

Treatment with IL-18 is associated with decreased numbers of $CD8^+$ T cells observed at the tumor site

To examine the effects of IL-18 on the characteristics of tumor-infiltrating lymphocytes, samples of IL-18-treated CL8-1 tumor cells were harvested from the animals and immunostained with anti-CD4, anti-CD8a, and anti-CD11b (anti-Mac-1) Abs (Fig. 6). Untreated tumor showed a moderate infiltration of $CD4^+$ (Fig. 6*A*) and $CD8^+$ (Fig. 6*D*) cells, and a dense penetration of Mac-1^+ cells (data not shown) into the tumor. Neither pretreatment nor post-treatment with IL-18 affected the population of $CD4^+$ cells (Fig. 6, *B* and *C*) or Mac-1^+ cells (data not shown). IL-18 treatment was associated with a reduced

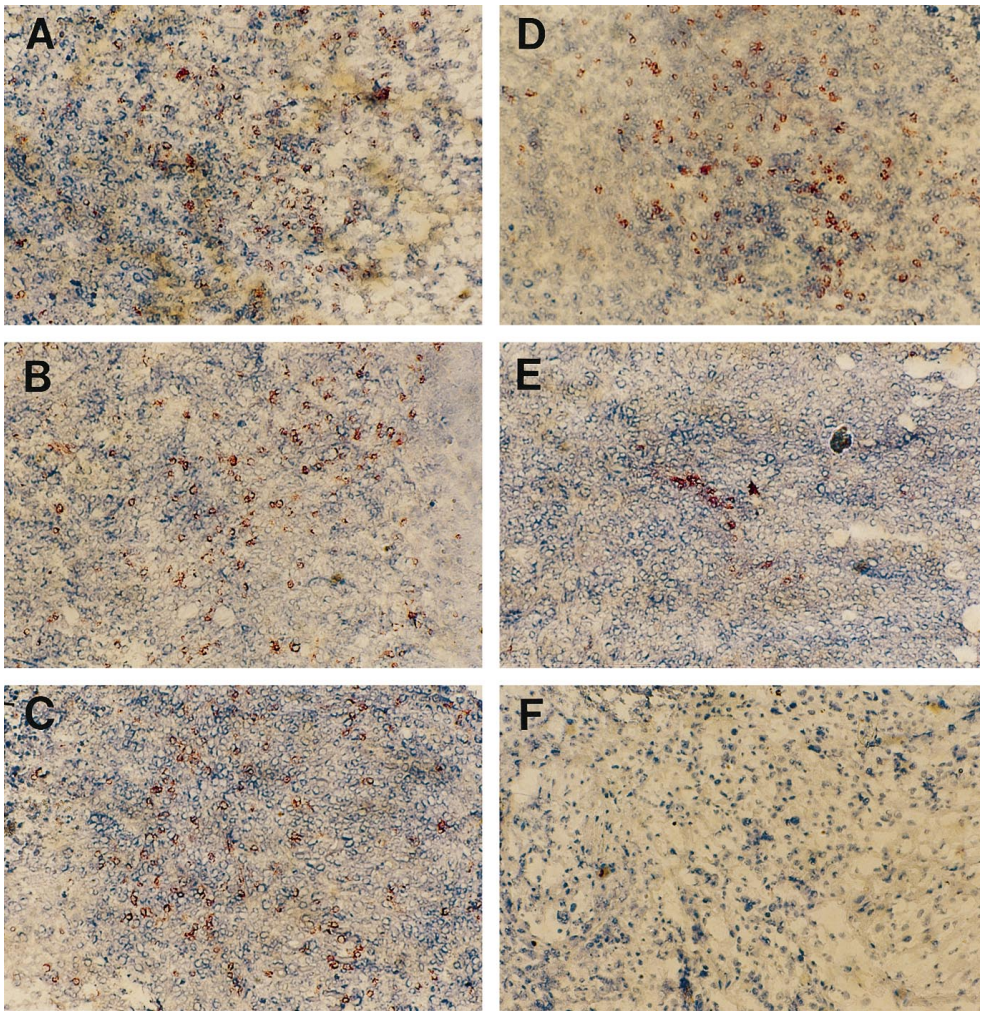

FIGURE 6. Treatment with IL-18 was associated with decreased number of $CD8^+$ cells within the tumor. Mice received i.d. inoculations of 1×10^6 of CL8-1 cells in the right flank on day 0 and i.p. injections of 1 μg of IL-18 from day -7 to -1 (pretreatment) or day 0 to 6 (post-treatment). Each group consists of three mice. Tumors were excised on day 6 and examined for infiltrating cells using immunohistochemistry. Each sample was immunostained with anti-CD4 (*A*, *B*, and *C*) or anti-CD8a (*D*, *E*, and *F*), respectively. Evaluation of the results was performed in blinded fashion. Representative photos are shown. Untreated tumor showed a moderate infiltrate with $CD4^+$ (*A*) and $CD8^+$ T cells (*D*) and a dense penetration of Mac-1^+ cells (data not shown) in tumor. While no significant changes in populations of $CD4^+$ T cells (*B*, *C*) or Mac-1^+ cells (data not shown) were observed following treatment with IL-18, a significant reduction of the numbers of infiltrating $CD8^+$ T cells in tumor was observed (*E*, *F*). Original magnification was $\times$100.

number of $CD8^+$ cells within tumors (Fig. 6, *E* and *F*), and this reduction was more apparent in the samples from the animals received post-treatment with IL-18 (Fig. 6*F*).

Expressions of IFN-γ and IP-10 in tumor were not altered following administration of IL-18

The antitumor effects of IL-12 are attributed in part to its ability to inhibit angiogenesis that is mediated in part by induction of IP-10, which in turn is induced by IFN-γ (13, 15). Thus, we examined the mRNA expression of IFN-γ and IP-10 on CL8-1 tumor in mice treated with IL-18. CL8-1 tumors were inoculated, treated with 1 μg of IL-18 for 6 days, and harvested to analyze the expression of IFN-γ and IP-10 using reverse-transcriptase PCR. IFN-γ and IP-10 were expressed constitutively and were not changed significantly following treatment with IL-18 (data not shown).

Discussion

IL-18 was identified as a novel cytokine that induces IFN-γ secretion from NK and T cells, and was initially termed IFN-γ-inducing factor (17). Although IL-18 has similarities with IL-1 in its structure and requirement for processing to an active molecule by an IL-1β-converting enzyme (18), IL-18 possesses its own receptor (19) and distinctive functions. IL-18 augments NK cytolytic activity and enhances proliferation of T cells (17). Administration of mouse rIL-18 protein has significant antitumor activities in murine tumor establishment and therapy models. The dose of IL-18 administered correlated well with the induced level of serum IFN-γ, as well as the magnitude of its inhibitory effects on tumor growth. The antitumor efficacy of IL-18 was enhanced by IL-12 administration. The antitumor effects of IL-18 were retained in IFN-γ or IL-12 gene-desrupted mice, but were abrogated when NK cells were depleted following anti-ASGM1 antiserum administration. Interestingly, reduced accumulation of $CD8^+$ T cell was observed in regressing tumors treated with IL-18.

The CL8-1 immunogenic tumor (22) was used to evaluate the antitumor effects of IL-18. Both pre- and post-treatment with IL-18 promoted rejection of this tumor. Since IL-18 enhances the cytotoxic activity of NK cells in vitro (17), we hypothesized that augmented NK cell activity enhanced in animals by pretreatment with IL-18 was central to the antitumor effects observed. Treatment

with IL-18 alone was also effective in suppressing the progression of the MCA205 tumor without apparent deleterious effects at both doses used. The ability to suppress tumor growth utilizing 1 μg of IL-18 was somewhat less than that observed with 0.1 μg of IL-12. Higher doses of IL-18 may be necessary to exert more potent antitumor effects. When the tumor is eradicated following IL-18 administration, these mice maintain a state of long lasting immunity. Thus, IL-18 possesses significant antitumor effects and induces a state of tumor-specific immunity that is mediated by activation of NK cells. NK-mediated tumor cell destruction, in turn, may play a significant role in the subsequent T cell response.

Additive antitumor effects of combination treatment with IL-18 and IL-12 were noted. These observations are consistent with in vitro studies that demonstrate that IL-18 cooperates with IL-12 to enhance production of Th1-type cytokines (17) and proliferation of Th1 cells (21). The level of serum IFN-γ detected in vivo was higher when animals were treated with a combination IL-18 and IL-12, and this appears to correlate with the antitumor effects. Moreover, NK depletion studies support the notion that they are important cells mediating in part the antitumor of IL-18. Nevertheless, some of the mice treated with combined therapy died of diarrhea and weight loss. Mice treated with both IL-12 and IL-18 developed severe hemorrhagic colitis with diffuse mucosal erosions as well as atrophic thymi. We did not observe diarrhea as a side effect of treatment with either IL-18 or IL-12 applied single. Levels of serum IFN-γ as high as 5,000 pg/ml with rIL-12 (9) or 15,000 pg/ml with injection of the IL-12 adenovirus vector (unpublished data) were observed in the past, but did not cause these gastrointestinal effects. Moreover, these side effects observed with treatment with 1 μg IL-18 and 0.01 μg IL-12 were not seen with treatment with 0.2 μg IL-18 and 0.01 μg IL-12. Both treatments elevated the level of the serum IFN-γ to the same level (500 pg/ml) (data not shown). We believe that it is unlikely that elevated serum IFN-γ is the sole cause of the side effect of combination treatment with IL-18 and IL-12. To optimize the antitumor effects of the combination of IL-18 with other cytokines, including IL-12, and to minimize the side effects of combination therapy, we plan to investigate modifications in the administration schedule and to more carefully define the mechanisms of toxicity.

IL-12 enhances the NK cytotoxic activity (26) and regulates differentiation of naive T cells into Th1 cells (27). IL-18 synergizes with IL-12 to enhance IFN-γ production, which also correlates with the extent of IL-18 antitumor effects. Furthermore, endogeneous expression of IL-12 induced by tumor challenge appears to be a key event in rejection of such a tumor in some murine tumors (Quan Cai, manuscript in preparation). Thus, it was of interest to determine whether intrinsic IL-12 production is necessary to mediate the antitumor effects of IL-18. In IL-12 gene ko mice, CL8-1 tumor grow more rapidly than in wild-type mice. Treatment with IL-18, however, still retained significant antitumor effects in these mice. These data suggest that endogeneous IL-12 production is not an absolute requirement for the IL-18 antitumor effects.

To examine whether IFN-γ is the main mediator of IL-18 antitumor effects or not, IL-18 treatment was performed in IFN-γ gene ko mice. The antitumor effects of IL-18, represented by delayed emergence and growth retardation, were abrogated only marginally in IFN-γ gene ko mice (Fig. 5). Thus, it appears that IL-18 administration mediates antitumor immune reaction independent of IFN-γ pathways, unlike IL-12.

IP-10 (28), a potent inhibitor of angiogenesis (29, 30), is known to mediate IL-12-induced anti-angiogenesis (13, 15). However, expressions of IP-10 were not different at the tumor site following treatment with IL-18 when compared with tumors in untreated animals, suggesting that IP-10 is not directly involved in antitumor effects of IL-18.

Immunostaining of CL8-1 tumor samples revealed dense infiltration of Mac-1^+ cells, which include macrophages, granulocytes, and NK cells. A significant difference in the number of Mac-1^+ cells was not detected in IL-18-treated tumors. Treatment with IL-18 reduced the number of $CD8^+$ cells found within the tumor, particularly in tumors from animals given IL-18 after establishment, but did not alter the numbers or distribution of $CD4^+$ or Mac-1^+ cells. Systemic treatment with IL-12 induces IL-10 production (31). IL-10 transduction of CL8-1 enhanced the number of $CD8^+$ T cells found within the tumor (22). Administration of IL-18 did not induce elevation of serum IL-10 levels in mice, which may explain the apparent reduction. Local production of IL-10 is possibly associated with accumulation of $CD8^+$ T cells within tumor. Micallef et al. prepared spleen cells from mice previously bearing the Meth A sarcoma following treatment with IL-18, and showed that $CD4^+$ T cells, but not $CD8^+$ T cells, were responsible for the cytotoxic activity in vitro (32). We speculate that treatment with IL-18 induced $CD4^+$ T cell immunity to tumor cells. These characteristics of tumor-infiltrating lymphocytes in IL-18-treated animals are different from those observed in animals treated with IL-12. $CD8^+$ T cells (8) or both $CD4^+$ and $CD8^+$ T cells (9) are the critical cells mediating the antitumor efficacy of IL-12. Dao et al. reported that treatment with IL-18 and IL-12 induced Fas ligand in activated murine Th1 cells in vitro (33), suggesting the possible role of Fas ligand expression on $CD4^+$ cells in tumor immunity induced by IL-18 administration.

In conclusion, we demonstrated that the administration of mouse rIL-18 has significant antitumor effects against the growth of murine tumor cell lines in vivo and also can induce specific immunity to the tumor. Synergistic antitumor effects of IL-18 with IL-12 in vivo were also observed with markedly elevated IFN-γ production. The antitumor effects of IL-18 are mediated by NK cells and $CD4^+$ cells, but in IFN-γ- and IL-12-independent pathways. These results indicate a possibility for the application of IL-18 in cancer therapy and warrant further investigation of the role of IL-18 in immunity.

Acknowledgments

We thank Dr. Yasuhiko Nishioka, Dr. Muneo Numasaki, Dr. Takashi Iwazawa, Dr. Toru Kitagawa, Dr. Andrea Gambotto, Dr. Jeanne Magram, Dr. JoAnne L. Flynn, and Dr. Hideho Okada for their valuable suggestions and assistance. We are also grateful to Susan F. Schoonover and Loraine R. McKenzie for their excellent technical assistance.

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