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Synergistic antitumor effects of interleukin-12 gene transfer and systemic administration of interleukin-18 in a mouse bladder cancer model

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Abstract We introduced the interleukin-12 (IL-12) gene into the mouse bladder cancer cell line (MBT2) to establish sublines that secrete bioactive IL-12. IL-12-secreting MBT2 (MBT2/IL-12) sublines were completely rejected when subcutaneously implanted into immunocompetent syngeneic C3H mice. Although this antitumor effect did not change when IL-12-secreting cells were injected into immunodeficient mice whose $CD8^+$ T or $CD4^+$ T cells had been depleted by the corresponding antibody, it was abrogated when natural killer cells were depleted by anti-asialoGM1 antibody. In addition, when parental MBT2 cells mixed with MBT2/IL-12 cells were subcutaneously injected into mice, admixed MBT2/IL-12 inhibited the growth of the parental tumor. Furthermore, this antitumor effect was enhanced by systemic IL-18 administration. This synergism was abrogated when the mice were treated with interferon-γ-neutralizing antibody in vivo. In conclusion, local secretion of IL-12 led to effective antitumor activity that was enhanced by systemic administration of IL-18. Interferon-γ plays an important role in the synergism of IL-12 gene transduction and systemic administration of IL-18.

Key words Bladder cancer · Interleukin-12 · Interleukin-18

Introduction

Interleukin-12 (IL-12), a bimolecular glycoprotein consisting of a 35- and 40-kDa subunit, was originally identified as a factor that stimulates natural killer cells (NK cells) and promotes maturation of cytotoxic T lymphocytes (CTL) [7, 8, 15, 16, 18]. This is the first cytokine to eradicate the established tumor by itself [2]. However, adverse effects turned out to be the main hindrance to the clinical use of IL-12, since a huge amount of IL-12 must be administered to maintain an effective concentration at the tumor site [4]. Thus, the introduction of an IL-12 gene into the tumor cells is one way to avoid severe adverse effects. We introduced the IL-12 gene into the mouse bladder cancer cell line (MBT2) and investigated the mechanism of tumor rejection by locally secreted IL-12, using immunodeficient mice treated with various kinds of antibodies. We also investigated whether IL-12-secreting MBT2 could inhibit the tumor growth of parental MBT2 cells.

Interleukin-18 (IL-18), previously called interferon γ (IFNγ)-inducing factor, is an 18.3-kDa cytokine produced by mature macrophages and related cells [12]. IL-18 augments NK cell activity and promotes the proliferation of stimulated T cells in the presence of concanavalin A, anti-CD3 mAb or IL-2 [13]. Although the function of IL-18 seems to be similar to that of IL-12, the mechanism of activation is different. Kohno et al. showed that IL-18 could augment IFNγ production, proliferation and IL-2 receptor α-chain expression of the Th1 clones even in the presence of saturating amounts of IL-12 [9]. With respect to the antitumor activity, IL-18 and IL-12 may have synergistic effects. In this study, we investigated whether systemic administration of IL-18 could enhance the antitumor effect of IL-12-secreting cells against parental MBT2.

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Materials and methods

Tumor cell lines

MBT2, a mouse bladder carcinoma of C3H origin, was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Expression plasmid and transfection to tumor cells

The expression plasmid used in this study was previously described [14]. The cDNA of the p35 and p40 subunits of mouse IL-12 were kindly provided by Dr. Maurice K.Gately (Hoffmann-La Roche, Nutley, N. J.) and were subcloned in the proper reading frame and in the appropriate orientation into the plasmid expression vector pCEXV3 [14]. DNA fragments encoding the p35 and p40 subunits were cloned into the *Sma*I site of pCEXV3 and named pCEXV3/p35 and pCEXV3/p40. In pCEXV3/p35 and pCEXV3/p40, standard calcium-phosphate-mediated transfection was performed to introduce the gene into the cell line. For drug selection, pSV2neo was co-transfected. Briefly, 1.8×10^5 MBT2 cells were plated in a 6-cm dish 1 day before transfection. A 9-μg sample of pCEXV3 (as a control) or 7 μg pCEXV3/p35 and 7 μg pCEXV3/p40 were co-transfected with 1 μg pSV2neo by the calcium phosphate method [17]. The concentration of gentamicin (Geneticin; Sigma, St. Louis, Mo.) was 0.25 mg/ml. After 2 weeks of drug selection, colonies were picked up with cloning cylinders and expanded to cell lines.

IL-12 assays

Parental or IL-12-gene-transfected MBT2 cells (3×10^5) were plated in six-well plates (9.6 cm^2/well) with 1.5 ml medium, and supernatants were collected 24 h later. IL-12 activity was measured by a lymphoblast proliferation assay, as previously described [14]. Briefly, human peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation, incubated with phytohemagglutinin (PHA) to a final concentration of 10 μg/ml for 48 h and subsequently incubated with human recombinant IL-2 to a final concentration of 50 U/ml for 24 h. In a 96-well plate, 50 μl PHA-activated human lymphoblast (2×10^4 cells) suspension was incubated with 50 μl sample supernatants or recombinant mouse IL-12 reference standard for 24 h. Then the cells were pulsed with 1 μCi [H^3] thymidine and cultured for 6 h. IL-12 activity of the sample supernatants was calculated by measuring [H^3] thymidine incorporation. An IL-12 enzyme-linked immunosorbent assay (Bio Source International Inc., Carmarillo, Calif.) was also performed to confirm the results.

Animal studies

C3H/He mice (female, 6–8 weeks old) and athymic nude mice (Balb/c nu/nu, female, 6–8 weeks old) were purchased from CLEA Japan Inc. (Tokyo). In all animal experiments for tumor growth measurement, each group consisted of five mice. Tumor cells were trypsinized and washed twice with phosphate-buffered saline (PBS) and cells were injected subcutaneously into the flank of each mouse. The growth of subcutaneous tumor was measured in millimeters, using a caliper. The longest surface length (*a*) and the perpendicular width (*b*) were measured, and tumor size was reported as the product of $a \times b$. Tumor growth was checked at least twice per week. To determine the metastatic potential, 5×10^5 cells of each subline were injected intravenously via the tail vein. Two weeks later, mice were sacrificed and the number of surface metastatic nodules in the lung was counted under a dissecting microscope.

All animal experiments were conducted according to the Guidelines for animal experimentation at Kobe University School of Medicine.

Antibody treatments in vivo

Depletion of T cells in vivo was accomplished by intraperitoneal administration of rat monoclonal antibody (mAb) GK1.5 (anti-CD4; IgG2b) or mAb 2.43 (anti-CD8; IgG2b) (both hybridomas were acquired from the ATCC). These mAb were used as ascites fluids (titer > 1:10 000 by staining mouse thymocytes by flow cytometry). These ascites fluids (0.2 ml) were injected i.p. on day −3 and every 7 days thereafter. For depletion of NK cells, 50 μl anti-asialoGM1 antiserum (Wako Fine Chemicals, Osaka, Japan) diluted to 200 μl in PBS was injected intraperitoneally on day −1 and this was repeated every 5 days. To neutralize IFNγ activity in vivo, the ascites fluid (0.2 ml) of the R4-6A2 (purchased from the ATCC) rat mAb against mouse IFNγ was injected i.p. on days −1, 0, 1 and repeated every 7 days.

IL-18 treatment in vivo

Recombinant murine interleukin-18 (IL-18) was a product of Hayashibara Biochemical Laboratories Inc. (Okayama, Japan) and was obtained by expressing murine IL-18 cDNA in *Escherichia coli* and purified by chromatographic methods [12]. A 1-μg sample of IL-18 diluted to 0.2 ml in PBS was injected intraperitoneally on day −1 and this was repeated every 3 days.

Results

In vitro studies of MBT2 sublines

The mouse bladder cancer cell line, MBT2, was co-transfected with pCEXV3/p35, pCEXV3/p40 and pSV2neo. After G418 selection, clones were picked up and the IL-12 activities of their culture supernatants were measured. Clones with high IL-12 secretion, MBT2/IL-12_1 and MBT2/IL-12_2, secreted approximately 500 pg/ml IL-$12/10^6$ cells in 24 h. Neither parental MBT2 cells nor MBT2 cells transfected with pCEXV3 (MBT2/Con) produced detectable amounts of IL-12. There was no significant difference in cell proliferation In vitro among parental and transfected MBT2 cells (data not shown).

IL-12 secretion by MBT2 inhibits tumor growth in vivo

To examine the effect of IL-12 secretion on tumor growth, 10^6 cells of MBT2/IL-12_1, MBT2/IL-12_2, MBT2/Con or parental MBT2 were injected into the right flank of syngeneic C3H mice. In each experiment, parental MBT2 appeared in most mice 10–15 days after tumor inoculation and grew quickly. MBT2/Con exhibited almost the same degree of tumor growth. On the other hand, in mice injected with clones transduced with the IL-12 gene MBT2, tumor did not grow; that is, it was completely rejected (Fig. 1). When the cells were injected intravenously, parental MBT2 or MBT2/Con made numerous metastatic nodules in the lung. By contrast, MBT2/IL-12_1 and MBT2/IL-12_2 made few metastatic nodules (Table 1).

Depletion of potential effector cells

To investigate the type of effector cells participating in rejection of MBT2 tumors with in situ IL-12 secretion, tumor-bearing hosts were depleted in vivo with selected effector cell populations. Depletion of $CD8^+$ T and $CD4^+$ T cells did not significantly influence tumor rejection (Fig. 2A, B). However, IL-12-secreting tumors

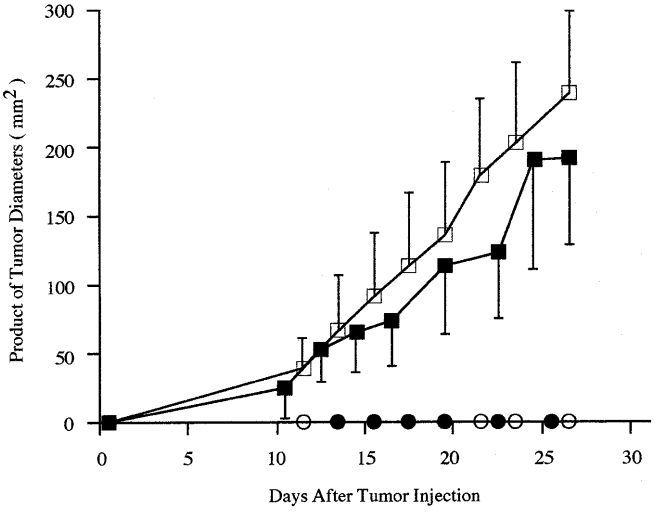

Fig. 1 Tumor growth in C3H mice of parental MBT2 (■ MBT2), control-vector-transfected MBT2 (□ MBT2/Con), or clones of MBT2 transfected with interleukin-12 (IL-12) cDNA (● MBT2/IL-12_1, ○ MBT2/IL-12_2). Mice were subcutaneously injected with 10^6 cells in the right flank on day 0. Tumor growth was measured as the product ($a \times b$) of the maximal diameter (a) and the perpendicular diameter (b). *Bars* standard deviations of tumor size with five mice per group

Table 1 Production of metastases by MBT2 sublines injected into the tail vein of mice. Production of lung metastasis by the MBT2 parental cell line, MBT2 transfected with interleukin-12 (*MBT2/IL-12_1*) and MBT2/IL-12_2 injected into the tail vein of mice. Cells (5×10^5) were injected, and the mice were killed 2 weeks after injection. We counted the number of surface metastatic nodules in the lung

Cells	No. of lung metastases
MBT2/P	246.7 ± 83.3*
MBT2/Con	281.7 ± 95.1
MBT2/IL-12_1	1.0 ± 2.2*
MBT2/IL-12_2	1.4 ± 2.6

* The mean number of metastases produced by MBT2 transfected with IL-12 was significantly lower than that produced by parental MBT2. $P < 0.001$ (Student's t-test)

grew faster in NK-cell-depleted mice than in immunocompetent mice, although the tumor did not grow as fast as parental MBT2 cells (Fig. 2C). This showed that NK cells partially participate in the antitumor effect of MBT2/IL-12.

Antitumor effect of MBT2/IL-12 for parental MBT2 and the synergistic effect with IL-18

Parental MBT2 (10^5 cells) mixed with 10^6 MBT2/IL-12_1 were injected subcutaneously. Admixed MBT2/IL-12 cells inhibited the tumor growth of 10^5 parental MBT2 cells (Fig. 3■, □). Moreover, this antitumor effect was enhanced by systemic administration of IL-18 (Fig. 3□, ○), although IL-18 alone did not affect the tumor growth of parental MBT2 (Fig. 3■, ●).

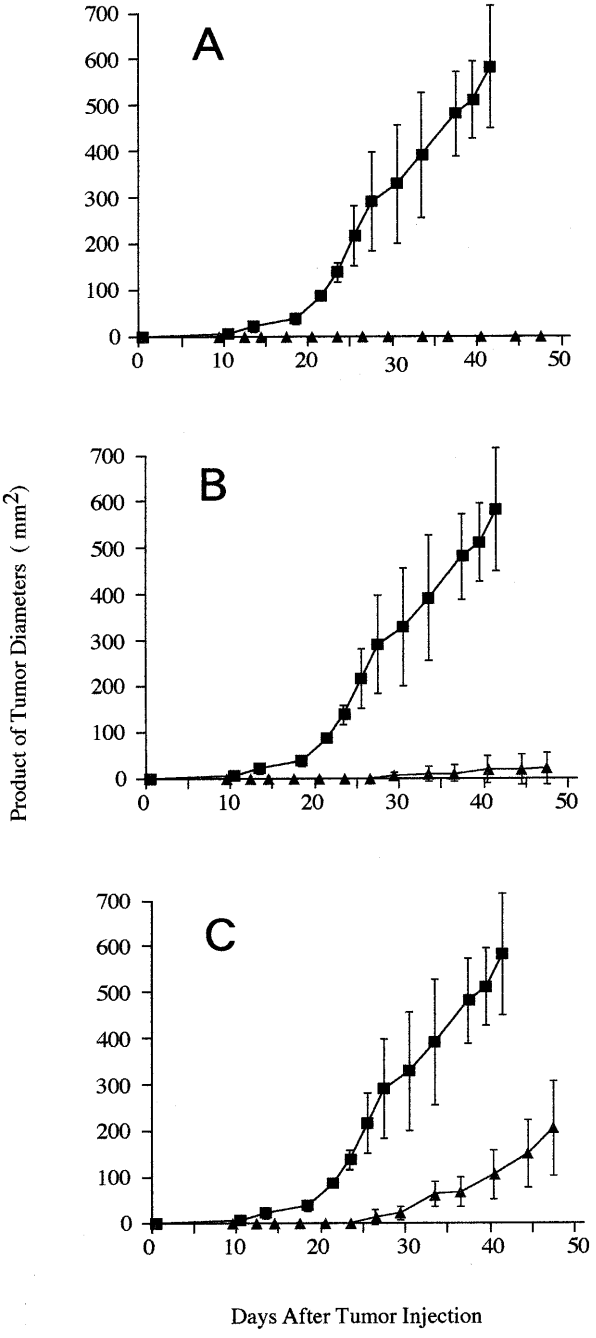

Fig. 2A–C Tumor growth in immunodeficient C3H mice of parental MBT2 (■ MBT2) and MBT2 transfected with IL-12 cDNA (▲ MBT2/IL-12_1). Mice were depleted of potential effector cell populations by mAb against CD4, CD8 and natural killer (NK) antigens, as described in Materials and methods. **A** C3H mice depleted of $CD8^+$ T cells. **B** C3H mice depleted of $CD4^+$ T cells. **C** C3H mice depleted of NK cells. Tumor measurements were performed as described in the legend to Fig. 1

Neutralization of IFNγ partially abrogates the antitumor effect of MBT2/IL-12 for parental MBT2 and the synergistic effect with IL-18

To examine the role of IFNγ in the antitumor effects of MBT2/IL-12 and the synergism with IL-18, IFNγ-

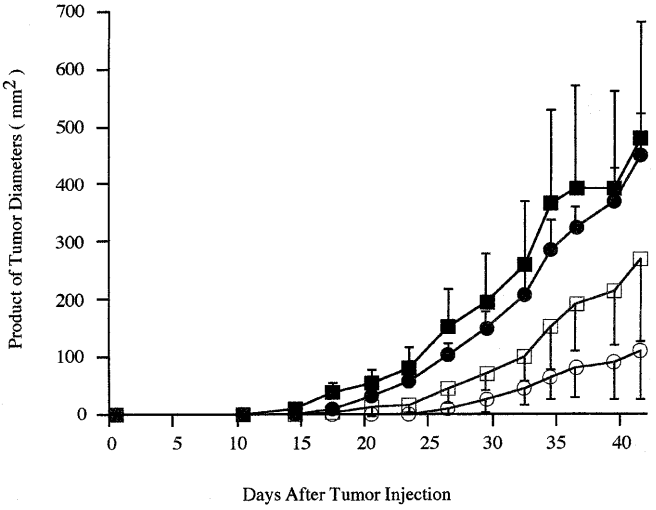

Fig. 3 Tumor growth of parental MBT2 mixed with or without MBT2/IL-12. The effect of systemic administration of IL-18 was also studied. Tumor growth of 10^5 parental MBT2 cells (■), 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 (□), 10^5 parental MBT2 cells with IL-18 (●) or 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 treated with IL-18 (○) in C3H mice. Mice were subcutaneously injected with 10^5 parental MBT2 cells or 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 in the right flank on day 0. For IL-18 treatment, 1 μg IL-18 diluted with 0.2 ml phosphate-buffered saline (PBS) was injected intraperitoneally on day−1 and this was repeated every 3 days. Tumor measurements were performed as described in the legend to Fig. 1

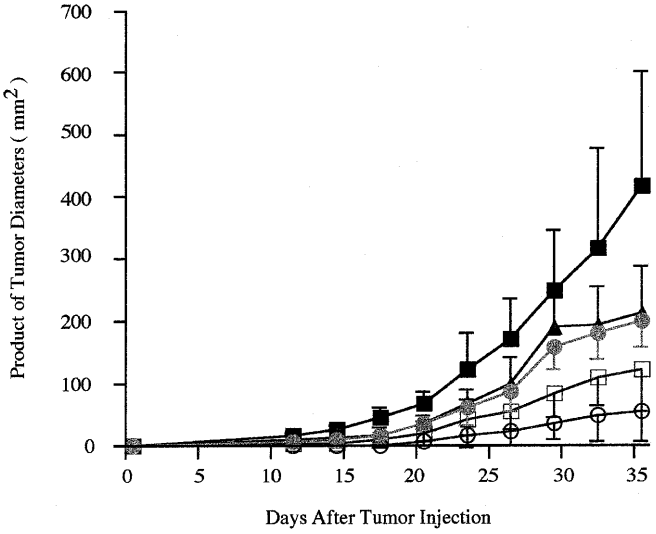

Fig. 4 Tumor growth of 10^5 parental MBT2 cells (■), 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 (□), 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 treated with IL-18 (○) in C3H mice and tumor growth in IFNγ-neutralizing mice of 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 (▲), 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 treated with IL-18 (●). Mice were subcutaneously injected with 10^5 parental MBT2 cells or 10^5 parental MBT2 cells mixed with 10^6 MBT2/IL-12 in the right flank on day 0. For IL-18 treatment, 1 μg IL-18 diluted with 0.2 ml PBS was injected intraperitoneally on day −1 and this was repeated every 3 days. IFNγ-specific neutralizing Ab, as described in Materials and methods, were administered. Tumor measurements were performed as described in the legend to Fig. 1

specific neutralizing Ab was administered. As shown in Fig. 3, admixed 10^6 MBT2/IL-12_1 inhibited the tumor growth of 10^5 parental MBT2 cells, and this antitumor effect was enhanced by systemic administration of IL-18 (Fig. 4■, □, ○). When IFNγ activity was inactivated in vivo, the antitumor effect of MBT2/IL-12_1 was partially inhibited and additional IL-18 administration did not affect the tumor growth of MBT2/IL-12 (Fig. 4●, ▲). This means that INFγ production is one of the mechanisms of the antitumor effect due to in situ secretion of IL-12, and that the synergistic antitumor effect with IL-18 mainly depends on IFNγ production.

Discussion

Recent studies have shown that cytokine-gene-modified tumor cells are capable of immunizing mice against parental tumor and many researchers have studied the antitumor effect of cytokine gene transfer to cancer cells. Transfer of cytokine genes into tumor cells more closely approximates the physiological mode of cytokine secretion. IL-12 exerts a variety of biological effects on T and NK cells in vitro and demonstrates potent antitumor effects when injected systemically [2, 6, 7, 15, 16]. IL-12 acts as a growth factor for NK cell and T cells, enhances NK/LAK cell cytolytic activity, augments cytolytic T cell responses, induces secretion of cytokines and particularly acts as the most potent inducer of IFNγ production by T and NK cells [3]. This cytokine consists of a disulfide-linked 35-kDa light chain (p35) and 40-kDa heavy chain (p40). Simultaneous transfection with the expression-vector-inserted cDNA fragment encoding p35 and p40 is thus necessary for production of biologically active IL-12. We introduced the IL-12 gene into the mouse bladder cancer cell line and IL-12-gene-transduced cells secreted bioactive IL-12.

In the present study, we demonstrated that introduction of the IL-12 gene into MBT2 inhibited tumor growth in mice, leading to complete rejection. This effect was observed not only after subcutaneous but also after intravenous injection. With respect to the mechanism of this antitumor effect, we showed that in vivo depletion of NK cells could partially abrogate the antitumor effect, whereas depletion of $CD8^+$ T cells or $CD4^+$ T cells did not change the antitumor effect. This suggested that NK cells play an important role in the antitumor effect of locally secreted IL-12. Brunda et al. demonstrated that, in RenCa renal cell carcinoma, the antitumor effect of exogenous IL-12 mainly involved $CD8^+$ T cells [2]. Tahara et al. suggested that NK cells play a role in the antitumor effects of IL-12-gene-transduced cells, principally during the early phase of antitumor reactivity, and T cells play a major role in the later stage, finally resulting in complete eradication [9]. Although it is not clear why there was a difference between these models, it might be attributable to differences in cell lines. The

immunogenicity and tumorigenicity of each cell line differs and such differences can result in different mechanisms of antitumor effect.

We also showed that MBT2/IL-12 mixed with parental MBT2 could inhibit the tumor growth of parental MBT2. However, MBT2/IL-12 injected into the left flank of the mice did not inhibit the tumor growth of parental MBT2 injected into the right flank (data not shown). This suggested that the injection of IL-12-producing cells did not lead to the acquisition of a systemic antitumor immunoresponse, but effector cells activated by locally secreted IL-12 effectively inhibited tumor growth of parental MBT2.

Since IL-12 is a strong inducer of IFNγ, we studied the influence of IFNγ on the antitumor effect using neutralizing antibody. When IFNγ was inactivated in vivo, the antitumor effect of MBT2/IL-12 was partially abrogated. This means that IFNγ participates in the antitumor effect to some extent.

IL-18 augments NK cell activity and IFNγ production by T cells and NK cells and enhances the Fas-ligand-mediated cytotoxicity of Th1 cells [5, 12]. IL-18 also has an antitumor effect in vivo [11]. These bioactivities were similar to those of IL-12. However, the mechanism of their action is thought to be different and they have a synergistic effect with respect to IFNγ production [9, 10, 12]. Even in the presence of saturating amounts of IL-12, IL-18 still augments IFNγ production and proliferation [9]. One of the mechanisms for the synergism is reported to be up-regulation of the IFNγ-inducing factor receptor by IL-12 [1]. In the present study, we showed that systemic administration of IL-18 enhanced the antitumor effect of IL-12-secreting cancer cells. Since systemic administration of IL-18 alone did not affect the tumor growth of parental MBT2, the synergistic antitumor effect of IL-18 is observed only when there is in situ secretion of IL-12. We also showed that the synergistic antitumor effect was totally abrogated by inactivation of IFNγ in vivo. We therefore assume that the synergistic antitumor effect of IL-12 and IL-18 is mediated mainly through synergistic IFNγ production.

As a result, we showed that local secretion of IL-12 could lead to effective antitumor activity that was enhanced by systemic administration of IL-18. Such a synergism was mainly related to the synergism of IFNγ induction. For the future clinical use of IL-12, IL-18 may play a critical role in enhancing the antitumor effect of IL-12 and may reduce the adverse effects by diminishing the required dosage of IL-12.

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