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ORIGINAL ARTICLE

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Application of interleukin 12 to antitumor cytokine and gene therapy

Abstract In vivo administration of interleukin 12 (IL-12) at 2000 U/mouse induced IL-12-activated killer (IL-12AK) cells in parallel with an elevation in serum interferon-γ (IFN-γ) activity. Although NK1.1$^+$CD3$^-$ natural killer cells are the major precursor of IL-12AK cells, asialoGM1$^+$CD8$^+$ T-cells were also demonstrated to be novel precursors. Such anomalous killer cells may play an important role in the early stages of the host defense mechanisms against tumors. It was also shown that IL-12 is effective in inducing tumor-specific cytotoxic T-lymphocytes. Consistent with these data, IL-12 had marked activity against various kinds of established tumors when given systemically. Mice cured of tumors by IL-12 treatment acquired tumor-specific T-cell immunity. Moreover, we initially demonstrated that IL-12 was effective in preventing and inhibiting the growth of primary tumors induced by the chemical carcinogen methylnitrosourea using c-Ha-*ras* transgeneic mice. Finally, we investigated the application of IL-12 to antitumor gene therapy. Transfer of the IL-12 gene into A20 B-lymphoma cells resulted in the continuous production of IL-12 and caused abrogation of in vivo tumorigenicity. Tumor cells transfected with the IL-12 gene are potentially a good tool as a tumor vaccine, as they effectively induced IL-12AK cells, IFN-γ production, and tumor-specific protective immunity. Although B16–BL-6 melanoma cells, which are a highly metastatic subclone of B16 melanoma cells, showed resistance to IL-12 gene therapy, combination therapy with the B7-1 gene and systemic IL-12 administration almost completely inhibited tumor metastasis. Similar results were obtained using B16–BL-6 melanoma cells transfected with both B7-1 and IL-12 genes. These results suggest that IL-12 is a promising cytokine for antitumor cytokine and gene therapy.

Key words IL-12 · Tumor · Cytokine therapy · Gene therapy

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Introduction

Evidence that cytotoxic T-lymphocytes (CTLs) recognize tumor-rejection antigen peptide bound to the major histocompatibility complex (MHC) and lyse autologous tumor cells encouraged us to develop new strategies for tumor-specific immunotherapy [23]. However, it has been considered difficult to induce tumor-specific immunity in vivo due to low tumorigenicity and strong immunosuppression [20].

Recent experiments have demonstrated that activation of the T-helper function at tumor sites (local help) is essential for induction of in vivo antitumor immunity [3, 4, 15]. Many investigators initially tried to induce local help using interleukin 2 (IL-2), but this was unsuccessful due to the short half-life of IL-2 in vivo. To overcome this problem we have tried several strategies to introduce local help in vivo [13]: targeting of both CD4$^+$ T-cells and CD8$^+$ T-cells using an antitumor × anti-CD3 bispecific antibody (helper/killer therapy) [16, 17], delivery of MHC-binding antigen using an antitumor monoclonal antibody (MAb) [7], and application of IL-12 in tumor immunotherapy [8].

The recently identified cytokine IL-12 has a longer half-life in vivo and has multiple immunoregulatory functions, including activation of natural killer (NK) cells, CTLs, and Th1 cells [22]. Therefore, use of IL-12 to induce local help in a tumor-bearing host appeared to be a rational strategy. In this paper we describe the characteristics of in vivo-induced IL-12-activated killer (IL-12AK) cells and the therapeutic effect of IL-12 on both transplantable and primary tumor cells. We also discuss the application of IL-12 to antitumor gene therapy.

Materials and methods

Mice

C57BL/6 mice and BALB/c mice were purchased from Charles River Japan (Yokohama, Japan) and used at 5–6 weeks of age.

Induction of IL-12AK cells in vivo

Normal C57BL/6 mice received an intraperitoneal injection of recombinant IL-12 (2000 U/mouse; kindly donated by Genetics Institute, Cambridge, Mass., USA) 1 day before the experiments. As a control, saline or IL-2 (2000 U/mouse; kindly supplied by Shionogi Pharmaceutical, Osaka, Japan) was used. Spleen cells from the treated mice were harvested and passed through a nylon-wool column. The nylon-passed spleen cells (NPSPL) from IL-12-injected mice were used as IL-12AK cells. Pooled NPSPL from three mice per group were used in all experiments. To define the characteristics of IL-12AK cells, spleen cells obtained from IL-12-injected mice were sorted into $NK1.1^+CD3^-$ NK cells, $NK1.1^-CD3^+$ T-cells, asialoGM1 $(ASGM1)^+CD8^+$ T-cells, and $ASGM1^-CD8^+$ T-cells. In some experiments, IL-12 was injected into tumor-immunized mice. The cytotoxicity of CTLs or IL-12AK cells was determined by 4-h $[^{51}Cr]$-release assay as described previously [14].

Tumor immunotherapy by systemic administration of IL-12

Mice were intradermally inoculated with tumor cells (2×10^6 cells) on day 0. When the inoculated tumor cells were readily palpable (6–8 mm), tumor-bearing mice received an intraperitoneal injection of saline, IL-2 at 2000 U/mouse per day (10^7 U/mg; kindly supplied by Shionogi Pharmaceutical), or IL-12 at 2000 U/mouse per day (5.6×10^6 U/mg; kindly donated by Genetics Institute) for 4 days. Each group consisted of ten mice. The antitumor activity of IL-12 was determined by monitoring of tumor growth by measuring the change over time in the mean of two perpendicular diameters. The survival rate was also determined. Mice that lived for more than 3 months after tumor regression were regarded as completely cured. Generation of systemic protective immunity was determined by rechallenge with the same tumor or by Winn's assay as described previously [13].

Inhibition of methylnitrosourea-induced primary tumor growth by IL-12

The therapeutic effect of IL-12 against primary tumor growth was investigated using c-Ha-*ras* transgeneic mice [1], in which forestomach papillomas are induced at an incidence of 100% after 2 months by a single intraperitoneal injection of methylnitrosourea (MNU) at 50 mg/kg. Our previous data also demonstrated that at least one papilloma can be detected at 1 month after MNU treatment [1].

IL-12 administration was initiated 30 days after MNU treatment. IL-12 at 2000 U/mouse suspended in 100 ml of saline was injected intraperitoneally into MNU-treated transgeneic mice four times a week for 4 weeks. As a control, saline was injected intraperitoneally into transgeneic mice. The therapeutic effect of IL-12 was determined by counting of the numbers of MNU-induced forestomach papillomas using a stereoscopic microscope. Seven mice per group were used for this experiment.

Establishment of tumor cells transfected with IL-12 genes or the B7-1 gene

Murine IL-12 genes and IL-12 p35 and p40 subunit genes were kindly donated by Genetics Institute. p40 cDNA was subcloned into the BCMGSNeo plasmid (generous gift from Dr. H. Karasuyama, Department of Immunology, Tokyo Metropolitan Cancer Institute, Japan) [6], which carries the neomycin phosphotransferase gene driven by a cytomegalovirus (CMV) promoter; the resulting plasmid was termed BCMGSNeo-p40. p35 cDNA was subcloned into the CEP4 plasmid (Invitrogen, San Diego, Calif., USA), which carries the hygromycin B phosphotransferase gene driven by a CMV promoter; the resulting plasmid was termed CEP4-p35. Tumor cells were transfected with BCMGSNeo-p40 by electroporation (Genepulser; Japan Bio-Rad Laboratory, Tokyo, Japan). Stable transfectants were selected by culture in RPMI 1640 medium containing G418 (0.5 mg/ml), 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (10 mg/ml). After cloning of p40-transfected cells, the cells were further transfected with the CEP4-p35 plasmid by electroporation. Tumor cells cotransfected with the p40 and p35 genes were selected by culture in medium containing G418 (0.5 mg/ml) and hygromycin B (0.5 mg/ml). After cloning, the expression of both genes was confirmed using a reverse transcription-polymerase chain reaction.

The B7-1 gene was transfected into B16–BL-6 melanoma cells using recombinant adenoviruses, Adex1CAwtB7-1, which encode the murine B7-1 gene. Detailed methods for the construction of Adex1-CAwtB7-1 have been described elsewhere [11, 19].

Results

Effect of IL-12 on generation of antitumor effector cells

C57BL/6 mice treated with IL-12 at 2000 U/mouse generated killer cells at 24 h after treatment; this occurred concomitantly with an elevation in serum IFN-γ levels (Fig. 1A). Killer-cell generation and IFN-γ production were not induced in control or IL-2-treated mice. The cytotoxic IL-12AK cells were enriched in $NK1.1^+CD3^-$ NK cells but not in $NK1.1^-CD3^+$ T-cells (Fig. 1B). However, when $ASGM1^+CD8^+$ T-cells were isolated from NPSPL, they showed higher cytotoxicity than $ASGM1^-CD8^+$ T-cells (Fig. 1C). These results demonstrated that NK cells are the major IL-12AK precursor cell and that $ASGM1^+CD8^+$ T-cells are a novel second-precursor cell type for IL-12AK cells.

Although IL-12AK cells were shown to be anomalous killer cells, which can lyse a variety of tumor cells (data not shown), IL-12 also induced $ASGM1^+CD8^+$ tumor-specific CTLs when injected into tumor-immunized mice. This result is not inconsistent with our previous finding that culture of human tumor-infiltrating lymphocytes with IL-2 plus IL-12 caused enhanced generation of autologous tumor-specific CTLs [8].

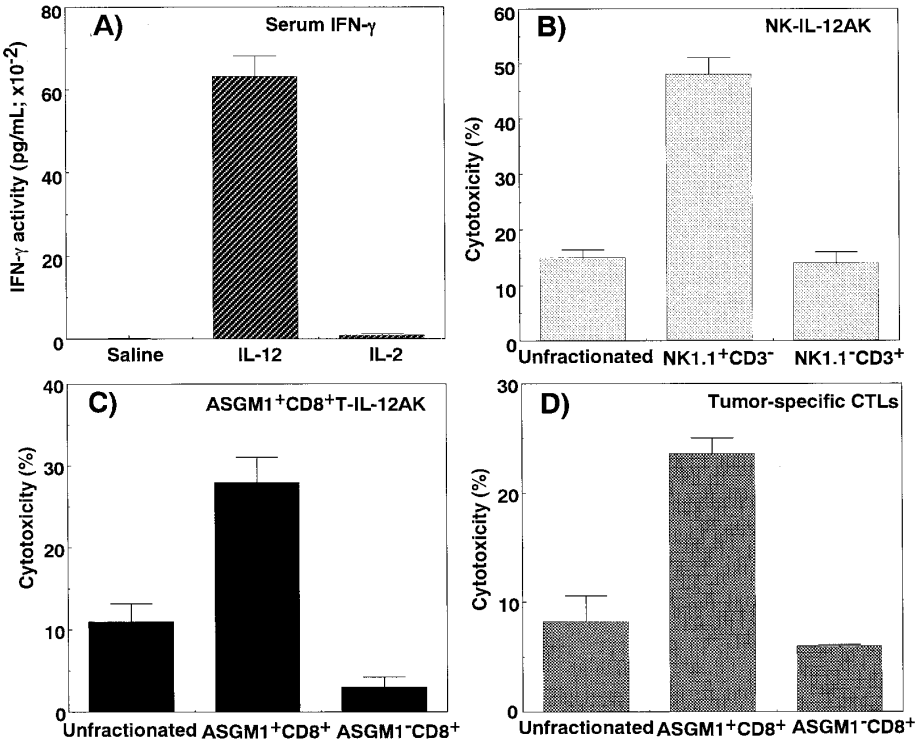

Fig. 1 **A** C57BL/6 mice received saline, IL-2, or IL-12 by intraperitoneal injection. After 24 h, serum IFN-γ activity was determined by ELISA (Endogen). **B** C57BL/6 mice were treated with IL-12 intraperitoneally. After 24 h, $NK1.1^+CD3^-$ NK cells and $NK1.1^-CD3^+$ T-cells were sorted by FACStar. Their cytotoxicity against an NK cell-resistant tumor, MBL-2, was then measured. **C** C57BL/6 mice were treated with IL-12 intraperitoneally. After 24 h, $CD8^+$ cells were separated into $ASGM1^+CD8^+$ T-cells and $ASGM1^-CD8^+$ T-cells. Their cytotoxicity against MBL-2 tumors was then determined. **D** C57BL/6 mice that had received 2 injections of MBL-2 cells were treated with 2 intraperitoneal injections of IL-12. At 1 day after the last IL-12 treatment, spleen cells were separated into $ASGM1^+CD8^+$ T-cells and $ASGM1^-CD8^+$ T-cells by FACStar and their cytotoxicity against MBL-2 tumors was determined

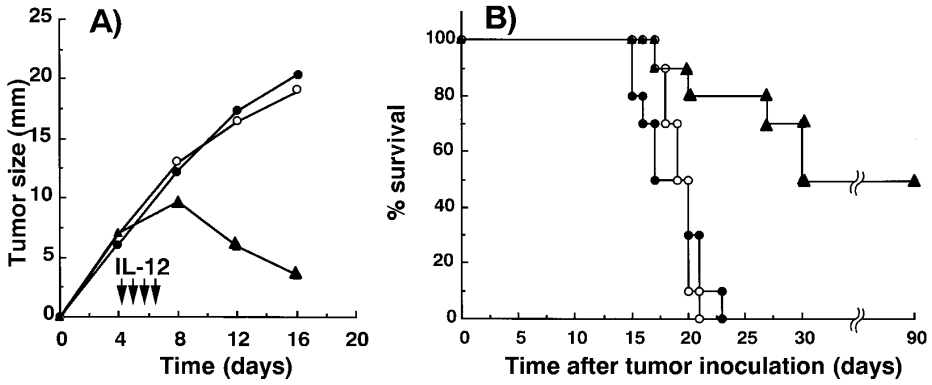

Fig. 2 A, B Tumor immunotherapy by systemic in vivo administration of IL-12. **A** C57BL/6 mice were inoculated intradermally with MBL-2 T-lymphoma cells (2×10^6 cells). When the inoculated tumor cells became palpable (day 4), the mice were treated with saline (*black circles*), IL-2 at 2000 U/mouse (*white circles*), or IL-12 at 2000 U/mouse (*black triangles*). In vivo tumor growth was then determined as described in Materials and methods. Tumor size was monitored by measuring the changes in the mean of 2 perpendicular diameters over time. **B** Survival curves generated for groups of 10 mice treated as described above. Mice cured of tumors within 90 days were regarded as completely cured. Data represent mean values ±SE

Tumor immunotherapy by systemic in vivo administration of IL-12

Systemic administration of IL-12 to mice with lymphomas caused by intradermal injection of MBL-2 lymphoma cells inhibited tumor growth, whereas IL-2 administration showed no significant in vivo antitumor activity (Fig. 2A). The effect of systemic administration of IL-12 on the survival rate of tumor-bearing mice was also examined (Fig. 2B). Mice treated with saline or IL-2 showed mean survival times of 18.5 ± 2.8 and 19.2 ± 1.2 days, respectively. However, intraperitoneal injection of IL-12 significantly prolonged the mean survival time to 24.4 ± 5 days for mice that were not cured; five of ten treated mice were completely cured.

To determine whether the disappearance of tumors following IL-12 treatment correlated with tumor-specific immunity, mice cured of MBL-2 lymphomas were rechallenged with an intradermal injection of MBL-2 cells or of unrelated B16 melanoma cells. As shown in Fig. 3A and 3B, mice cured of MBL-2 lymphomas by IL-12 treatment totally rejected the rechallenge with MBL-2 cells but not that with B16 melanoma cells. Moreover, nylon-passed splenic T-cells isolated from MBL-2 lymphoma-cured mice possessed potent tumor neutralization activity in an in vivo Winn assay (Fig. 3C).

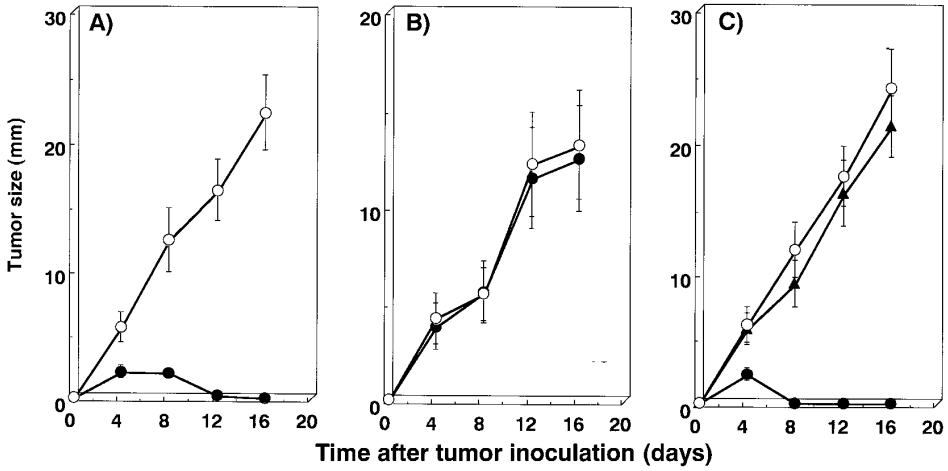

Fig. 3A–C Acquisition of systemic tumor-specific protective immunity by immunotherapy with IL-12. **A** MBL-2 T-lymphoma cells or **B** B16 melanoma cells were used to challenge untreated C57BL/6 mice (*white circles*) or C57BL/6 mice cured of MBL-2 tumors using IL-12 (*black circles*). Tumor growth was monitored as described in the legend to Fig. 1. Data represent mean values ±SE for 5 mice. **C** In vivo tumor-neutralization activity of nylon-passed spleen T-cells obtained from normal mice (*white circles*) or mice cured of MBL-2 tumors using IL-12 (*black circles*) was determined using the Winn assay as described in Materials and methods. As a control, tumor cells alone were injected into mice (*black triangles*). Data represent mean values ±SE for 5 mice

We next investigated mechanisms of in vivo induction of tumor-specific immunity following IL-12 treatment using various antibodies. As summarized in Table 1, in vivo administration of either anti-ASGM1 antibody, anti-CD4 MAb, or anti-CD8 MAb caused inhibition of IL-12-mediated antitumor activity. Such inhibition was not observed after administration of normal rabbit serum or rat Ig (data not shown). These results indicate that IL-12-mediated antitumor immunity is associated with the induction of both tumor-specific $CD8^+$ CTLs and a $CD4^+$ T-helper cell response. It was also suggested that IL-12-responsive $ASGM1^+$ cells ($ASGM1^+$ NK cells and $ASGM1^+CD8^+$ T-cells) may play an important role in the induction of tumor-specific immunity.

IL-12 inhibits the growth of both transplantable and primary tumor cells

In addition to MBL-2 T-lymphoma cells, we confirmed the systemic antitumor activity of IL-12 against Meth A sarcoma cells and B16 melanoma cells (Fig. 4A, B). Moreover, we also investigated the therapeutic effect of IL-12 against chemically induced primary tumors using c-Ha-*ras* transgeneic mice. As previously demonstrated [1], the majority of c-Ha-*ras* transgeneic mice form forestomach papillomas at 1 month after MNU treatment. In this experiment, transgeneic mice were treated with either saline or IL-12 starting at 30 days and ending at 60 days following MNU treatment. IL-12 at 2000 U/mouse was injected intraperitoneally four times a week. As shown in Fig. 4C, it was demonstrated that IL-12 inhibited the growth of primary tumor cells.

Table 1 Inhibition of IL-12-mediated in vivo antitumor activity by administration of antibodies against ASGM1, CD4, or CD8 antigen[a]

Time after tumor inoculation (days)[b]	Tumor size (mm)				
			IL-12 + antibody against[d]:		
	None	IL-12[c]	ASGM1	CD8	CD4
4	6.6±1.4	7.4±0.7	7.1±1.4	6.2±0.8	7.5±1.1
6	9.6±0.4	7.6±0.5	9.1±2.3	9.2±2.8	7.7±0.8
8	10.8±1.8	9.6±1.1	11.5±2.4	11.7±1.7	10.1±0.5
10	12.2±1.9	9.5±0.8	14.0±3.7	15.0±5.5	11.5±1.6
12	15.7±4.4	7.6±1.3	16.0±3.6	17.2±4.7	13.7±2.5
14	19.2±4.7	5.5±2.2	17.2±4.4	18.0±4.5	16.2±3.4
16	21.5±5.0	3.0±0.0	Died	20.5±5.5	19.0±3.9

[a] Data represent mean values ±SE of the measurements of 2 perpendicular diameters in 5 mice/group
[b] MBL-2 (2×10^6) cells were inoculated intradermally into the abdominal skin of C57BL/6 mice
[c] IL-12 at 2000 U/mouse was injected intraperitoneally into tumor-bearing mice on days 4, 5, 6, and 7 after tumor inoculation
[d] Anti-ASGM1 antibody, anti-CD4 MAb, or anti-CD8 MAb (500 mg/mouse) was injected intraperitoneally into tumor-bearing mice on days 0 and 1 before in vivo IL-12 administration

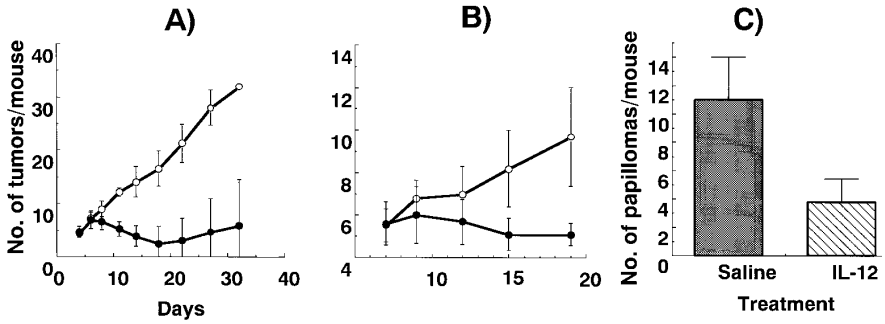

Fig. 4A–C Systemic antitumor activity of IL-12 against both transplantable and primary tumors. **A** BALB/c mice or **B** C57BL/6 mice were inoculated with 2×10^6 **A** Meth A fibrosarcoma cells or **B** B16 melanoma cells. After the tumor mass had become palpable, IL-12 at 2000 U/mouse (*black circles*) or saline (*white circles*) was injected intraperitoneally daily for 4 days. **C** c-Ha-*ras* transgeneic mice were treated intraperitoneally with MNU at 50 mg. At 1 month after MNU treatment, mice were treated with saline or IL-12 at 2000 U/mouse for 4 times a week for 4 weeks. At 2 months after MNU treatment, all the mice were killed and the numbers of forestomach papillomas were counted under stereoscopic microscopy. Average numbers of papillomas per mouse (7 mice/group) are indicated

Application of IL-12 to antitumor gene therapy

A20 B-lymphoma cells genetically engineered to secrete IL-12 were shown to lose their tumorigenicity and induce tumor-specific antitumor immunity in vivo (Fig. 5A, B). Mitomycin C-treated tumor cells transfected with the IL-12 gene and injected intraperitoneally into mice induced in vivo IFN-γ and IL-12AK cell production (Fig. 5C). Moreover, intradermal injection of IL-12 gene-carrying A20 cells into the skin of the left flank caused complete regression of parental A20 cells injected into the right flank of mice (Fig. 5D). These data suggest that tumor

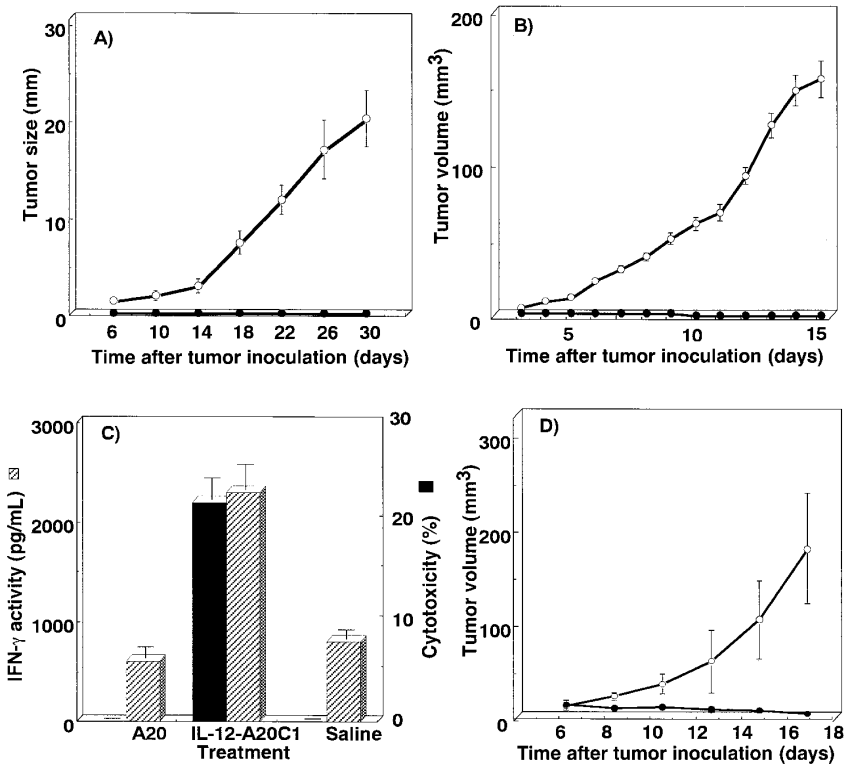

Fig. 5A–D Application of IL-12 to antitumor gene therapy. **A** BALB/c mice were inoculated with 2×10^6 A20 B-lymphoma cells transfected with both IL-12 p40 and p35 genes (IL-12-A20C1 cells, *black circles*) or with control A20 cells transfected with the IL-12 p40 gene (*white* circles). **B** BALB/c mice that had rejected IL-12-A20C1 cells were rechallenged with parental A20 cells (*black circles*). As a control, A20 cells were injected intradermally into untreated BALB/c mice (*white circles*). Thereafter, tumor growth was measured. **C** BALB/c mice were treated intraperitoneally with mitomycin C-treated parental A20 or IL-12-A20C1 cells or with saline. At 1 day after treatment, serum IFN-γ activity (*hatched bars*) and the cytotoxicity of NPSPLs against YAC-1 cells (*solid bars*) were measured. **D** To investigate the possibility that IL-12 transfectants could be used as a tumor vaccine, BALB/c mice were treated by intradermal injection of 2×10^6 parental A20 cells into the right-flank skin and were simultaneously treated by intradermal injection of 2×10^6 IL-12-A20C1 cells in the left-flank skin (*black circles*). As a control, BALB/c mice were inoculated intradermally with 2×10^6 A20 B lymphoma cells alone (*white circles*). The growth of parental A20 cells was monitored

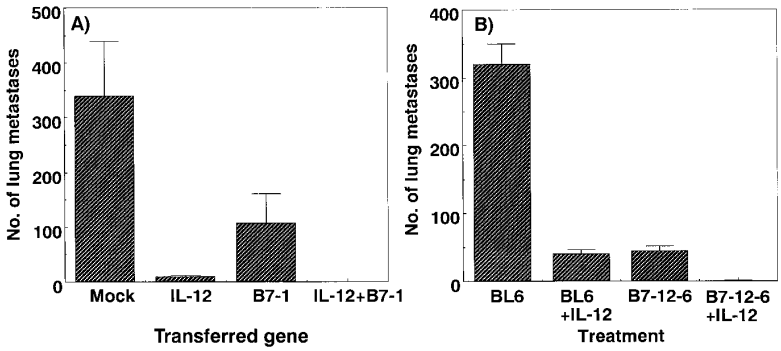

Fig. 6 **A** C57BL/6 mice were injected intravenously with 5×10^4 B16–BL-6 cells mock-transfected with adenovirus vector (*Mock*), B16–BL-6 cells transfected with IL-12 genes (*IL-12*), B16–BL-6 cells transfected with the B7-1 gene using an adenovirus vector (*B7-1*), or B16–BL-6 cells transfected with IL-12 genes and the B7-1 gene (*IL-12+B7-1*). At 3 weeks after tumor inoculation, the numbers of lung metastases were determined. **B** B16–BL-6 melanoma cells (*BL6*) or B16–BL-6 cells transfected with the B7-1 gene (*B7-12-6*; 2×10^4 cells) were injected intravenously into C57BL/6 mice, which were then treated with saline (BL6 and B7-12–6) or IL-12 at 1000 U/mouse (BL6+IL-12 and B7-12-6+IL-12) for 4 days. At 3 weeks after tumor inoculation, the mice were killed and the numbers of lung metastases were calculated. All data represent mean values ±SE for 5 mice

cells transfected with IL-12 genes have potential as a tumor vaccine in vivo.

It was also demonstrated that IL-12 gene therapy is effective for the inhibition of tumor metastasis. As shown in Fig. 6A, mice injected with B16–BL-6 melanoma cells transfected with IL-12 genes developed fewer lung metastases than those injected with parental B16–BL-6 cells. The transfer of the B7-1 gene into tumors was also effective in inhibiting lung metastasis. Cotransfection of the B7-1 and IL-12 genes was most effective in inhibiting tumor metastasis. Such synergistic therapeutic effects against lung metastasis were also observed after combination therapy consisting of systemic IL-12 administration and B7-1 gene transfer into tumors (Fig. 6B).

Discussion

The present data show that IL-12 has significant antitumor effects in vivo against both transplantable and primary tumors. It is noteworthy that mice cured of tumors by IL-12 treatment can reject rechallenge with the same tumor cells and acquire tumor-specific protective immunity.

One of the most important issues in tumor immunology is how to overcome immunological suppression in the tumor-bearing host [20] and induce tumor-specific immunity, which inhibits tumor recurrence or metastasis. Consistent with other reports [2, 12, 21], we have demonstrated that IL-12 overcomes immunosuppression and induces tumor-specific immunity in vivo. Although the detailed mechanisms underlying IL-12-induced antitumor activity remain unclear, the activation of Th1-dominant immunity by IL-12 appears to be important for the induction of tumor-specific cellular immunity. As shown in Fig. 1, IL-

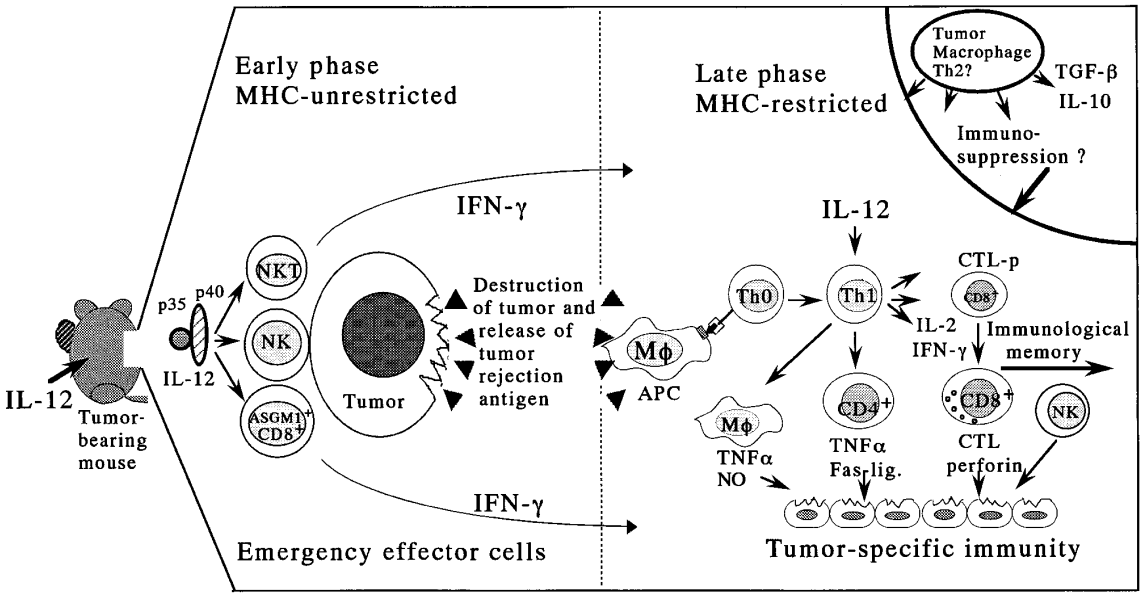

Fig. 7 Possible mechanisms underlying IL-12-induced antitumor activity in vivo

12 injection in vivo caused activation of IL-12AK cells, which consist of ASGM1$^+$NK1.1$^+$CD3$^-$ NK cells and ASGM1$^+$CD3$^+$CD8$^+$ T-cells. Both of these effector cells produce large amounts of IFN-γ in response to IL-12 [9]. The evidence that in vivo treatment with anti-ASGM1 antibodies caused abrogation of IL-12-induced in vivo antitumor activity strongly suggests that ASGM1$^+$ NK or ASGM1$^+$ CD8$^+$ T-cells may play an essential role in the generation of tumor-specific T-cell-mediated immunity in vivo. It is hypothesized that IL-12-responding emergency effector cells, such as NK cells, ASGM1$^+$CD8$^+$ T-cells, or NKT-cells [5], may produce IFN-γ and destroy tumor cells. The tumor antigen derived from the killed tumor may then be processed by antigen-presenting cells and stimulate Th1-dominant immunity, which facilitates the generation of tumor-specific CTLs (Fig. 7) [22, 23].

The overall goal of IL-12 therapy would be to reduce primary tumor growth or tumor metastasis. For antigenic tumors, IL-12 may show potent antitumor activity and induce a tumor-specific response. However, for nonimmunogenic or weakly immunogenic tumor cells, combination therapy with tumor antigen peptide [18, 23] or costimulatory molecules [10] may be required. As shown in Figs. 5 and 6, IL-12 gene transfer into tumors induced tumor-specific protective immunity in vivo. Moreover, combination therapy consisting of the IL-12 gene and the B7-1 gene has been demonstrated to be the most effective regimen for inhibition of lung metastasis by weakly immunogenic B16–BL-6 melanoma cells. The synergistic effect of the IL-12 gene and B7-1 gene appears to be derived from augmented induction of IFN-γ and enhanced generation of tumor-specific CTLs (data not shown).

These results suggest that IL-12 is a promising cytokine for antitumor cytokine and gene therapy. We are currently trying to develop an efficient method to induce human CTLs specific for autologous tumor cells using both the IL-12 and B7-1 genes.

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References

1. Ando K, Saitoh A, Hino O, Takahashi R, Kimura M, Katsuki M (1992) Chemically induced forestomach papillomas in transgeneic mice carry mutant human c-Ha-*ras* transgene. Cancer Res 52: 978
2. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med 178: 223
3. Fearon ER, Pardoll DM, Itaya T, Golombek P, Levitsky HI, Simins JW, Karasuyama H, Vogelstein B, Frost P (1990) Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell 60: 397
4. Hamaoka T, Fujiwara H (1987) Phenotypically and functionally distinct T-cell subsets in anti-tumor responses. Immunol Today 8: 267
5. Hashimoto W, Takeda K, Anzai R, Ogasawara K, Sakihara H, Sugiura K, Seki S, Kumagai K (1995) Cytotoxic NK1.1 Ag+ alpha beta T-cells with intermediate TCR induced in the liver of mice by IL-12. J Immunol 154: 4333
6. Karasuyama H, Melcher F (1988) Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vector. Eur J Immunol 18: 97
7. Kuge S, Miura Y, Nakamura Y, Mitomi T, Habu S, Nishimura T (1995) Superantigen-induced human CD4$^+$ helper/killer T-cell phenomenon: selective induction of Th1 type of helper/killer T-cells and application to tumor immunotherapy. J Immunol 154: 1777
8. Kuge S, Watanabe K, Makino K, Tokuda Y, Mitomi T, Kawamura N, Habu S, Nishimura T (1995) Interleukin-12 augments the generation of autologous tumor-reactive CD8+ cytotoxic T lymphocytes from tumor-infiltrating lymphocytes. Jpn J Cancer Res 86: 135
9. Lee U, Santa K, Habu S, Nishimura T (1996) Murine asialoGM1+CD8+ T-cells as novel interleukin-12-responding killer T-cell precursors. Jpn J Cancer Res (in press)
10. Murphy EE, Terres G, Macatonia SE, Hsieh C-S, Mattson J, Lanier L, Wysocka M, Trinchieri G, Murphy K, O'Garra A (1994) B7 and interleukin 12 cooperate for proliferation and interferon γ production by mouse T helper clones that are unresponsive to B7 costimulation. J Exp Med 180: 223
11. Nakamura Y, Wakimoto H, Abe J, Kanegae Y, Saito I, Aoyagi M, Hirakawa K, Hamada H (1994) Adoptive immunotherapy with murine tumor-specific T lymphocytes engineered to secrete interleukin 2. Cancer Res 54: 5757
12. Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Storkus WJ, Lotze MT (1994) Recombinant IL-12 administration induces tumor regression in association with IFN-γ production. J Immunol 153: 1697
13. Nishimura T, Togashi Y, Goto M, Yagi H, Uchiyama Y, Hashimoto Y (1986) Augmentation of the therapeutic efficacy of adoptive tumor immunotherapy by in vivo administration of slowly released recombinant interleukin 2. Cancer Immunol Immunother 21: 12
14. Nishimura T, Burakoff SJ, Herrmann SH (1987) Protein kinase C required for cytotoxic T lymphocyte triggering. J Immunol 130: 2888
15. Nishimura T, Uchiyama Y, Hashimoto Y (1988) In vivo generation of lymphokine-activated killer cells by sensitization with interleukin 2-producing syngeneic T-lymphoma cells. Cell Immunol 112: 220
16. Nishimura T, Nakamura Y, Takeuchi Y, Tokuda Y, Iwasawa M, Kawasaki A, Okumura K, Habu S (1992) Generation, propagation, and targeting of human CD4$^+$ helper/killer T-cells induced by anti-CD3 monoclonal antibody plus recombinant IL-2: an efficient strategy for adoptive tumor immunotherapy. J Immunol 148: 285
17. Nishimura T, Nakamura Y, Tsukamoto H, Takeuchi Y, Tokuda Y, Iwasawa M, Yamamoto T, Masuko T, Hashimoto Y, Habu S (1992) Human c-*erbB*-2 proto-oncogene product as a target for bispecific-antibody-directed adoptive tumor immunotherapy. Int J Cancer 50: 800
18. Noguchi Y, Richards EC, Chen Y-T, Old LJ (1995) Influence of interleukin 12 on p53 peptide vaccination against established Meth A sarcoma. Proc Natl Acad Sci USA 92: 2219
19. Saito I, Oya Y, Yamamoto K, Yuasa T, Shimojo H (1985) Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. J Virol 54: 711
20. Tada T, Ozeki S, Utsumi K, Takiuchi H, Muramatsu M, Li X-F, Shimizu J, Fujiwara H, Hamaoka T (1991) Transforming growth factor-β-induced inhibition of T-cell function: susceptibility dif-

ference in T-cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. J Immunol 146: 1077

21. Tahara H, Zer HJ III, Storkus WJ, Pappo I, Watkins SC, Gubler U, Walf SF, Robbins PD, Lotze MT (1994) Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. Cancer Res 54: 182
22. Trinchieri G (1995) Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 84: 4008
23. Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plean E, Van Den Eynde B, Knuth A, Boon T (1991) A gene coding an antigen recognized by cytotoxic T lymphocytes on a human melanoma. Science 254: 1643