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Synergistic Suppressive Effect of Double Transfection of *Tumor Necrosis Factor-α* and *Interleukin 12* Genes on Tumorigenicity of Meth-A Cells

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Tumor necrosis factor-α (TNF-α) and interleukin 12 (IL-12), both potent antitumor cytokines, are known to be involved in the host's antitumor immune surveillance in tumor bearers, via different mechanisms. The former enhances the activities of dendritic cells, natural killer/lymphocyte-activated killer (NK/LAK) and cytotoxic T lymphocyte (CTL), while the latter induces Th1-type cellular immunity and enhances the activities of natural killer T (NKT), NK/LAK and CTL. In the present study, in the expectation of synergistic actions of these cytokines in stimulating the host's immune responses, we investigated the feasibility of a cancer vaccine involving double transfection with both genes in a murine model. The expression of major histocompatibility complex (MHC) class I, class II and B7.1 on the surface of the double transfectants was enhanced as revealed by FACS analysis. A significant decrease in tumorigenicity was observed in mice inoculated with the double transfectants. Cytotoxicity assay revealed that the activities of NK/LAK and CTL from spleens of mice bearing the double transfectants were enhanced. The induction of tumor-specific immunity was confirmed by rechallenge with parental Meth-A cells in mice that had rejected the double transfectants. Thus, double transfection of *TNF-α* and *IL-12* genes was considered to bring about synergistic suppressive effects on the tumorigenicity of transfectants through the activation of killer cells by produced cytokines and the enhancement of expression of MHC class I, II and B7.1 molecules.

Key words: TNF-α — IL-12 — MHC class I — B7.1 — Cancer gene therapy

Tumors grown *in vivo* are usually endowed with the ability to escape the host's immunological surveillance via various mechanisms. First, tumor cells produce immunosuppressive factors by which they inhibit each process involved in the induction of antitumor cell-mediated immunity. For example, transforming growth factor-β (TGF-β) inhibits the differentiation and proliferation of cytotoxic T lymphocytes (CTL) and the production of interleukin 2 (IL-2) and IL-12.[1–3] IL-10 inhibits cytokine production by Th1 cells and the antigen presentation capacity of monocytes via downregulation of major histocompatibility complex (MHC) class II expression.[4–6] Prostaglandin E2 (PGE2) inhibits IL-2 production, IL-2 responses and MHC class II expression.[7,8] Some tumor cells suppress the differentiation of dendritic cells, potent antigen-presenting cells, by secreting vascular endothelial growth factor (VEGF).[9]

Second, tumors are frequently defective in presenting immunogenicity, having low expression of MHC class I, class II or costimulatory molecules such as B7. Therefore, it is suggested that they cause decreased recognition of antigens by T cells and the induction of peripheral tolerance.[10] Furthermore, it is reported that abnormality of the ζ chain which constitutes T cell receptor (TCR) complex causes dysfunction of effector T cells themselves in a tumor-bearing host.[10] Accordingly, a method of enhancing the immunogenicity of tumor cells or stimulating the host's antitumor immunity will be required to overcome the escape of tumor cells from the host's immunological surveillance.

Tumor necrosis factor-α (TNF-α) and IL-12, which are both potent antitumor cytokines, are known to be involved in antitumor immunological surveillance of the host via different mechanisms. TNF-α possesses such immunostimulatory activities as enhancement of macrophage, natural killer/lymphokine-activated killer (NK/LAK) and CTL activities[11,12] and the antigen-presenting capacity of

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dendritic cells.[13] We previously reported that Meth-A cells transfected with *TNF* gene showed reduced tumorigenicity and exhibited therapeutic vaccination effects against pre-existing tumors.[14] We have also found that an important aspect of the mechanisms behind these antitumor effects is augmentation of the cytotoxic effects of such killer cells as CTL and LAK and enhancement of MHC class I expression on tumor cells. Moreover, we demonstrated that *TNF* gene transfection of tumor cells freshly isolated from the ascitic fluids of patients with gastrointestinal cancers enhanced the expression of MHC class I and/or intercellular adhesion molecule-I (ICAM I), and we suggested that this would be involved in augmentation of the killer activity of tumor-infiltrating lymphocytes (TIL) induced by coculturing with autologous tumor cells transfected with *TNF* gene.[15] On the other hand, IL-12 is a cytokine which potently induces Th1-type cell-mediated immunity[16] and enhances the activities of natural killer T (NKT),[17] NK/LAK[18] and CTL.[19] Through these immunostimulatory actions, IL-12 is reported to exert potent antitumor and antimetastatic effects via systemic administration[20, 21] or upon local production induced by cancer vaccines.[22]

In the present study, we investigated the feasibility of cancer vaccines using Meth-A cells double-transfected with *TNF-α* and *IL-12* genes in a murine model, in the expectation that synergistic or supplementary actions of both cytokines would broadly stimulate the host's immune network.

MATERIALS AND METHODS

Cell lines and culture The 3-methyl-cholanthrene-induced murine fibrosarcoma cell line, Meth-A (M0), murine colon adenocarcinoma cell line, colon 26 and murine lymphoma cell line, YAC-1, were obtained from the American Type Culture Collection (ATCC). These cell lines were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, North Ryde, Australia), 100 U/ml penicillin and 100 mg/ml streptomycin, and maintained at 37°C under 5% CO_2. Each transfectant derived from M0 (see below) was also cultured in the same way.

Animals Female BALB/c mice were purchased from Charles River Japan (Tokyo) and fed in a specific pathogen-free environment. Only mice older than 8 weeks were used in this experiment.

***TNF* gene transfection** The human TNF expression vector (pLJ-TNF) was constructed by inserting human TNF cDNA derived from pcDV-TNF[23] into a unique *Bam*HI site of the retroviral vector pLJ.[24] Cell lines that produce modified viruses had been previously established.[14] Meth-A cells (M0) were incubated with a viral solution of virus-producing clones (pLJ-TNF introduced) in the presence of 8 μg/ml of polybrene for 2 h at 37°C. *TNF* gene transfectants (M-TNF) were selected by incubation with 400 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) for 14 days. *LacZ* gene-transfected cells were produced as mock transfectants using the same procedures.

***IL-12* gene transfection** The retroviral vector TFG-mIL-12-neo was generously provided by Dr. Tahara (University of Pittsburgh).[22] This vector carries genes for murine IL-12 and neomycin linked with IRES (internal ribosome entry site) sequences from the encephalo-myocarditis virus, allowing expression of a single polycistronic transcript. Using this retroviral vector, murine *IL-12* gene was transfected into M0 (M-IL-12) and M-TNF (M-TNF/IL-12) as described earlier.

Cytokine assay IL-12 concentration in the culture medium of *IL-12* gene-transfected tumor cells was measured with a mouse IL-12 immunoassay kit (Biosource, Camarillo, CA). TNF concentration in the culture medium of *TNF* gene-transfected tumor cells was measured with a TNF ELISA kit provided by Asahi Chemical Industry (Tokyo).

Flow cytometric analysis for MHC class I, class II and B7.1 molecules on tumor cells M0 and its transfectants (5×10^5 cells) were incubated for 30 min at 4°C with FITC-conjugated-anti-class I monoclonal antibody (H-$2K^d$, Meiji Nyugyo, Tokyo), FITC-conjugated-anti-class II monoclonal antibody (I-A, Seikagaku, Tokyo) or FITC-conjugated-anti-B7.1 monoclonal antibody (Sumitomo Denko, Yokohama). The cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Tumor inoculation To examine the tumorigenicity of transfectants, BALB/c mice were s.c. inoculated with 1×10^5 cells of either parental Meth-A (M0) or transfectants (M-TNF, M-IL-12, and M-TNF/IL-12) into the right flank and mice were monitored for tumor growth (each group consisted of 7 mice). Another group of mice was administered i.p. with 100 μg of anti-CD8 monoclonal antibody (Lyt 2.2, 2.43 hybridoma, ATCC) before s.c inoculation of double transfectants. To examine the immunological specificity of vaccination using M-TNF/IL-12, 1×10^6 cells of M0 or colon 26 were rechallenged s.c. at the left flank of mice two weeks after the complete regression of previously inoculated M-TNF/IL-12 (1×10^6 cells). The tumor size was calculated with the following formula: Tumor size (mm) = $(a + b)/2$, where a is the larger and b is the smaller diameter.

MLTC (mixed lymphocyte tumor cell culture) Tumor cells (1×10^6) were inoculated into BALB/c mice as described above. One week later, the spleen was removed aseptically and splenocytes were harvested. Splenocytes (5×10^6) were cocultured with M0 cells (1×10^5) pretreated for 1 h with mitomycin C (MMC) (100 μg/ml) in 2 ml of RPMI 1640 medium, in a 24-well plate, followed by addition of 100 JRU/ml IL-2 on day 2 of culture. Five days

after incubation, stimulated splenocytes were collected for evaluation of their killing activity.

Cytotoxicity assay M0 and YAC-1 cells (1×10^5) radiolabeled with ^{51}Cr were incubated with killer cells obtained from MLTC in various ratios in a 96-well plate. After incubation for 4 h, the plate was centrifuged and the radioactivity in the supernatant was counted using a γ-counter. Percent specific lysis was calculated as follows: % specific lysis=(mean cpm from test supernatants−mean cpm from supernatants of target cells alone (spontaneous release))/(mean cpm after target cell lysis with lysis buffer (total release)−spontaneous release)×100.

A blocking experiment was also conducted with anti-MHC class I monoclonal antibody (H-2K^d, Meiji Nyugyo) to clarify the involvement of CTL in the cytotoxicity of these killer cells against M0. ^{51}Cr-labeled parental cells were pretreated with a saturating amount of the monoclonal Ab at 4°C for 1 h, washed twice with phosphate-buffered saline (PBS) and then used in the cytotoxicity assay.

Table I. TNF and IL-12 Production of Gene-transfected Meth-A Cells

Cells	*In vitro* cytokine production (ng/10^5 cells/48 h)	
	TNF	IL-12
M0	<0.2	<0.2
M-LacZ	<0.2	<0.2
M-TNF	8.7±1.5	<0.2
M-IL-12	<0.2	21.1±2.1
M-TNF/IL-12	5.6±1.2	10.2±2.0

Cells (1×10^5 cells/ml) were incubated for 48 h and their supernatants were assayed for TNF and IL-12 using the methods described in "Materials and Methods."
M0, parental cells; M-LacZ, *LacZ* gene-transfectant; M-TNF, *TNF* gene-transfectant; M-IL-12, *IL-12* gene-transfectant; M-TNF/IL-12, *TNF* and *IL-12* gene-transfectant.

RESULTS

Production of TNF and IL-12 by the gene transfectants TNF or IL-12 production in the culture medium of transfectants was measured by ELISA (Table I). Neither of the cytokines was detected in the culture medium of parental cells (M0) and *LacZ* gene-transfectants (M-LacZ). TNF and IL-12 concentrations in the culture medium of transfectants (M-TNF and M-IL-12) were 8.7±1.5 ng/10^5 cells/48 h and 21.1±2.1 ng/10^5 cells/48h, respectively. Meth-A cells double-transfected with *TNF* and *IL-12* genes were found to secrete lower levels of TNF (5.6±1.2 ng/10^5 cells/48 h) and IL-12 (10.2±2.0 ng/10^5 cells/48 h) than those transfected with a single gene. In particular, the levels of IL-12 in culture medium of double transfectants were almost one-half of those of the corresponding single gene-transfectants.

Expression of MHC class I, II and B7.1 on the surface of *TNF* and/or *IL-12* gene-transfected tumor cells Flow cytometry was performed to investigate whether transfection of *TNF* and *IL-12* genes into tumor cells altered the expression of MHC class I, class II or B7.1 antigens on the cell surface (Fig. 1). The expression of MHC class I, class II and B7.1 on transfectants with *TNF* gene (M-TNF) was enhanced compared to that on parental cells (M0) or mock transfectants (M-LacZ). On transfectants with *IL-12* gene (M-12), expression of MHC class I, class II and B7.1 was also enhanced compared to that on M0, though not so substantially as on M-TNF. The enhancement of expression of these antigens was more apparent on double transfectants (M-TNF/IL-12) than on single transfectants with either gene.

Tumorigenicity of *TNF* and *IL-12* gene-transfected tumor cells M0 and its transfectants (1×10^5) were s.c. inoculated into BALB/c mice to examine the tumorigenicity (Fig. 2 and Table II). Progressive tumor growth was observed in mice inoculated with parental cells (M0) and

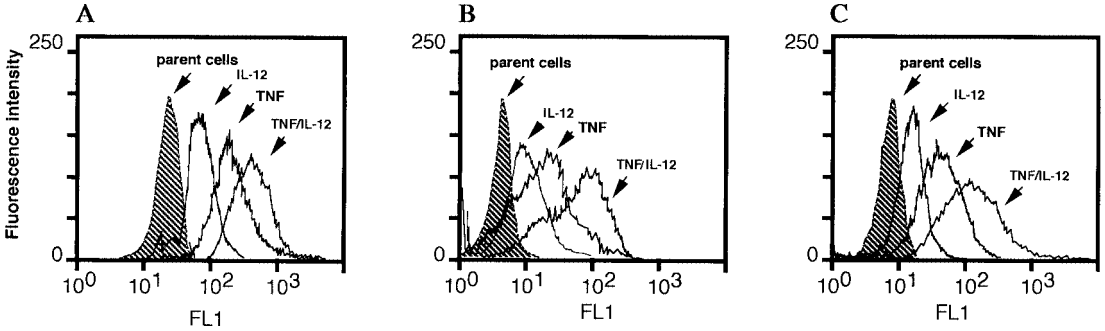

Fig. 1. Analysis of surface molecules expressed on *TNF* and *IL-12* gene-transfected tumor cells. The changes of MHC class I (A), II (B) and B7.1 (C) expression were examined by FACS analysis. Tumor cells were stained with anti-MHC class I monoclonal antibody, anti-MHC class II monoclonal antibody or anti-B7.1 monoclonal antibody. The expression on parental cells is indicated by the shaded histogram.

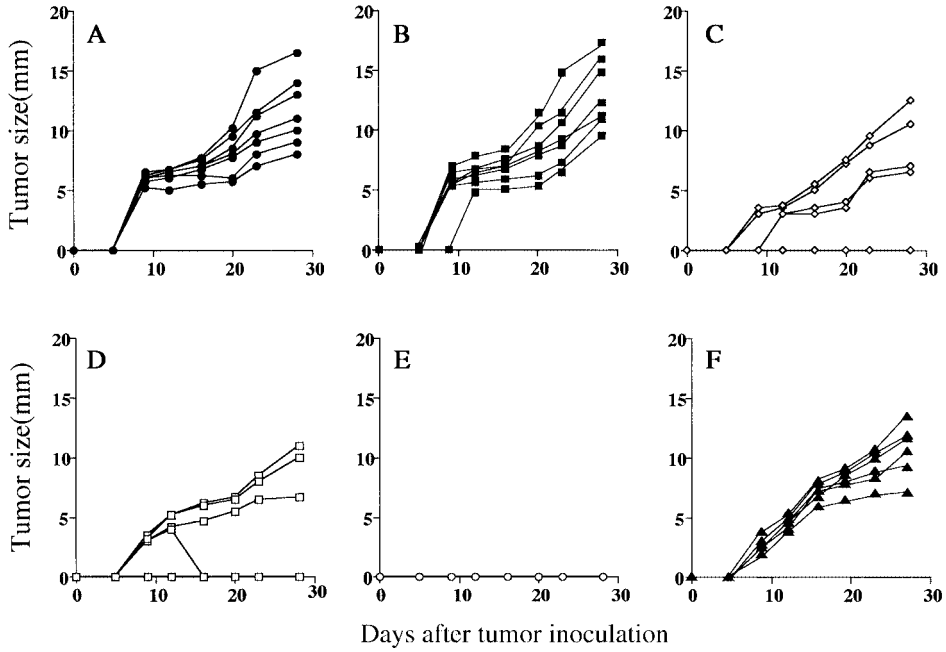

Fig. 2. Tumorigenicity of *TNF* and *IL-12* gene-transfected tumor cells. BALB/c mice were injected s.c with 1×10^5 cells of parental or gene-transfected Meth-A cells and the tumor growth was observed for 4 weeks. Tumor sizes of individual mice in each group (n=7) are indicated as the mean of perpendicular diameters. (A) M0, (B) M-LacZ, (C) M-TNF, (D) M-IL-12, (E) M-TNF/IL-12, (F) M-TNF/IL-12+anti-CD8 Ab.

Table II. Tumorigenicity of Meth-A Cells Transfected with *TNF* and/or *IL-12* Gene

Tumor cells	Tumor emergence[a)] (%)	Tumor size[b)]
M0	7/7 (100)	11.6±3.0
M-LacZ	7/7 (100)	11.3±2.5
M-TNF	4/7 (57.1)	9.1±2.9
M-IL-12	3/7 (42.9)	9.3±2.2
M-TNF/IL-12	0/7 (0)	0
M-TNF/IL-12 +anti-CD8 Ab	7/7 (100)	9.7±2.8

BALB/c mice (7 mice/each group) were s.c inoculated with 1×10^5 cells of parental or gene-transfected Meth-A cells into the right flank. Tumor take and tumor size 28 days after tumor inoculum.

a) No. of mice with tumor/No. of mice inoculated.

b) Mean±SD of tumor size of individual mice with tumor in each group.

mock transfectants (M-LacZ). In 3/7 (42.9%) or 4/7 (57.1%) of mice inoculated with M-TNF or M-IL-12, respectively, complete regression of tumors was observed. In mice inoculated with double transfectants with both genes (M-TNF/IL-12), complete regression was obtained in 100% of mice. This antitumor effect was inhibited by treatment with anti-CD8 antibody (Fig. 2). The *in vitro* growth rate was almost the same for parental cells and transfectants (data not shown).

Induction of specific immunity against rechallenged tumors Parental cells (M0) or colon 26 cells (1×10^6) were inoculated into the opposite flank of mice which had undergone complete regression of previously inoculated *TNF* or *IL-12* gene-transfected Meth-A cells (M-TNF or M-IL-12). Colon 26 tumor grew progressively, whereas M0 tumor was completely rejected after forming a palpable tumor, indicating the induction of tumor-specific immunity (data not shown). Moreover, we also observed the induction of tumor-specific immunity with double transfectants (M-TNF/IL-12) (Fig. 3).

Enhanced induction of killer cells by *TNF* and *IL-12* gene-transfected tumor cells We investigated the cytotoxic activities of killer cells induced from spleen cells of BALB/c mice inoculated with parental or transfected Meth-A cells by coculturing with parental cells (M0) (Fig. 4). Cytotoxicity of killer cells against M0 was enhanced in mice inoculated with single transfectants with *TNF* (M-TNF) or *IL-12* (M-IL-12) gene compared to that in mice inoculated with parental cells or mock transfectants. In mice inoculated with double transfectants (M-TNF/IL-12), more potent cytotoxicity was observed compared to that in mice inoculated with single transfectants with either gene. Furthermore, this cytotoxicity was significantly inhibited

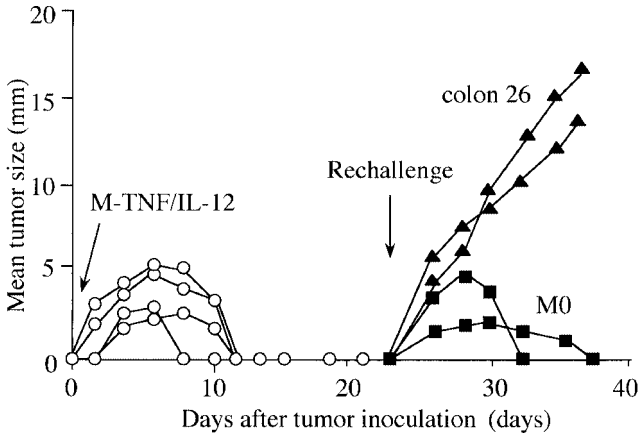

Fig. 3. Rechallenge with Meth-A and colon 26 cells in mice vaccinated with TNF and IL-12 gene-transfected Meth-A cells. BALB/c mice (n=4) were injected s.c with 1×10^5 cells of M-TNF-12 in the right flank. On day 10 following complete regression of M-TNF-12 tumor, 1×10^6 cells of parental Meth-A cells (M0, n=2) or colon 26 cells (n=2) were rechallenged in the opposite flank.

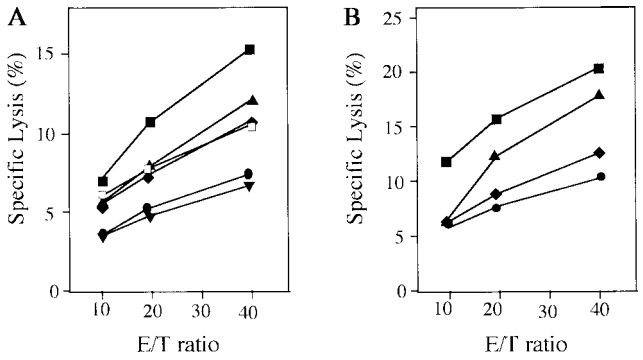

Fig. 4. Cytotoxicity of killer cells induced from spleen cells of mice vaccinated with *TNF/IL-12* gene-transfected Meth-A cells. The pooled splenocytes of mice (n=3) vaccinated with parental and gene-transfected Meth-A cells were isolated on day 7 following vaccination. They were then cocultured with irradiated M0 in the presence of IL-2 100 U/ml for 5 days. The cytotoxic activities of killer cells against parental Meth-A cells (M0) and YAC-1 cells were examined using 4-h ^{51}Cr release assay. (A) M0, (B) YAC-1. Effector cells: ■ IL-12/TNF, ▲ IL-12, ◆ TNF, □ anti-class I Ab, ▼ Meth A, ● M-LacZ.

by anti-class I monoclonal antibody. In addition, cytotoxicity of killer cells against YAC-1 cells was also enhanced in mice inoculated with double transfectants compared to either of the single transfectants.

DISCUSSION

In the present study, we attempted to obtain efficient antitumor effects by transducing the genes of two antitumor cytokines which have different mechanisms into tumor cells, and utilizing them as a vaccine to broadly stimulate the antitumor immune network. Double transfection with *TNF-α* and *IL-12* genes apparently induced synergistic antitumor effects, and these effects were supported by cytotoxicity assay. Splenocytes induced from mice bearing double transfectants showed more potent cytotoxicity against the parental cells compared to those from mice bearing single transfectants. It was clear that Meth-A cell-specific CTL was critically involved in this cytotoxicity, as it was blocked by anti-MHC class I antibody, and its antitumor effect was inhibited by anti-CD8 antibody. Moreover, rechallenged Meth-A cells, but not colon 26 cells, were rejected. However, as splenocytes induced from mice bearing double transfectants also showed considerable cytotoxicity against YAC-1 cells, NK in addition to CTL was suggested to play an important role in this cytotoxicity. In fact, TNF-α and IL-12 are reported to synergistically enhance NK activity.[25]

There seem to be two possible mechanisms for the enhancement of this killing activity. One is that the two cytokines secreted from double transfectants induced additive or synergistic activation of effector cells, such as CTL and LAK. The other possibility is that the expression of tumor surface molecules was enhanced by gene transfection. We previously reported that the transfection of *TNF* gene could enhance the expression of MHC class I and ICAM I on tumor cells.[14, 15] Then, we examined the expression of surface antigens on double transfectants by flow cytometry. Transfection with *IL-12* gene enhanced MHC class I expression and furthermore, slightly enhanced the expression of MHC class II and B7.1. On the other hand, double transfection with *TNF* and *IL-12* genes significantly enhanced the expression of MHC class I, class II and B7.1 compared to the transfection with either gene alone. Also, because tumor-related antigenic peptides such as GP110 have been identified on Meth-A cells,[26] synergistic effects might be caused by upregulation of tumor antigens. We conducted similar experiments using mice inoculated with a murine melanoma cell line, B16. The expression of MHC class I, class II or B7.1 was enhanced on double transfectants (B-TNF/IL-12) compared to that on single transfectants with either gene. Nevertheless, no synergistic antitumor effects were observed compared with single transfectants (data not shown). This is presumably due to inherently low expression of tumor antigens, leading to insufficient induction of CTL.

On the other hand, it has been demonstrated that IL-12 activates not only CTL,[19] but also NKT.[17] As NKT exerts potent antimetastatic effects, in the case of B-16 cells, when we use the model of metastatic tumors, it is possible

that double transfectants afford better vaccination effects compared to either of the single transfectants.

In conclusion, these results demonstrate that tumor vaccination by using double transfection with *TNF-α* and *IL-12* genes shows antitumor effects via not only activation of effector cells (paracrine effects), but also increased immunogenicity of the gene-transfected tumor cells themselves.

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