Article

Interleukin-12 Gene Modification Exerts Anti-Tumor Effects on Murine Mammary Sarcoma Cell Line *in vivo*

Dan Li[1], Hong Yu[1], Tengfei Xu[1], Jinghua Li[2], Yunfang Sun[3] and Wenqing Zhang[1, 4]

The aim of this project was to investigate the anti-tumor effect of an IL-12 gene modified mammary sarcoma murine cell line, EMT6/IL-12, in mouse model. In this study, we transfected the recombinant eukaryotic plasmid encoding IL-12 gene (pcDNA6-p70) into EMT6 and obtained the IL-12 expressing EMT6/IL-12 cell line. Then EMT6/IL-12 cells were *s.c.* inoculated into mice. The recombinant vector treatment group was set as control. We then evaluated the inhibition of tumor growth and the anti-tumor immunity function *in vivo* such as cytotoxicity, proliferation of splenocytes and serial IFN-γ level. And the percentage of IFN-γ producing CD4 or CD8 T cells among splenocytes was also analyzed in tumor bearing mice. Our results showed that the growth of tumors was obviously inhibited in EMT6/IL-12 group. Moreover, the capacities of anti-tumor immunity were all significantly higher in EMT6/IL-12 group compared to the controls. The results of the present investigation support the notion that EMT6/IL-12 could exert gene therapy in tumor model by improving the anti-tumor cellular immunity. *Cellular & Molecular Immunology*. 2008;5(3):225-230.

Key Words: interleukin-12, transformation, genetic, gene therapy, mammary neoplasm

Introduction

Since the major characterization of cancer is recognized by incompetence of cellular immunity, the aim of active specific cancer immuno-therapy is to induce or stimulate tumor-specific immunity (1, 2). Numerous studies have shown that IL-12, a key cytokine for the induction of Th1-oriented immune responses, is one of the most potent molecules in cancer therapy. IL-12 is a heterodimeric immunoregulatory cytokine composed of covalently linked p40 and p35 subunits and exhibits anti-tumor activities in a variety of laboratory models (3-5). Analysis of the anti-tumor mechanism of IL-12 has shown that CTLs are critical immune effectors. Others, such as promoting the proliferation of T cells, NK cells and tumor-infiltrating lymphocytes were involved. In addition to developing strong anti-tumor CTL responses, the augmented production of other cytokines including IFN-γ, and TNF-α induced by IL-12 has been implicated in its anti-tumor effectors (6). Whereas, administration of IL-12 protein was directly limited by severe toxicity *in vivo*. The genetic therapy approach provides a strategy to achieve temporary and high local concentrations of cytokines within a tumor with less risk of systemic toxicity (7). The efficiency of local secretion of cytokines at the tumor site using genetically modified fibroblasts or tumor cells or recombinant vectors has been demonstrated effectively in a variety of tumor models (8-10).

We constructed a recombinant eukaryotic plasmid containing a fusion gene encoding a linearized single chain of human IL-12, pcDNA6/v5-his-p70 (pcDNA-p70) (11). Previous investigations have shown that the expressed IL-12 revealed expected biological activities both *in vitro* and *in vivo*. When pcDNA-p70 was injected directly into the transplanted murine tumor (S180, a murine sarcoma cell line) on mouse model, the tumor regression appeared clearly (12). In this study, pcDNA-p70 was transfected into EMT6, a murine mammary carcinoma cell line. The genetically modified tumor cells (EMT6/IL-12) were transplanted into

[1]Department of Microbiology, Medical College of Qingdao University, 308 Ningxia Road, Qingdao 266071, China;

[2]Department of Central Laboratory, Qingdao Municipal Hospital, 1 Jiaozhou Road, Qingdao 266011, China;

[3]Department of Clinical Laboratory Medicine, Shandong Medical College, 39 Qingnian Road, Linyi 276002, China;

[4]Corresponding to: Dr. Wenqing Zhang, Department of Microbiology, Medical College of Qingdao University, 308 Ningxia Road, Qingdao 266071, China. Tel: +86-532-8378-0015, E-mail: zhangwenqing01@yahoo.com.cn

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Abbreviations: CTL, cytotoxic T lymphocyte; NK cell, natural killer cell; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-α; LDH, lactate dehydrogenase; NBT, nitroblue tetrazolium; NAD, nicotinamide adenine dinucleotide; CFC, cytokine flow cytometry.

mouse model to evaluate its capacity in tumor genetic therapy.

Materials and Methods

Plasmid
Recombinant eukaryotic plasmid containing a fusion gene encoding a linearized single chain of human IL-12, pcDNA6/v5-his-p70 (pcDNA-p70) has been described previously (11).

Cell line
EMT6 cell line (BALB/c mammary adenocarcinoma cell) was purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). Cells were cultured in RPMI 1640 (GIBCO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sijiqing Company of Hangzhou, China) at 37°C in 5% CO_2.

Mice
Thirty female SPF Kunming mice (weight from 18 g to 20 g) were purchased from Qingdao Experimental Animal Center. They were kept (ten mice per cage) in isolation under specific pathogen-free conditions, exposed to 12-h light/12-h dark cycles, and provided with standard feed and water.

Cell modification
EMT6 cell line was transfected with pcDNA6-p70 by polyethyleneimine (PEI, Sigma, USA) as described previously (11). The modified cell line (EMT6/IL-12) was selected in 10% FBS/RPMI 1640 with Blasticidin S HCl (final concentration 10 mg/ml, Invitrogen, Carlsbad, CA) for 14 days and cultured in maintenance solution (Blasticidin S HCl, 2 mg/ml).

Identification of EMT6/IL-12
IL-12 fragments were amplified from DNA of EMT6/IL-12 cells. The sequences of the primers are as follows: forward primer: 5'-GCG GTA CCA CCA TGT GTC ACC AGC AGT TGG TCA TCT C-3' (37 bp), reverse primer: 5'-TTG CGG CCG CGG AAG CAT TCA GAT AGC TCA TCA CTC-3' (36 bp). Amplified fragment was separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

The total RNA was extracted from the 5×10^6 EMT6/IL-12 using TRIZOL (Invitrogen, Carlsbad, CA) and reverse transcribed into first-strand cDNA using oligo-dT primers and the AMV reverse transcriptase (Promega, USA) from 5 μg of RNA sample. IL-12 fragments were amplified from the cDNA and analyzed by electrophoresis as described above.

Tumor models and in vivo treatment
Thirty mice were randomly divided into 3 groups (10 mice a group): EMT6/IL-12, pcDNA6-p70, EMT6. Mice in EMT6/IL-12 group were inoculated *s.c* in the right prothorax with EMT6/IL-12 cells (2×10^6 cells/ml, 100 μl). On days 4, 7, 10, 14 and 17 after inoculation, saline (100 μl/mice) was injected on the primary injected site. Mice in pcDNA6-p70 group were inoculated with EMT6 cells (2×10^6 cells/ml, 100 μl) and pcDNA6-p70 was injected in the same way as the EMT6/IL-12 group. EMT6 group was inoculated with EMT6 cells (2×10^6 cells/ml, 100 μl) and saline was injected as described above. All mice were sacrificed on day 21, and subcutaneous tumors, spleens, thymus glands were weighted, and tumor inhibitory rate [(weight of control group - weight of experimental group) / weight of control group], splenic index (weight of spleen / weight of mouse), and thymus index (weight of thymus / weight of mouse) were calculated.

Tumor growth curve
The growth status of tumors on days 4, 7, 10, 14, 17 and 20 was observed, and the diameters of the tumors were acquired by vernier caliper to build the tumor growth curve.

Lymphocyte proliferation
Mouse splenocytes were isolated by Ficoll (TBD sciences, Haoyang Biological Manufacture Company of Tianjin, China). A total of 2×10^6 cells/ml was cultured in 96-well (100 μl/well) plates with RPMI 1640, 5 μg/ml Con A, and 10% FBS for 72 hours. Three parallel pores were performed in each sample. MTT (final concentration 0.5 mg/ml) was added into each well for the last 4 h, and DMSO (120 μl) was added to each well in the end. Absorbance was evaluated at 570 nm. Proliferation rate = A of experimental well minus A of control well.

Cytotoxicity of splenocytes
Splenocytes were isolated and adjusted into 2×10^6 cells/ml as the effector, and EMT6 cells were adjusted into 2×10^5 cells/ml as the target. Both of them (100 μl) were co-cultured in 96-well U-shaped plate (effector: target = 50:1) for 4 hours. Meanwhile maximum releasing (100 μl target cells plus 100 μl 2% Triton-X 100) and spontaneously releasing (100 μl target cells plus 10% FBS/RPMI 1640 100 μl) wells were set. Then, supernatants were harvested and 100 μl freshly prepared LDH substrate (NBT0.032 mg, NAD^+ 0.08 mg, PMS 0.008 mg, 1 mol/L sodium lactate 4 μl) was added to each well, avoiding light at room temperature for 10-15 min, the reaction was stopped by adding 30 μl Citric acid/well. Absorbance was evaluated at 570 nm. Cytotoxity of splenocytes = (A of experimental group minus A of spontaneously releasing) / (A of maximum releasing minus A of spontaneously releasing) × 100%.

IFN-γ assay
Blood was collected from the orbit before sacrificed. Serum was prepared. IFN-γ was measured by ELISA kit according to the manufacturer's instruction. The absorbance (A value) was detected at 490 nm. Concentrations of IFN-γ in the serum were expressed as pg per milliliter with reference to a standard IFN-γ curve.

Cytokine flow cytometry (CFC) analysis
Cells were prepared for cytokine following cytometry analysis according to the BD Biosciences Inc. protocol, with modifications (13). Approximately 1×10^6 mouse splenocytes

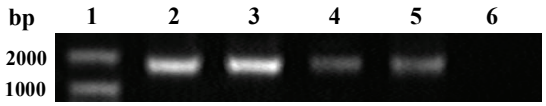

Figure 1. Agarose gel electrophoresis of PCR products of IL-12 gene fragment. Lane 1, DL2000 Marker (Takara, Dalian, China); Lane 2 and 3, The target amplification fragments of IL-12 gene from DNA; Lane 4 and 5, RT-PCR products; Lane 6, negative control (ddH_2O).

were prepared and cultured in RPMI 1640 with 10% FBS and Con A (final concentration of 5 μg/ml), at 37 °C for 72 h. Brefedin A (final concentration 10 μg/ml) was added in the last 16 h. Washing the cells with ice-cold PBS and re-suspending them in 50 μl PBS containing 0.1 mM EDTA and 0.5% BSA, incubating for 10 min at 30 min. PE-anti-mouse-CD4/CD8 monoclonal antibody (10 μl, Immunotech Company, France) was added, and cells were incubated at 37°C for 30 min in the dark. The cells were washed twice by PBS. Permeabilization reagent A was added (100 μl, Beckman Coulter, USA), and incubated at room temperature for 30 min, then washed again. Then permeabilization reagent B (100 μl) and 10 μl FITC-anti-mouse-IFN-γ antibody (Immunotech Company, France) were added, mixed gently and incubated at room temperature in the dark for 15 min. The cells were washed twice and analyzed by flow cytometry (Beckman Coulter, USA). The data were analyzed by WinMDI 2.9 software and shown in dot plot for FITC (FL1) and PE (FL2) fluorescence. Cell gate regions were drawn around viable blast cells based on their forward scatter (FSC) versus side scatter (SSC) properties. Double negative, PE^+, $FITC^+$ mono-positive cells were also set as match control.

Statistical analysis

The one-way ANOVA was used to test for differences among experiments by SPSS 11.5. p value < 0.05 was considered statistically significant.

Results

Identification of transfection and expression of EMT6/IL-12 cell line

The methods of PCR, RT-PCR and ELISA were utilized to test the expression of IL-12 by the modified cell line EMT6/IL-12, from DNA, RNA and protein levels. As shown in Figure 1, the fragments amplified from the DNA and cDNA template of EMT6/IL-12 have the same nucleotide sequence as IL-12 (1.8 kb). Meanwhile, EMT6/ IL-12 secreted 0.9-1.6 ng/ml IL-12 (assessed in supernatant from 2×10^5 cells grown for 48 h in 2 ml of medium) as measured by ELISA (data not shown). These results indicated that pcDNA6-p70 was successfully expressed in EMT6/IL-12 cells.

Inhibition of tumor growth in EMT6/IL-12 mice

In order to investigate the tumor growth in the three groups,

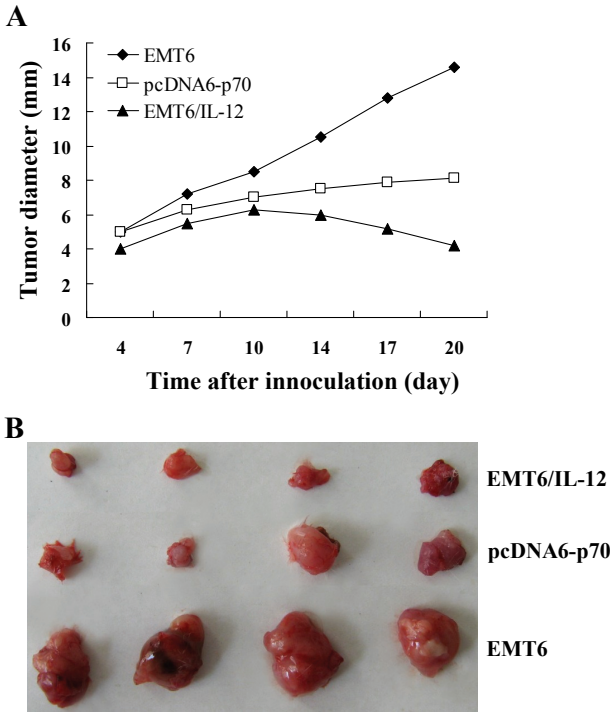

Figure 2. Tumor regression in the EMT6/IL-12 mice. (A) The diameter of tumor was measured by vernier caliper on days 4, 7, 10, 14, 17 and 20 to build the tumor growth curve. (B) Mice were sacrificed on day 21. The representative photograph were shown for limited subcutaneous tumor size in EMT6/IL-12 group.

the tumors were detected from day 4 in mice inoculated with tumor cells. Mice in EMT6/IL-12 group developed palpable tumors which growing continuously from day 0 to day 7. After that time, tumors began to regress progressively. Five mice were tumor free on day 21. Tumor growth was inhibited to a certain extent in the group pcDNA6-p70. While in the EMT6 group tumors were growing progressively until the sacrificed day (Figures 2 and Table 1). These results suggested that EMT6/IL-12 cell line has the ability to inhibit the tumor growth in mice.

EMT6/IL-12 enhances the anti-tumor immune function in vivo

To check the capacities of anti-tumor immunity *in vivo*, we studied some immune indexes. Compared to the control

Table 1. Weight of tumors and inhibitory rates

	Weight of tumors (g)	Inhibitory rate (%)
EMT6/IL-12	$0.014 \pm 0.02^{*,\#}$	97.08
pcDNA6-p70	$0.114 \pm 0.08^{\dagger}$	76.25
EMT6	0.480 ± 0.07	0

Subcutaneous tumors were weighted and inhibitory rate were calculated. $^*p < 0.001$ *vs* EMT6; $^{\#}p < 0.001$ *vs* pcDNA6/IL-12; $^{\dagger}p < 0.01$ *vs* EMT6.

Table 2. Anti-tumor immune functions

Groups	Lymphocyte proliferation (%)	Cytotoxicity of splenocytes (%)	IFN-γ (pg/ml)	Thymus index	Spleen index
EMT6/IL-12	13.56 ± 0.9*#	56.77 ± 3.34*#	31.95 ± 2.22*#	4.86 ± 0.72*	7.93 ± 0.74*#
pcDNA6-p70	7.44 ± 0.8†	41.18 ± 4.3†	24.24 ± 3.30†	4.19 ± 0.41†	5.33 ± 0.55†
EMT6	3.43 ± 0.6	28.14 ± 2.8	14.96 ± 2.46	2.13 ± 0.44	4.23 ± 0.85

The anti-tumor immune functions *in vivo* and the serial IFN-γ levels were evaluated. *$p < 0.01$ *vs* EMT6, #$p < 0.01$ *vs* pcDNA6/IL-12, †$p < 0.01$ *vs* EMT6, n = 10 in each group.

groups, the indexes of spleen and thymus in EMT6/IL-12 group increased significantly. Moreover, the cytotoxicity of splenocytes, lymphocyte proliferation and the level of serial IFN-γ were also elevated obviously (Table 2). These data confirmed that EMT6/IL-12 could induce higher anti-tumor immunity.

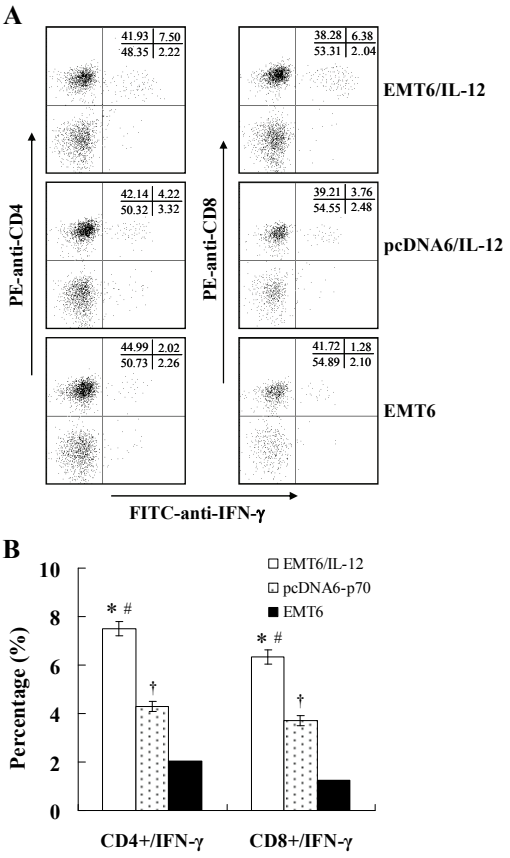

Figure 3. The frequencies of IFN-γ producing CD4 and CD8 T cells increased in the EMT6/IL-12 mice. (A) Splenocytes (1×10^6) were prepared and stimulated by Con A for 72 h, and Brefedin A was added at the last 16 h. Cells were harvested, stained with fluorochrome-conjugated mAbs against CD4, CD8 and IFN-γ and analyzed by double-color flow cytometry. Double negative, and mono-positive cells were set as control. (B) The percentages of double positive cells were shown as mean ± SD for ten samples of each group. *$p < 0.01$ *vs* EMT6, #$p < 0.01$ *vs* pcDNA6/IL-12, †$p < 0.01$ *vs* EMT6.

Effects of EMT6/IL-12 cell line on enhancing the functional T cells

We then analyzed the percentage of functional T cells, which plays an important role in the anti-tumor immune response. After stimulated with Con A, the frequencies of IFN-γ producing CD4 and CD8 T cells in splenocytes raised significantly in the group of EMT6/IL-12 (Figures 3). It is worthy noted that there were significantly much functional T cells in the splenocytes of EMT6/IL-12 group.

Discussion

IL-12 is a potent central cytokine to regulate innate and adaptive immune responses. Previous reports showed that it is one of the most effective multi-functional cytokines with immuno-competence and regulation effect (14). The efficacy of IL-12 as a therapeutic cytokine has been studied in various experimental murine tumor models and it has been used in clinical trials for the treatment of certain human cancers.

IL-12 could exert a number of regulatory effects on splenocytes (15-17). These include facilitating specific $CD8^+$ T cell responses, promoting the development of Th1-type T cells, enhancing the cytotoxity of splenocytes, and inducing the secretion of IFN-γ. All the mechanisms above appear to underlie the well-known anti-tumor effects of IL-12. In fact, systemic administration of IL-12 has been shown to significantly suppress the growth of a variety of established murine tumors and prolong the survival of tumor-bearing mice (18-20).

Despite the efficacy of IL-12, the appropriate approach for delivery of IL-12 *in vivo* remains to be determined. Although the pharmacokinetics of IL-12 is more favorable than those of many other cytokines, repeated systemic administration is required for maximal therapeutic activity in mice. The systemic administration of IL-12 is known to be associated with significant toxicity, which has essentially precluded its use in clinical practice (21). Alternative approaches for IL-12 delivery include viral-mediated gene therapy and gene-modified tumor cell vaccines and others (22-24). Unfortunately, viral-mediated gene therapy is associated with the generation of neutralizing antibodies (25). The development of reliable and inexpensive techniques for the sustained local delivery of cytokines could facilitate the clinical application of IL-12.

Our research team has successfully constructed a eukaryotic expression vector, pcDNA6-p70 and primarily

studied its anti-tumor effect on experimental mice through intro-tumor injection of the vector (11, 12). In this investigation, we have extended these observations. We used IL-12 modified tumor cell line, EMT6/IL-12 to increase the concentration of IL-12 at the tumor site and demonstrated this could be more effective in inhibiting the tumor growth.

In our study, we demonstrated the EMT6/IL-12 could generate a high production of IL-12, which is similar to James's results (26). EMT6/IL-12 tumors grew progressively for 10 days and then rapidly began to regress. On day 21, five of the mice remained tumor free, which indicates that locally secreted IL-12 could mediate strongly anti-tumor immunity.

It is indicated that locally produced IL-12 controls the growth of solid tumors through at least 3 distinct mechanisms. Firstly, stimulation of T cell response: the frequencies of $CD4^+$/IFN-γ, $CD8^+$/IFN-γ double positive cells were elevated, which made the tumor diminish or disappear. Secondly, activation of splenocytes mediated tumor cell lyses (28). Thirdly, the higher level of serum IFN-γ under the effect of IL-12 promoted anti-tumor immune response through variety of approaches (27). Previous studies have clearly identified these mechanisms in different tumor models (26). However, few have assessed the combined potential contributions of each mechanism within an individual tumor model.

Our study showed that the oncogenicity of modified tumor cell line, EMT6/IL-12 *in vivo* decreased and it induced persistent anti-tumor immunity. The weights of spleen and thymus in EMT6/IL-12 tumor-bearing mice increased significantly compared to the direct injection of recombinant vector. Moreover, because of the steady and persistent IL-12 expression in tumor site, it was more convenient to avoid the vector injection repeatedly. Besides, this approach had less side effects and lower price. So, we have soundly reasons to believe that this genetic engineered regime could become a more effective strategy and contribute to the improvement of long-term recipient survival.

In conclusion, although genetically engineered tumor cell could steadily produce IL-12 and yield anti-tumor immunity in mice, such an approach, however, may not be clinically feasible because it is labor-intensive and autologous tumor cells are not always readily available (29). So eliminating the oncogenicity of modified tumor cell lines and raising its immunogenicity by combining it with the tumor specific gene vaccine are what we need to do in the future. Meanwhile, the anti-tumor effect is complex as affected by multiple factors (4), thus we should combine it with other means of traditional operation, chemotherapy, radiotherapy and hematopoietic stem cells in the further investigation.

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References

1. Robert MS, David MG. Targeted therapy of cancer: new prospects for antibodies and immunoconjugates. Cancer J Clin. 2006;56:226-243.
2. Robert DL, Deborah AB, Scott AT, Arul MC, Kenneth JP. The lethal phenotype of cancer: the molecular basis of death due to malignancy. Cancer J Clin. 2007;57:225-241.
3. Angelica SIL, Moa EF, Thomas THT. AdCD40L gene therapy counteracts T regulatory cells and cures aggressive tumors in an orthotopic bladder cancer model. Clin Cancer Res. 2005;11: 8816-8821.
4. Xian J, Yang H, Lin Y, Liu S. Combination nonviral murine interleukin 2 and interleukin 12 gene therapy and radiotherapy for head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2005;131:1079-1085.
5. Mitrus I, Delić K, Wróbel N, Missol-KE, Szala S. Combination of IL-12 gene therapy and CTX chemotherapy inhibits growth of primary B16 (F10) melanoma tumors in mice. Acta Biochim Pol. 2006;53:357-360.
6. Maheshwari A, Han S, Mahato RI, Kim SW. Biodegradable polymer-based interleukin-12 gene delivery: role of induced cytokines, tumor infiltrating cells and nitric oxide in anti-tumor activity. Gene Ther. 2002;9:1075-1084.
7. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-γ production. Blood. 1997;90:2541-2548.
8. Tae HK, Hua J, Hyun WK, Myung-HC, Chong SC. Mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in BALB/c mice bearing CT-26 carcinoma cells. Mol Cancer Ther. 2006;5:1723-1732.
9. Hisae I, Kota O, Ryoji F, et al. Superior protective and therapeutic effects of IL-12 and IL-18 gene-transduced dendritic neuroblastoma fusion cells on liver metastasis of murine neuroblastoma. J Immunol. 2006;176:3461-3469.
10. Chen Y, Emtage P, Zhu Q, et al. Induction of ErbB-2/neu-specific protective and therapeutic antitumor immunity using genetically modified dendritic cells: enhanced efficacy by cotransduction of gene encoding IL-12. Gene Ther. 2001;8: 316-323.
11. Zhang WQ, Wang LN, Liu ZJ. Functional evaluation of recombinant human IL-12 *ex vivo* with cytokine follow cytometry (CFC). Zhong Hua Wei Sheng Wu He Mian Yi Xue Zha Zhi. 2006;26:383-384.
12. Sun YF, Zhang WQ, Wang LN, Yu H, Li D. Curative effect of a recombinant plasmid coding human interleukin-12 on tumor bearing mice. Zhong Hua Zhong Liu Fang Zhi Zha Zhi. 2007;14: 349-351.
13. Maecher HT, Maino VC. Analyzing T-cell responses to cytomegalovirus by cytokine flow cytometry. Hum Immunol. 2004;65:493-499.
14. Trinchieri G. 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. 1994;84:4008-4027.
15. Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity. 2003;19:641-644.
16. Kobayashi K, Kai M, Gidoh M, et al. The possible role of interleukin (IL)-12 and interferon-γ-inducing factor/IL-18 in protection against experimental *Mycobacterium* leprae infection in mice. Clin Immunol Immunopathol. 1998;88:226-231.
17. Rakhmilevich AL, Hooper AT, Hicklin DJ, et al. Treatment of experimental breast cancer using interleukin-12 gene therapy combined with anti-vascular endothelial growth factor receptor-2 antibody. Mol Cancer Ther. 2004;3:969-976.
18. Gautam SC, Xu YX, Dumaguin M, et al. Interleukin-12 (IL-12)

gene therapy of leukemia: immune and anti-leukemic effects of IL-12-transduced hematopoietic progenitor cells. Cancer Gene Ther. 2000;7:1060-1068.
19. Marina NT, Xue QX, Williams H, Shry Y, Shulin L. Stat1 deficiency in the host enhances interleukin-12-mediated tumor regression. Cancer Res. 2006;66:4461-4467.
20. Robertson MJ, Cameron C, Atkins MB, et al. Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. Clin Cancer Res. 1999;5:9-16.
21. Manuel RG, Íñigo TF de la C, Ainhoa AI, Miguel NCL, Javier ZF. Use of gene therapy in a subcutaneous murine model of lung cancer. Arch Bronconeumol. 2006;42:526-532.
22. Loree H, KathleenM, Jeffrey W, et al. Evaluation of toxicity following electrically mediated interleukin-12 gene delivery in a B16 mouse melanoma model. Clin Cancer Res. 2006;12:3177-3183.
23. Hwang KS, Cho WK, Yoo J, et al. Adenovirus-mediated interleukin-12 gene transfer combined with cytosine deaminase followed by 5-fluorocytosine treatment exerts potent antitumor activity in Renca tumor-bearing mice. BMC Cancer. 2005;5:51.
24. Colombo MP, Vagliani M, Spreafico F, et al. Amount of interleukin 12 available at the tumor site is critical for tumor regression. Cancer Res. 1996;56:2531-2534.
25. James PM, Scott AG, Celeste AM, John GF, Edith ML. Transfection of the genes for interleukin-12 into the K1735 melanoma and the EMT6 mammary sarcoma murine cell lines reveals distinct mechanisms of antitumor activity. Int J Cancer. 2003;106:690-698.
26. Jeff JS, Veronica LH, Timothy CB, et al. Enhanced antitumor response by divergent modulation of natural killer and natural killer T cells in the liver. Cancer Res. 2006;66:11005-11012.
27. Alessandro L, Eduardo MN, Laura V, et al. Natural killer cells in vernal keratoconjunctivitis. Mol Vis. 2007;13:1562-1567.
28. Tuting T, Gambotto A, Baar J, et al. Interferon-α gene therapy for cancer: retroviral transduction of fibroblasts and particle-mediated transfection of tumor cells are both effective strategies for gene delivery in murine tumor models. Gene Ther. 1997;4:1053-1060.