

Preliminary Study on Mouse Interleukin-21 Application in Tumor Gene Therapy

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Interleukin-21 (IL-21) is a recently characterized T cell-derived cytokine with a significant homology to IL-2, IL-4 and IL-15. To determine whether IL-21 has broad immunoregulatory activity and can stimulate durable antitumor responses, we constructed mouse IL-21 (mIL-21) recombinant plasmid and evaluated its antitumor efficacy. Mouse IL-21 cDNA was amplified from Con A-activated mouse T cells by RT-PCR. Recombinant pcDNA3.1/mIL-21 was constructed and transfected into Sp2/0 cells. Mouse IL-21 expression was analyzed by Western blotting and its activities were detected by ³H-TdR incorporation and MTT assay. The recombinant pcDNA3.1/mIL-21 was injected *s.c.* into tumor lump. Tumor size, weight, the activities of CTLs, NK cells and LAK cells and serum IFN- γ level were measured for evaluating mIL-21 mediated antitumor responses. The results indicated that mIL-21 was correctly expressed in Sp2/0 cells and it can improve the proliferation of T cells and B cells, and enhance NK cytotoxic activity *in vitro*. The activities of CTL and NK cells, and serum IFN- γ level were significantly improved, furthermore the tumor growth was obviously suppressed in pcDNA3.1/mIL-21 treated mice. However, the LAK activity did not alter significantly. Taken together, this study suggests that the injection with recombinant plasmid containing mIL-21 is a potential approach for tumor gene therapy. *Cellular & Molecular Immunology*. 2004;1(6):461-466.

Key Words: IL-21, tumor, gene therapy, CTL, NK, IFN- γ

Introduction

Interleukin (IL)-21 is a T cell-derived cytokine with a four-helix-bundle structure (1), regulates innate and adaptive immune responses (2). It is most closely homology to IL-2 and IL-15, secreted by activated natural killer (NK) and CD4⁺ T cells. IL-21 can improve the proliferation and maturation of NK cell populations from bone marrow, proliferation of mature B-cell populations co-stimulated with anti-CD40, and proliferation of T cells co-stimulated with anti-CD3 (1). IL-21, like IL-2 and IL-15, has an ability to regulate antitumor activity in mice that has generated considerable interest in understanding their mode of action (3-7). IL-21R complex composed with a ligand

binding subunit and an indispensable signal transduction subunit, the common γ chain (8, 9). The γ c chain is also the signal transduction subunit of IL-2, IL-4, IL-7, IL-9 and IL-15 (3). Most of the cytokines of the γ c family, including IL-21, have shown significant effectors in immunotherapy or immunogene therapy application. Common γ chain network cytokines gene therapy may represent significant progress in immuno therapy against malignancies.

To further investigate the antitumor responses of IL-21, we construct eukaryotic expression plasmid encoding mouse IL-21 (mIL-21), and apply it to mice tumor model by intratumoral injection and evaluate its antitumor efficacy and possible antitumor mechanisms in tumor-bearing mice.

Materials and Methods

Mice and cell lines

BALB/c mice of 6-8 weeks of age were purchased from the University of Science and Technology of China. All mice were housed under pathogen-free condition.

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Abbreviations: mIL-21, mouse interleukin-21; Con A, concanavalin A; NK, natural killer; CTL, cytotoxic T lymphocyte; LAK, lymphokine activated killer cell; IFN- γ , interferon gamma; *s.c.*, subcutaneous injection; OD, optical densities; ELISA, enzyme linked immunosorbent assay; RT-PCR, reverse transcription polymerase chain reaction; cpm, counts per minute; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DC, dendritic cell.

The Sp2/0 cell line (BALB/c mice myeloma cells), YAC-1 cell line (Moloney leukemia-induced T-cell lymphoma of A/Sn mouse origin) and Raji (hematopoietic malignant cells) were obtained from Cellular Institute in Shanghai and were cultured at 37°C in 5% CO₂ in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate.

Plasmids and primers

Plasmid pcDNA3.1 was gifted by Professor Chen Zhi in Institute of Infectious Diseases, Zhejiang University. Sense primer for specific mIL-21 5'-TGT CGC TAG CTC CTG GAG ACT CAG TTC TG-3' and antisense primer 5'-CCG GGA TAT CCT AGG AGA GAT GCT GAT G-3' according to the published sequence and PCR product is around 460 bp (1).

Generation of recombinant plasmid pcDNA3.1/mIL-21

Total RNA from activated spleen cells with 5 µg/ml concanavalin A (Con A), was prepared with RNeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions and then was transcribed cDNA. The purified mIL-21 cDNA was inserted into plasmid pcDNA3.1 in two different polylinker sites by T4 DNA ligase (Promega, USA) and formed the recombinant pcDNA3.1/mIL-21 constructs, which was sequenced by Shenneng Company, Shanghai.

Development of stable expression Sp2/0 cell line

Sp2/0 cells were transferred to 6-well plate after 24 hours splitting and transfected by mixing the Lipofectamin™ 2000 (Invitrogen, CA), serum-free medium with recombinant pcDNA3.1/mIL-21, or blank plasmid as control respectively. Mixtures were incubated for 20 minutes at room temperature and then put the mixture directly into Sp2/0 cells after the cell medium was removed from plates. Growth medium may be replaced after 4 hours, followed by selection with 600 µg/ml of G418 (Clontech, CA). About 7-10 days, G418-resistant clones were selected, clonally isolated and screened for mIL-21 expression by Western blotting.

Western blotting analysis

Whole cell extracts were prepared by protein extraction buffer (Novagen, Germany) according to manufacturer's protocol. Western blotting was performed after 12% SDS-polyacrylamide gel electrophoresis and then using WesternBreeze Kit (Invitrogen, CA). The anti-mIL-21 antibody was purchased from Genway company (USA). All steps were performed according to manufacturer's instructions.

Proliferation assay

T cells and B cells were isolated from mouse spleen as previously described (10). T-cell proliferation assay were performed on 96-well plastic culture plate. T cells (1×10^6) were seeded in each well and total volume was 200 µl with or without the presence of mIL-21 Sp2/0 supernatant containing. The well containing 10 µg/L Con A was as positive control and coordinated factor control. Each well was cultured at 37°C in 5% CO₂ atmosphere in RPMI 1640 for 48 hours. One µCi ³H thymidine (³H-TdR, purchased from Institute of Radionuclide, Chinese Academy of

Medicine, Shanghai, China) was added to each well for additional 6 hours incubation and the cells were harvested with a Micromate 96-well harvester. Radioactivity was measured by liquid scintillation counting. All proliferation assays were performed in triplicate. The data represent the mean counts per minute (cpm) (11). B cells proliferation assay were performed as described above.

Cytotoxicity assay

NK cytotoxicity assay was measured by the MTT colorimetry assay (12, 13). In briefly, YAC-1 cells (1×10^4) as target cells were seeded in 96-well plastic plate. Spleen cells as effector cells, were prepared from the mice and simultaneously seeded with YAC-1 at 50:1 ratios of effector to target (E:T) and total of volume was 200 µl with supernatant of mIL-21 or without supernatant of mIL-21. After the plate was incubated at 37°C in 5% CO₂ for 20 hours, the supernatant was discarded, and then each well was added with 200 µl MTT for additional 4 hours incubation. The supernatants were discarded and 20 µl dimethylsulfoxide was added and shaken for a minute, then optical densities were measured with the plate reader (Bio-rad) at OD₅₆₀. CTL and LAK activities were also measured by MTT colorimetry method as described as NK cytotoxicity assay. In CTL assay, the effector cells from mouse spleen cells were incubated for 24 hours with Sp2/0 target cells transfected with pcDNA3.1/mIL-21 at 50:1 ratios of E:T, and in LAK assay, the effector cells from mouse spleen cells were incubated with 1000 U/ml rIL-2 for three days and then Raji target cells was added in 96-well plastic plate at 50:1 ratios of E:T. All cytotoxic activity assays were performed in triplicate.

Treatment of established tumor with pcDNA3.1/mIL-21

BALB/c mice were injected *s.c.* with 5×10^5 Sp2/0 cells in flank in logarithmic growth phase. On day 14 when tumors were established, the intratumoral injections of 100 µg pcDNA3.1/mIL-21, 100 µg pcDNA3.1 and 100 µl PBS were applied in each group with 8 mice respectively.

Tumor size and lump tissue measurement

Tumor size were measured every week, and figured as (long distance \times short distance²). After six weeks observation, all mice were killed to isolate the spleen cells as effector cells of cytotoxic assay, and lumps were weighed in the scales.

Determination of IFN- γ by ELISA

Serum IFN- γ levels were detected by ELISA according to Kit's protocol (R&D Systems, Inc., Minneapolis, MN).

Statistical analysis

Statistical analysis was performed using the Student's *t* test for the difference between experiment group and control group. The data were expressed as mean \pm SD from eight mice per group, and the *p* values < 0.05 is taken as statistically significant.

Results

Recombinant protein mIL-21 was successfully expressed in

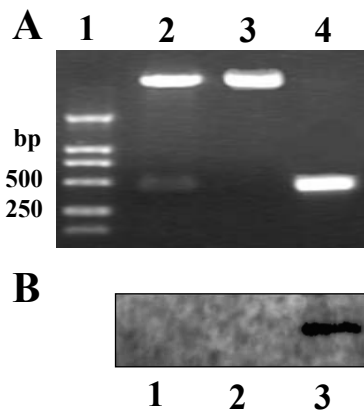


Figure 1. Identification and expression of recombinant mIL-21 in Sp2/0 cells. (A) Identification of recombinant pcDNA3.1/mIL-21. Lane 1, DNA marker; lane 2, pcDNA3.1/mIL-21 cut with Nhe I and EcoR V; lane 3, pcDNA3.1 cut with Nhe I and EcoR V; lane 4, PCR product of mIL-21. (B) Identification of recombinant protein mIL-21 in Sp2/0 cells. Lane 1, Sp2/0 cells transfected with plasmid pcDNA3.1; lane 2, Sp2/0 cells; lane 3, Sp2/0 cells transfected with plasmid pcDNA3.1/mIL-21.

Sp2/0 cells

The mIL-21 cDNA fragment was amplified from the Con A activated mouse T cells by RT-PCR and it was inserted into plasmid pcDNA3.1 and recombinant pcDNA3.1/mIL-21 was digested with restriction endonuclease. The resultant fragments indicated the recombinant pcDNA3.1/mIL-21 was constructed successfully. As shown in Figure 1A, the sequencing analysis indicated that although there were three differences in base pairs between our presented sequence and the reported sequence of accession number AF254070 in GenBank, the amino acid sequence had no change (data not shown).

To obtain recombinant protein mIL-21, Sp2/0 cells were transfected with recombinant pcDNA3.1/mIL-21 by Lipofectamin™ 2000 and the positive clones were selected by G418. Western blotting was performed on Western-Breeze Kit after 12% SDS-polyacrylamide gel electrophoresis. As shown in Figure 1B, there was an anti-mIL-21 response band on nitrocellulose membrane and no band was found in extracts from Sp2/0 cells transfected with blank plasmid, which suggested the mIL-21 was expressed correctly in Sp2/0 cells.

Identification of bioactivities of recombinant mIL-21

T cells proliferation was remarkably increased in present mIL-21 supernatant of Sp2/0 cells origin and Con A. The result suggested that mIL-21 could enhance T-cell proliferation with Con A co-stimulation. Although mIL-21 alone enhanced T-cell proliferation compared with no mIL-21 in supernatant of Sp2/0 cells origin, the enhancing efficacy was lower than that of mIL-21 plus Con A (Figure 2A). The supernatant from pcDNA3.1/mIL-21 transfected Sp2/0 could also stimulate B cell proliferation (Figure 2B).

As shown in Figure 2C, NK cytotoxic activity was increased obviously in supernatant of Sp2/0 cells transfected with recombinant pcDNA3.1/mIL-21, but not in supernatant of Sp2/0 cells transfected with blank

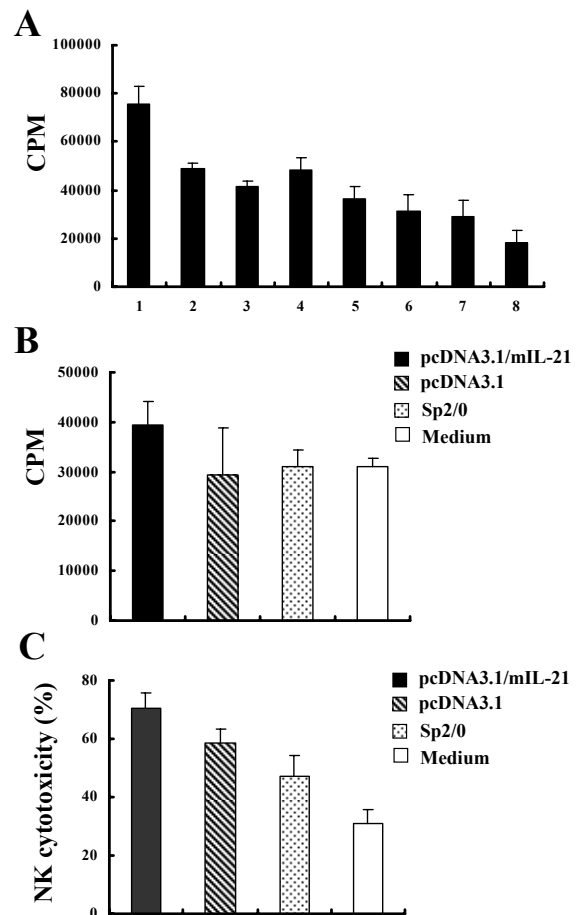


Figure 2. Identification of bioactivities of recombinant mIL-21. (A) T cells proliferation response was enhanced by mIL-21. Group 1, with supernatant of Sp2/0 cells transfected with pcDNA3.1/mIL-21 plus Con A; group 2, with supernatant of Sp2/0 cells transfected with pcDNA3.1 plus Con A; group 3, with supernatant of Sp2/0 cells plus Con A; group 4, with supernatant of Sp2/0 cells transfected with pcDNA3.1/mIL-21; group 5, with supernatant of Sp2/0 cells transfected with pcDNA3.1; group 6, with supernatant of Sp2/0 cells; group 7, with Con A; group 8, with 1640 medium. The results showed that mIL-21 can enhance T-cell purification significantly with ConA co-stimulation compared with other different proliferation responses ($p < 0.05$). (B) B cells proliferation response was enhanced by mIL-21. (C) NK cells cytotoxicity was increased by mIL-21. The results suggested that the mIL-21 could enhance NK cytotoxic activity significantly compared with other group mice ($p < 0.05$).

plasmid. The result suggested that mIL-21 in the supernatant could increase NK cytotoxic activity *in vitro*.

Inhibition of tumor growth in tumor-bearing mice injected intratumorally with recombinant expressed mIL-21

Lump tissue in tumor-bearing mice were directly injected *s.c.* with 100 μ g recombinant pcDNA3.1/mIL-21. Tumor size was measured each week and after the therapy was lasted 4 weeks, the tumor-bearing mice were killed and tumor weight was isolated and weighed in the scales. As shown in Figures 3A and 3B, mIL-21 could inhibit the tumor growth and make lump tissue regress in tumor-bearing mice treated with recombinant pcDNA3.1/mIL-21.

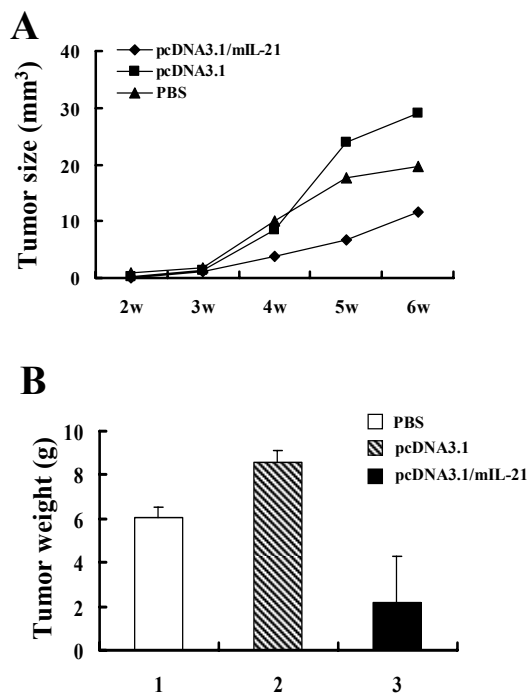


Figure 3. Antitumor effect was promoted by injection with pcDNA3.1/mIL-21. BALB/c mice were inoculated with 5×10^5 Sp2/0 cells for 14 days and tumor-bearing mice were divided randomly into group pcDNA3.1/mIL-21 mice immunized with 100 μ g recombinant pcDNA3.1/mIL-21, group pcDNA3.1 mice immunized with 100 μ g blank plasmid, group tumor control mice immunized with 100 μ l PBS without any treatment and each group had 8 mice. The mice were killed and tumor volume (A) and weight (B) were obtained.

Improvement activities of CTL and NK as well as IFN- γ level in tumor-bearing mice treated with recombinant pcDNA3.1/mIL-21

As shown in Figure 4, the CTL and NK activities were significantly improved in tumor-bearing mice injected with recombinant pcDNA3.1/mIL-21 compared with other three group mice ($p < 0.05$). Although the LAK activity was also enhanced in the pcDNA3.1/mIL-21-treated mice, the diversity had no obvious significant difference compared with other three group mice. The level of serum IFN- γ in pcDNA3.1/mIL-21-treated mice was higher than that of control mice, especially in three mice having distinct curative effect (Figure 5).

Discussion

A number of methods have been devised to augment antitumor immunity in tumor-bearing animal (5, 6) and patients with malignancies (14) as potential therapeutic interventions for the neoplasms. These methods include intratumoral or systemic administration of immunostimulatory molecules, antitumor vaccines, adoptive transfer of immune effector cells, and dendritic cells (DCs) vaccination. Molecules with immunomodulatory functions play key roles in many of these protocols, augmenting antitumor immune responses *in vivo* or activating immune-

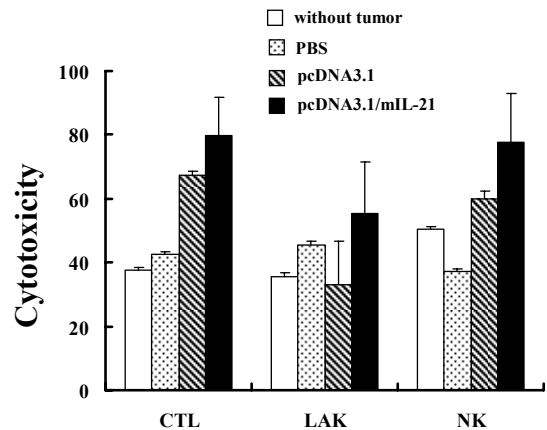


Figure 4. Cytotoxic activities of CTL and NK were improved in pcDNA3.1/mIL-21 treated mice. The cytotoxic activities were measured by the MTT colorimetry assay. There were four group mice in independent experiment and three kind of cytotoxic activities were shown in each group, in where each kind of rectangle chart represented a different cytotoxic activity. The cytotoxic activities of CTL and NK cells were significantly increased in group mice treated with pcDNA3.1/mIL-21 compared with other three groups ($p < 0.05$) and though the LAK activity was also enhanced in group mice treated with pcDNA3.1/mIL-21, the diversity had no obvious significant difference compared with other three group mice.

competent cells *ex vivo*. Therefore, it is important to screen and test potential therapeutic molecules that may be applicable to immunotherapy or immunogene therapy. Although many preclinical and clinical studies had been performed to examine the effects of various cytokines, most of such studies performed to date have had a little problem, such as low efficacy or induction of intolerable adverse effects (15). A novel cytokine is required for the development of a feasible immunotherapeutic strategy against malignancies. In the present study, we investigated the role of mIL-21 in the treatment of mice lymphoma using intratumoral injection of recombinant plasmid encoding IL-21 cDNA.

The results suggested that the cloning of mIL-21 cDNA was correct and mIL-21 cDNA sequence had been accepted by GenBank with the accession number AY428162 and the recombinant plasmid pcDNA3.1/mIL-21 had also been constructed successfully, and it could express mIL-21 protein with bioactivity, which were reflected in co-stimulating T cell proliferations and enhancing NK cytotoxic activity *in vitro* (Figure 2). In application mIL-21 for tumor gene therapy experiment, recombinant plasmid pcDNA3.1/mIL-21 was directly injected into lump tissue and the therapeutic outcome indicated that the tumor inhibition mediated by mIL-21 gene transfection in lump tissue is a good method and it can elicit powerful immune responses against malignant lymphoma *in vivo*. The efficacy of mIL-21 antitumor embodied that the activity of cellular immunity in tumor-bearing mice was improved obviously, especially NK and CTL activities. These results strongly suggested that recombinant pcDNA3.1/mIL-21 inoculated intratumorally was a potential approach for gene therapy.

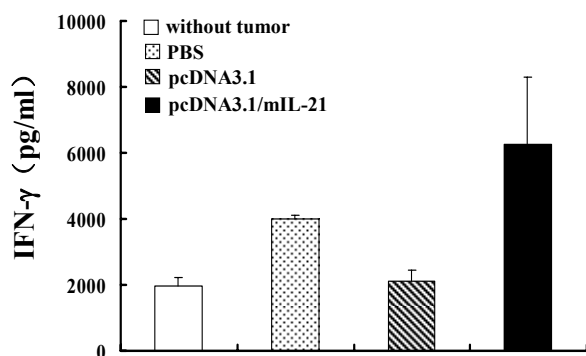


Figure 5. Serum IFN- γ was increased in pcDNA3.1/mIL-21-treated mice. Serum IFN- γ levels in mice treated with different methods were detected by ELISA and all steps were performed according to Kit's protocol. The IFN- γ level in the mice treated with pcDNA3.1/mIL-21 was highest among all groups and the IFN- γ level was increased significantly compared with normal mice, tumor control mice and mice treated with blank plasmid respectively ($p < 0.05$).

Mouse IL-21 secreted locally by transfected tumor cells induce NK cell, DC and CTL accumulation at the tumor site, which make the NK cell directly attack the tumor cell and DC effectively present tumor antigen to CTL and activate CTL cytotoxic activity. Data showed that IL-21 could induce secondary cytokine production, particularly IFN- γ (6) and that IL-21 promoted IFN- γ production by CTLs and NK cells (16). So, IFN- γ secreted by NK cells and CTLs can react on NK cells and CTLs, enhance their cytotoxic activities. In Figure 4 and Figure 5, the cytotoxic activities of NK cells, CTLs and IFN- γ level were significantly increased in mice immunized with pcDNA3.1/mIL-21, in which three mice out of eight mice immunized with pcDNA3.1/mIL-21, their clump tissue ceased growth or regress and curative effect was very significant (data not shown). We also found that mIL-21 had no great effect on LAK activity although it was little higher than that of control mice, and yet there were no significant difference between mice treated with pcDNA3.1/mIL-21 and normal control mice or mice treated with blank plasmid ($p > 0.05$). The level of anti-Sp2/0 cell antibody did not alter significantly in mice treated with recombinant pcDNA3.1/mIL-21 (data not shown). In view of these analyses, we think the mechanism of mIL-21 antitumor may be associated with the cell mediated immunity and Th1 related cytokine IFN- γ in tumor-bearing mice.

Our studies demonstrated that delivery of IL-21 genes suppressed tumor growth in tumor-bearing mice *via* lump-located injection. Although several previous reports had documented immunomodulatory functions of IL-21 *in vitro* or *in vivo*, the results of those studies were a little diversity. Most data showed that IL-21 could co-stimulate T cells, B cells proliferation (1, 6, 17), and NK cytotoxic activity (5, 18) *in vitro*, and promoted NK and CTL cytotoxic activities *in vivo*, however, Kishida's report suggested that IL-21 alone failed to affect NK activity (4). Our current results are compatible with most of previous reports that IL-21 can activate NK and CTL cytotoxicity, and

in especially, IL-21 activates prestimulated but naïve NK cells (15). This is important for antitumor innate immune when NK cells has activated in early neoplasm.

In summary, current experiment data suggest that mIL-21 represent a suitable immune adjuvant in CTL response and activate NK cell as well as induce cytokine IFN- γ production, based on their bioactivities, mIL-21 plays an important antitumor function in tumor-bearing mice. It is also a good way that the recombinant encoding IL-21 protein is directly injected into lump tissue in tumor gene therapy and IL-21 can stimulate robust cellular immune against tumor cells *in vivo*.

References

- Parrish-Novak J, Dillon S R, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 2000;408:57-63.
- Moroz A, Eppolito C, Li Q, et al. IL-21 enhances and sustains CD8⁺ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. *J Immunol*. 2004;173:900-909.
- Ugai S, Shimozato O, Kawamura K, et al. Expression of the interleukine-21 gene in murine colon carcinoma cells generates systemic immunity in the inoculated hosts. *Cancer Gene Ther*. 2003;10:187-192.
- Kishida T, Asada H, Itokawa Y, et al. Interleukin (IL)-21 and IL-15 genetic transfer synergistically augments therapeutic antitumor immunity and promotes regression of metastatic lymphoma. *Mol Ther*. 2003;8:552-558.
- Wang G, Tschoi M, Spolski R, et al. *In vivo* antitumor activity of interleukin 21 mediated by natural killer cells. *Cancer Res*. 2003;63:9016-9022.
- Di Carlo E, Comes A, Orenco AM, et al. IL-21 induces tumor rejection by specific CTL and IFN- γ -dependent CXC chemokines in syngeneic mice. *J Immunol*. 2004;172:1540-1547.
- Shan B, Yu L, Shimozato O, et al. Expression of interleukin-21 and -23 in human esophageal tumors produced antitumor effects in nude mice. *Anticancer Res*. 2004;24:79-82.
- Asao H, Okuyama C, Kumaki S, et al. Cutting edge: the common γ -chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol*. 2001;167:1-5.
- Habib T, Senadheera S, Weinberg K, et al. The common gamma chain (γ_c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation *via* JAK3. *Biochemistry*. 2002;41:8725-8731.
- Sheng GX, Zhou RL. T cell and B cell isolation. In: Guanxin Sheng, eds. *Experiment technique of modern immunology*. Wuhan: Hebei people's publishing; 2002:382-390.
- Dou J, Liu KZ, Chen Z, et al. Study on Peripheral blood mononuclear cell (PBMC) proliferation responses to different HCV antigens in patients with hepatitis C. *Mian Yi Xue Za Zhi*. 1999;15:5-10.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55-63.
- Chen GB, Dou J, Chen JS, Hong XW. Cloning and eukaryotic expression of mouse interleukin 21. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi*. 2004;20:49-52.
- Tagawa, M. Cytokine therapy for cancer. *Curr Pharm Des*. 2000;6:681-699.
- Gionanni C, Nanni P, Forni G. The prospects for cancer gene therapy. *Int J Immunopharmacol*. 2000;22:1025-1032.
- Kasaian MT, Whitters MJ, Carter LL, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity.

- Immunity. 2002;16:559-566.
17. Strengell M, Sareneva T, Foster D, et al. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. *J Immunol.* 2002;169:3600-3605.
 18. Strengell M, Matikainen S, Siren J, et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN- γ production in human NK and T cells. *J Immunol.* 2003;170:5464-5469.