

Interleukin-15: A potent adjuvant enhancing the efficacy of an autologous whole-cell tumor vaccine against Lewis lung carcinoma

XI CHEN^{*}, JIE NI^{*}, HUI MENG, DANDAN LI, YUQUAN WEI, YAN LUO and YANG WU

State Key Laboratory of Biotherapy, West China Hospital, West China Medical School,
Sichuan University, Chengdu, Sichuan 610041, P.R. China

Received January 15, 2014; Accepted February 18, 2014

DOI: 10.3892/mmr.2014.2474

Abstract. Lung cancer is a major cause of cancer-associated mortality worldwide due to its limited response rate to current chemotherapy and radiation, thus immunotherapy is rapidly becoming the most promising approach. Although the highly specific tumor-associated antigen of lung cancer has been found, autologous whole-cell tumor vaccines remain indispensable in the development of therapeutic cancer vaccines. Interleukin (IL)-15 is a T helper type 1 cytokine that has been demonstrated to have a marked antitumor immune response and the potential ability to reverse the host tolerance of tumor antigens in certain preclinical trials. In the present study, a cationic liposome encapsulating IL-15 gene-loaded plasmid acted as an adjuvant of an autologous whole-cell tumor vaccine by subcutaneous injection. The combination immunotherapy resulted in significant inhibition of tumor growth without side effects in the preventive tumor inhibition and adoptive therapy study. Cytotoxic lymphocyte assay detection of the serum antigen and cytokines using an enzyme-linked immunosorbent assay suggested that the IL-15 gene can significantly improve the cellular immune response and humoral immune response provoked by autologous whole-cell tumor vaccines. These results demonstrated that the IL-15 gene was an effective adjuvant of autologous whole-cell tumor vaccines against mouse lung cancer and may provide an attractive vaccine strategy for cancer immunotherapy.

Introduction

Biotherapy is a relatively new approach against cancer compared with conventional surgery, radiation therapy or chemotherapy. Therapeutic cancer vaccines are one of the most important approaches in cancer biotherapy, which aims to induce the immune response of the host to antigens that are selectively expressed by the tumor (1). The optimal cancer vaccine induces an effective antitumor response as well as avoiding unintended tissue damage; therefore, immunogenicity, tumor-specific antigens and suitable delivery systems are all necessary. Current vaccine strategies tend to screen out effective antigens against specified tumors as well as avoid unspecific autoimmunity (2), however, several types of cancer, including lung cancer, lack highly specific biomarkers. Therefore, owing to the complete antigen-spectrum of tumor cells, autologous whole-cell tumor vaccines remain indispensable in the development of therapeutic cancer vaccines (3).

Previous studies have suggested that autologous whole-cell tumor vaccines failed to provoke strong antitumor responses expected in large animal models and humans due to the poor immunogenicity of autologous whole-cell tumor vaccines *in vivo* (4). In order to overcome this, several approaches have been investigated, including genetic engineering, adjuvant exploitation and prime-boost vaccination (5).

Interleukin (IL)-15 has long been considered as one of the most promising cytokines to enhance antitumor activity in several models, as it is important in the innate and adaptive immune system. Since 1994, IL-15 has been understood to have a similar ability to IL-2 in increasing CD8⁺ T-cell expansion and the antitumor activity of tumor-specific T cells, as it shares the same receptor β and γ chains as IL-2 (6). However, certain studies have demonstrated that IL-15 has another unique chain that is responsible for the promotion of survival and maintenance of memory T cells (7-9). In addition, the development, homeostasis, function and survival of natural killer (NK) cells are closely associated with IL-15 (10,11). These differences in characteristics from IL-12 may lead to IL-15 offering more promise in cancer immunotherapy. A previous study demonstrated that an IL-15 gene-modified autologous whole-cell tumor vaccine improved its antitumor efficacy against melanoma (12). This result provided further evidence towards the potential for IL-15 to enhance the immunogenicity of autologous whole-cell tumor vaccines.

Correspondence to: Professor Yang Wu or Professor Yan Luo, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, 1 Keyuan Road 4, Chengdu, Sichuan 610041, P.R. China
E-mail: wuyang@scu.edu.cn
E-mail: yanluo300@sina.com

^{*}Contributed equally

Key words: cell vaccines, interleukin-15, cationic liposome, lung cancer, immunotherapy

A suitable delivery system is another important aspect of vaccine design. Cationic liposomes remain at the forefront of vaccine design, not only owing to their well-documented abilities to act as delivery vehicles, but also due to their immunostimulatory ability as an adjuvant (13). In addition, previous studies indicated that IL-15 may be used as an immunological co-adjuvant for cationic liposomal antigens *in vivo* (14). Based on these findings, rather than modifying an autologous whole-cell tumor vaccine with the IL-15 gene using transgenic technology, the present study used a cationic liposome as a carrier of the IL-15 gene-loaded plasmids owing to the possibility of a co-adjuvant action.

Based on these earlier findings, the present study aimed to investigate the efficacy of cationic liposomal IL-15 DNA as an adjuvant for an autologous whole-cell tumor vaccine and evaluated whether the IL-15 DNA-combined autologous whole-cell tumor vaccine enhances the immunogenic response and antitumor efficacy against lung cancer in mice.

Materials and methods

Cell culture. Lewis lung carcinoma (LL2) cells (purchased from the American Type Culture Collection, Manassas, VA, USA), a cell line passaged routinely in C57BL/6 mice, were cultivated in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL) at 37°C in a humidified incubator with 5% CO₂.

Animal care. C57 male mice aged ~4–6 weeks were purchased from Sichuan University Animal Centre (Sichuan, China) and housed in cages with free access to food and water 1 week prior to experiments. All animal experiments were performed with approval from the Sichuan University Animal Care and Use Committee.

Autologous whole-cell tumor vaccine and cationic liposomal IL-15 plasmid preparation. LL2 cells in the logarithmic growth phase were collected and fixed using 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA). Following being completely fixed, all paraformaldehyde was removed by washing with normal saline (NS; Chengdu Qingshan Li Kang Pharmaceutical Co., Ltd., Sichuan, China) and the whole-cell tumor vaccines were stored at -20°C.

The IL-15-plasmid open reading frame (pORF) and IL-15-free pORF plasmids were purchased from InvivoGen (San Diego, CA, USA) and amplified using *Escherichia coli in vitro*. Plasmids were purified using an Endofree Plasmid Giga kit (Qiagen, Valencia, CA, USA) following verification by restriction enzyme digestion (*Hind*III and *Nhe*I; Takara, Dalian, China) and DNA agarose gel electrophoresis. Fresh positive ion lipoplexes were mixed with plasmids within 30 min prior to immunization to ensure no deposition was present within the mixture.

Immunization. Animals were randomly divided into six groups: Combination group (autologous whole-cell tumor vaccine + IL-15-pORF plasmid), tumor vaccine (TV) group (autologous whole-cell tumor vaccine), IL-15-pORF group (cationic IL-15-pORF plasmid), pORF group (cationic

IL-15-free pORF plasmid), liposome group (positive ion lipoplexes) and NS (normal saline) group. In the combination group, the cationic IL-15-pORF plasmid (25 µg/mouse) was injected at the point of the autologous whole-cell tumor vaccination (10⁶ cells) via a subcutaneous multipoint injection. The other groups were immunized subcutaneously with equal doses of autologous whole-cell tumor vaccine or cationic IL-15-pORF plasmid. Immunizations were administered a total of four times (day 1, 14, 21 and 28).

Serum was obtained from the tail veins of all animals for the detection of antibodies and cytokines at set time points [pre-immune, D0 (the day of tumor inoculation) and D25 (25 days after tumor inoculation)].

Preventive tumor inhibition study. All animals were subcutaneously injected with 2x10⁵ LL2 cells to establish the lung cancer models 7 days after the fourth immunization. Tumor volumes were measured twice a week using calipers and calculated using the following formula: (length x width²) / 2. The tumor inhibition rate was calculated using the following formula: [(Average tumor volume of NS group - average tumor volume of experimental group) / average tumor volume of NS group] x 100%. Animal body weights were also measured twice weekly in order to detect any experimental abnormalities. Three tumor-bearing mice in each group were sacrificed by decapitation following being anesthetized with chloral hydrate to perform a cytotoxicity assay and obtain tumor tissues, whilst five mice in each group were kept for determining the survival rate.

Adoptive therapy study. For passive immunotherapy, splenic lymphocytes were obtained by density gradient centrifugation at 800 g for 30 min using iodixanol solution and nylon wool (Dakewe Biotech Co., Ltd., Shenzhen, China). The LL2 lung cancer models were established, as described previously. Mice were injected with adoptive spleen cells (10⁶-10⁷/mouse) twice a week into the tail vein for 3 weeks. Animals were sacrificed 3 weeks after challenge by tumor cells and the tumor weight was measured.

Cytotoxic lymphocyte (CTL) assay *in vitro*. A lactate dehydrogenase (LDH)-release assay (15,16) was performed to measure the antigen-specific cytotoxicity of splenic lymphocytes *in vitro* using a Non-radioactive Cytotoxicity assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the LL2 cells were seeded in a 96-well plate as target cells. Subsequently, the splenocytes obtained from the immunized or the control mice, as previously described, were added to the plate at different ratios of effector/target (E:T) cells, followed by additional incubation in a humidified chamber at 37°C and 5% CO₂ for 4–6 h. Lysis solution and assay buffer was added at set time points. The absorbance was recorded at 490 nm within 1 h after the addition of 50 µl/well of stop solution. The cytotoxicity was calculated using the following formula: %Cytotoxicity = (experimental - effector spontaneous - target spontaneous) / (target maximum - target spontaneous) x 100.

Measurement of antibodies and cytokines. A cell enzyme-linked immunosorbent assay (ELISA) (17) was performed to measure the number of antibodies against the whole-tumor cell vaccine.

LL2 cells were seeded at a density of 1×10^4 /well into a 96-well plate. Following incubation overnight, the cells were fixed using 4% polysaccharide for 15 min. The fixed tumor cells were incubated with serum from the different groups at several dilutions (for the antibodies, the dilutions were 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000 and for the cytokines, the dilution was 1:5) for 2 h after blocking unspecific antigens with 1% bovine serum albumin for 1 h. Horseradish peroxidase-labeled goat polyclonal anti-mouse immunoglobulin G and 3,3',5,5'-tetramethylbenzidine (Neobioscience, Shenzhen, China) were used for coloration. The result was measured using a microplate reader (3550-UV; Bio-Rad, Hercules, CA, USA) at 450 nm.

The levels of IL-4 and interferon- γ (IFN- γ) in the serum from the subcutaneous tumor inhibition study were determined using specific ELISA kits (Neobioscience, Shenzhen, China) according to the manufacturer's instructions.

Histopathology. The heart, liver, spleen, lungs and kidneys from the mice were fixed in 4% neutral-buffered formalin solution and embedded in paraffin for observation of any potential side effects in the treated mice. Paraffin sections (3-5 mm) of the embedded tumor tissues from each group were stained using hematoxylin and eosin (Sigma-Aldrich).

Immunohistochemical analysis of the tumor sections was performed to investigate the CD4⁺ T cells, CD8⁺ T cells, B cells and NK cells in the tumor tissues from the immunized and control mice with CD4, CD8, CD24 and CD57 antibodies, respectively.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Analysis of variance and an unpaired Student's t-test were performed for comparison of the individual time points. Survival analysis was computed using the Kaplan-Meier method and compared by the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IL-15 gene enhances the efficacy of the autologous whole-tumor cell vaccine against LL2 lung cancer in vivo. In order to determine whether the IL-15 gene can improve the effects of an autologous whole-cell tumor vaccine against murine lung cancer *in vivo*, the effect of the prophylactic combined treatment of the cell vaccine and the IL-15 gene on pulmonary tumorigenesis was evaluated in a subcutaneous LL2 lung cancer model. Combined immunization demonstrated a significant inhibition of tumor growth and the maximum tumor inhibition rate of the combined immunized mice reached 92.9% 15 days after tumor incubation, which was higher than the TV group (70.3%) and the IL-15-pORF group (58.9%; Fig. 1A). Furthermore, the combination group demonstrated apparent advantages in prolonging the median survival time compared with the other groups (Fig. 1B). In addition, no significant abnormal change was identified in the weight of the mice, suggesting no overt systemic toxicity (data not shown).

Combined immunization induces robust humoral responses. ELISA was performed to assess the humoral response in mice. The combination group and the TV group elicited vigorous

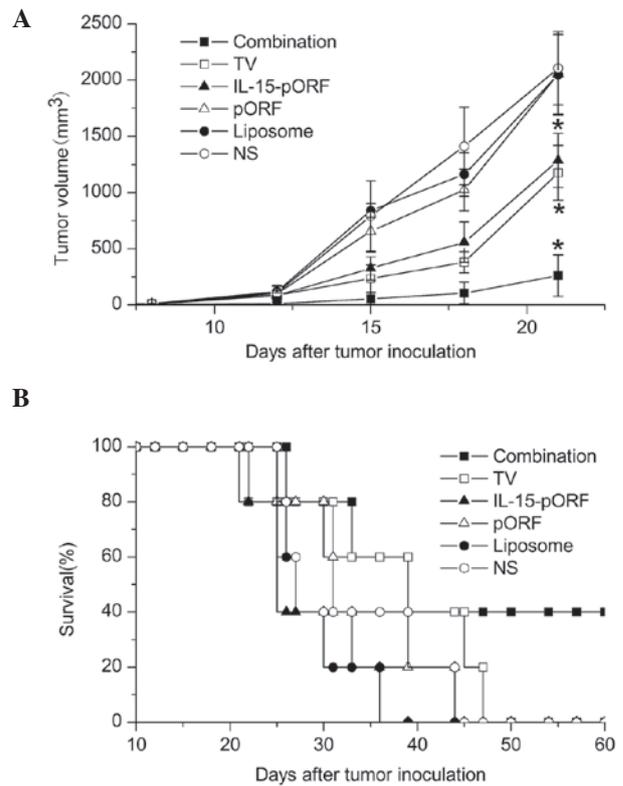


Figure 1. Induction of protective antitumor immunity by combined immunization of the autologous whole-cell tumor vaccine with the IL-15 gene. Mice were randomly divided into six groups (five in each group) and immunized with the autologous whole-cell tumor vaccine ($10^6/100 \mu\text{l}$) + cationic liposomal IL-15-pORF plasmid ($25 \mu\text{g}/100 \mu\text{l}$), autologous whole-cell tumor vaccine ($10^6/100 \mu\text{l}$), cationic liposomal IL-15-pORF plasmid ($25 \mu\text{g}/100 \mu\text{l}$), cationic liposomal IL-15-free pORF plasmid ($10 \mu\text{g}/100 \mu\text{l}$), cationic liposome (same dose as other experimental groups) or NS (0.9% sodium chloride, $100 \mu\text{l}$), respectively. Subcutaneous tumor models were established 7 days after the final vaccination (four in total). (A) Average tumor volume of combined immunized mice was significantly smaller than that of the other groups ($P < 0.05$, compared with the NS group). (B) Median survival percentage of combined immunized mice was prolonged compared with the other groups. TV, tumor vaccine; IL, interleukin; pORF, plasmid open reading frame; NS, normal saline.

production of antibodies following vaccination compared with the other groups and the antibodies persisted at a high level for 25 days after tumor challenge, as shown in Fig. 2A. Notably, the combination group demonstrated a significantly slower reduction and higher titer of antibodies compared with the TV group (Fig. 2B and C). This result indicated that the IL-15 gene succeeded in enhancing the humoral response to the autologous tumor cell vaccine *in vivo*.

Combined immunization induces more marked cellular responses. In order to evaluate the cellular responses, splenic lymphocytes from the groups were harvested 25 days after tumor inoculation. The antigen-specific cytotoxicity of the CD8⁺ T cells was assessed using an LDH-release assay *in vitro*, as described previously. The results demonstrated that potent cellular responses were induced in the combination group, reaching a peak cytotoxicity (E:T ratio=1:100) at 58.17%. The TV group and IL-15-pORF group demonstrated similar cytotoxicities of 33.51 and 31.47%, respectively, while the control groups demonstrated low cytotoxicity at all E:T ratios (Fig. 3).

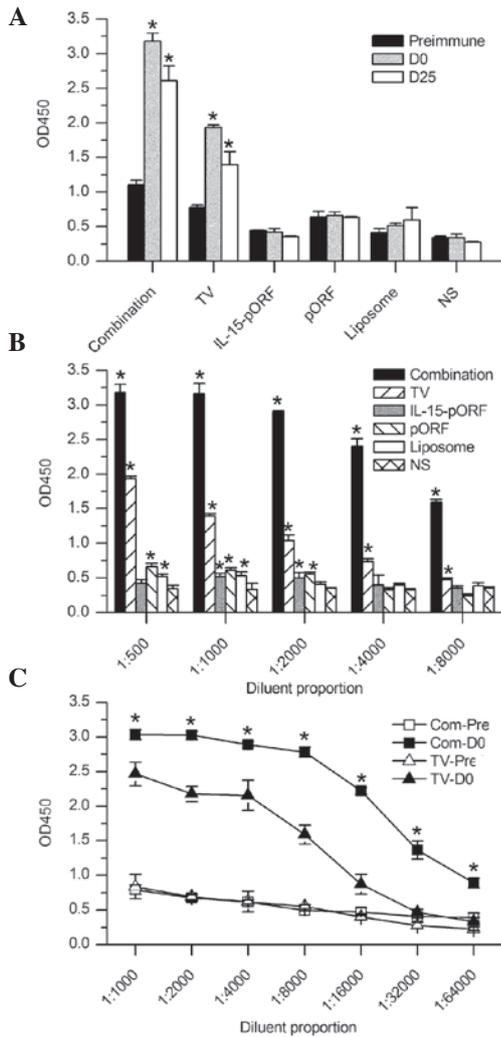


Figure 2. ELISA analysis of the humoral immune response in mice. Serum samples from the mice were analyzed for antibodies against Lewis lung carcinoma cells by cellular ELISA. (A) Antibody titers were determined at different time points, including prior to immunization, the day of tumor inoculation (7 days after the final vaccination) and 25 days after tumor challenge. All serum samples were diluted 500-fold. The combination group elicited a significantly higher production of antibodies following vaccination than the other groups ($P < 0.05$, compared with those pre-immunized). (B) Antibody titers in serum samples at different dilutions on the tumor inoculation day. A significantly higher antibody titer was detected in the combination group compared with the NS group ($P < 0.05$). (C) Semi-quantitative comparison of antibody titer between the combination group and the autologous whole-cell tumor vaccine group on the tumor inoculation day. The combination group demonstrated a significantly slower reduction at the different serum dilution rates than the other groups ($P < 0.05$, compared with the TV-Pre). D, day; Pre, pre-immunization; TV, tumor vaccine; IL, interleukin; pORF, plasmid open reading frame; NS, normal saline; Com, combination group; OD, optical density; ELISA, enzyme-linked immunosorbent assay.

Combined immunization induces a higher IFN- γ response.

To further examine the type of immune response, the secretion of cytokines in the serum was estimated using ELISA. Significantly higher levels of IFN- γ were detected in the mice immunized with the combined vaccine compared with the other groups (Fig. 4A). The IL-15-pORF group also demonstrated a considerable elevation in IFN- γ , which was higher than in the TV group but less than in the combination group. In addition, there was a mild increase in the secretion of IL-4 in the combination group, TV group and IL-15-pORF

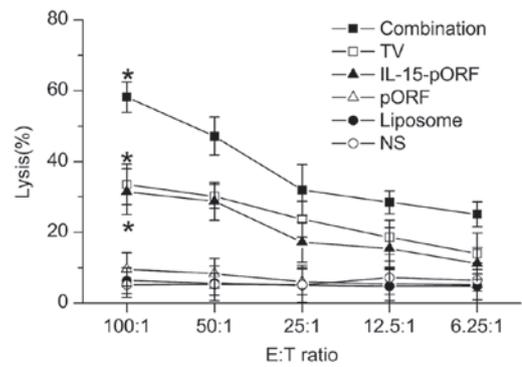


Figure 3. Lactate dehydrogenase release assessment of cytotoxic lymphocyte response *in vitro*. Lewis lung carcinoma cells were used as target cells and splenic lymphocytes from mice 25 days after tumor inoculation were isolated as effector cells. Cytotoxicity was measured and calculated at different E:T ratios. The highest lysis rate was detected in the combination group ($P < 0.05$, compared with the NS group). TV, tumor vaccine; IL, interleukin; pORF, plasmid open reading frame; NS, normal saline; E:T, effector/target cell.

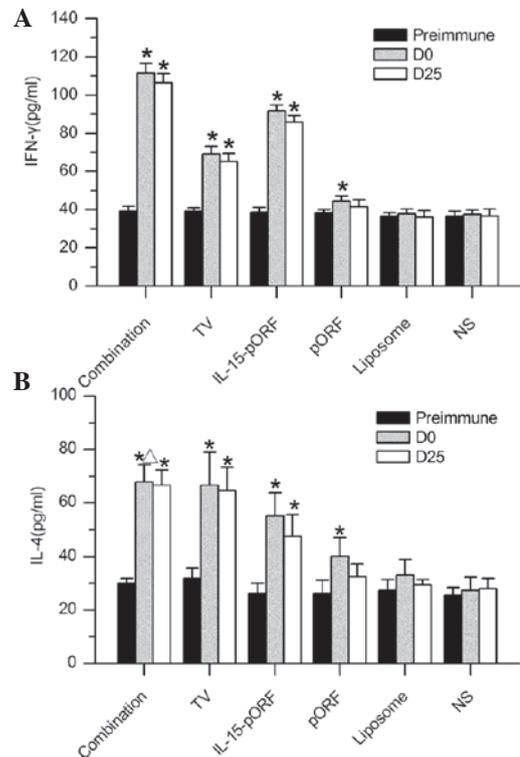


Figure 4. ELISA analysis of cytokine production in mice. Serum samples from mice in each group were analyzed for levels of IFN- γ and IL-4 by ELISA. (A) IFN- γ production in the combination group was significantly higher than the other groups ($P < 0.05$, compared with the pre-immune group). (B) Levels of IL-4 were not significantly different in the combination group, TV group or IL-15-pORF group ($P > 0.05$), but remained higher than in the three control groups ($P < 0.05$, compared with the pre-immune group, $P > 0.05$, compared with the TV group). IFN- γ , interferon- γ ; TV, tumor vaccine; IL, interleukin; pORF, plasmid open reading frame; NS, normal saline; D, day; Pre-immune, preimmunization; ELISA, enzyme-linked immunosorbent assay.

group following immunization, however, this was not significantly different between the three groups (Fig. 4B). The three control groups, including the pORF, liposome and NS group, demonstrated no change in cytokine production.

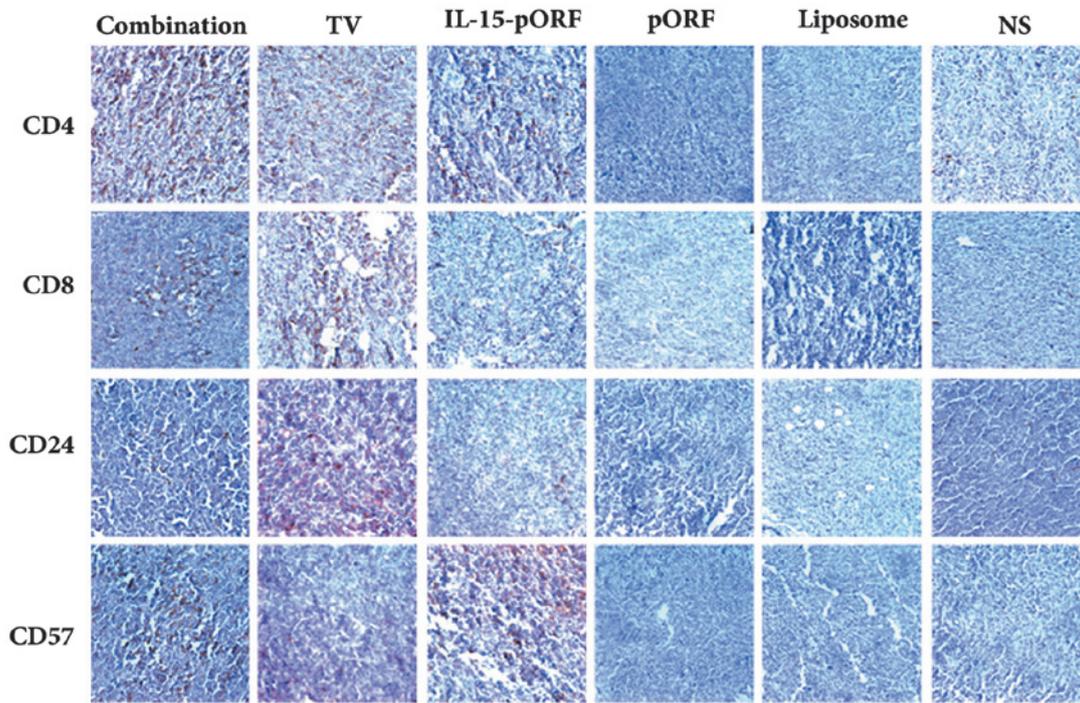


Figure 5. Immunohistochemical staining of the Lewis lung carcinoma tumor samples for CD4, CD8, CD24 and CD57 cells from the preventive tumor inhibition study. CD4⁺ T cells were clearly present in the tumor sections from the combination group and the TV group, followed by the IL-15-pORF group. The number of CD8⁺ T cells were significantly increased in the combination group and the TV group while few were found in the control groups. The number of CD24⁺ B cells increased in the combination group and the TV group. CD57⁺ NK cells were observed in the combination group and the IL-15-pORF group but few NK cells were found in the TV group or other control groups. TV, tumor vaccine; IL, interleukin; pORF, plasmid open reading frame; NS, normal saline.

Combined immunization induces the concentrated gathering of immune cells in tumor tissues. The number of CD4⁺ or CD8⁺ T cells and CD24⁺ B cells in the tumor sections from the TV group notably increased compared with the control groups, while the CD57⁺ NK cells were limited in number in this group. Of note, few CD4⁺ T cells, CD8⁺ T cells or CD24⁺ B cells were observed in the tumor sections from the IL-15-pORF group compared with the control groups. In addition, the number of CD57⁺ NK cells increased significantly compared with the TV group (Fig. 5). Taken together, the sections from the combination group demonstrated the highest density of all types of cells mentioned and the number of immune cells observed in the control groups was low.

Adoptive therapy with lymphocytes from combined immunized mice induces significant inhibition of tumor growth in vivo. Subcutaneous LL2 lung cancer models were established to evaluate whether the combination of the IL-15 gene with an autologous whole-tumor cell vaccine enhances antitumor efficacy with adoptive lymphocytes *in vivo*. As shown in Fig. 6, the maximum tumor inhibition rate of the combined group reached 94.7% 9 days after tumor incubation, which was markedly higher than that observed in the IL-15-pORF group (78.9%) or the TV group (42.1%). This result suggested that the adoptive therapy of lymphocytes from the combined immunized mice induced more marked inhibition of the established tumor growth *in vivo*.

Discussion

Autologous whole-cell tumor vaccines and IL-15 have been used extensively in the treatment of various types of tumor

in pre-clinical studies. Based on previous studies, the present study hypothesized that these two agents could co-stimulate the host immune response against cancer. In the present study, the IL-15 gene was used more widely than the IL-15 protein, as the IL-15 gene can persistently induce the protein expression of IL-15 *in vivo*. It can also be directly co-administered with an autologous whole-cell tumor vaccine for preventive anti-tumor investigation. The present study demonstrated that combined vaccination improved the inhibitory efficacy against subcutaneous lung cancer models and prolonged the median survival time of the tumor-bearing mice.

The mechanisms underlying the robust antitumor efficacy of the combined immunization appears to be complex. Using ELISA, the present study observed that combined immunized mice produced markedly more IFN- γ than those immunized with the tumor cell vaccine or the IL-15 gene alone, however, no significant difference in IL-4 generation was observed between the three experimental groups. This result suggested that the liposomal packaging IL-15 gene combined with a whole-tumor cell vaccine induced a predominantly cellular immune response, as IFN- γ is mainly associated with Th1 and CD8⁺ T cells (18).

Previous studies have reported that IL-15 demonstrates potent antitumor potential, not only by eliciting the priming and proliferation of CD8⁺ T cells, but also by improving the maintenance of memory CD8⁺ T cells (18). In addition, it has been reported that IL-15 was able to reverse the host tolerance of tumor antigens (18), which is commonly observed in patients treated with a tumor vaccine, by increasing the sensitivity of CD8⁺ T cells to tumor antigens. This evidence suggests that CD8⁺ T cells may be important in the antitumor efficacy of

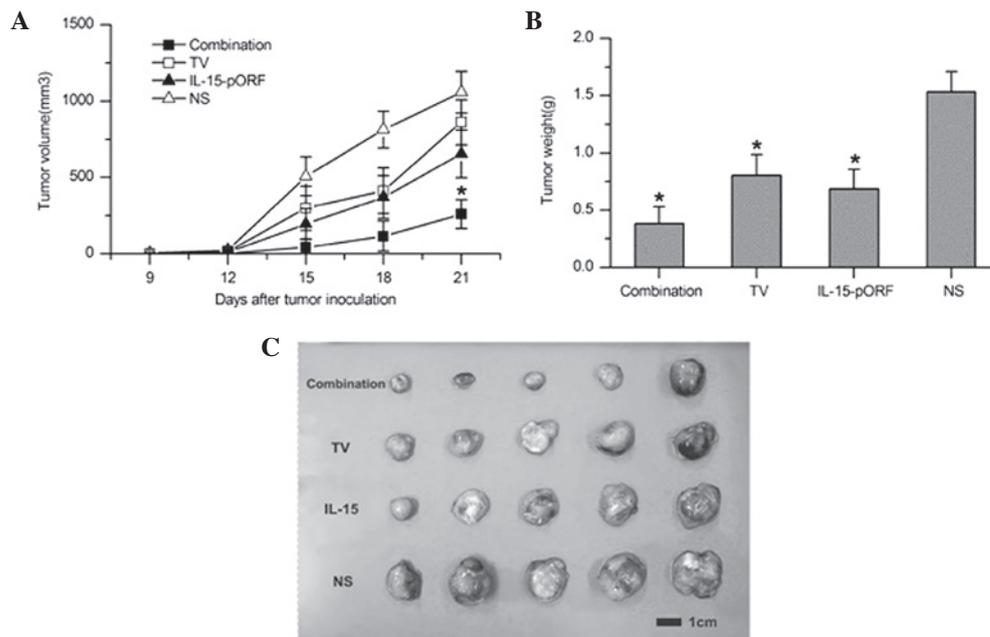


Figure 6. Combined immunization of autologous whole-cell tumor vaccine and the IL-15 gene enhances the adoptive protection of lymphocytes against LL2 lung cancer *in vivo*. Subcutaneous LL2 models were established, as described previously, and randomly divided into four groups (five mice in each). Splenic lymphocytes from the immunized and control mice were separated and transferred adoptively to tumor-bearing mice (10^6 - 10^7 each) twice a week via the tail vein for 3 weeks. (A) Average tumor growth of mice treated with lymphocytes in the combination group was significantly slower than in the other groups ($P < 0.05$, compared with the NS group). (B) Tumors were dissected 21 days after tumor challenge and the tumor weight was measured. Tumors in the combination group were significantly lighter than in the other groups ($P < 0.05$, compared with the NS group). (C) Subcutaneous gross tumor specimens. TV, tumor vaccine; IL, interleukin; pORF, plasmid open reading frame; NS, normal saline; LL2, Lewis lung carcinoma.

IL-15. Since autologous whole-cell tumor vaccines exhibit a considerable efficacy against various types of tumor, mainly through the activation of T cells observed in previous studies, it has been hypothesized that IL-15 may principally coordinate with the whole-tumor cell vaccine through enhancing the function of CD8⁺ T cells. Evidence from a previous study on murine melanoma supported this hypothesis (18). In the present study, data from the CTL assay *in vitro* revealed that the combined immunization induced substantially higher activity against LL2 cancer than the other groups in splenic lymphocytes 25 days after tumor inoculation. Since CD8⁺ T cells are principally involved in CTL activity and, as the subset of memory CTLs generated in response to a vaccination may determine the ultimate effectiveness of the vaccine in eliciting immune protection against cancer, the present study confirmed that combined immunization primarily improved antitumor efficacy by promoting the function of CD8⁺ T cells.

While the major role of CD8⁺ T cells in the antitumor activity of IL-15 and autologous whole-cell tumor vaccines has been established, the antitumor effect of CD4⁺ T cells cannot be excluded. In the present study, IL-15 was also found to regulate CD4⁺ T cells, which were activated by CD3⁺/CD28⁺ and increase the production of IFN- γ , TNF- α and IL-10 (19). Despite a previous report suggesting that IL-15 promotes the apoptosis of CD4⁺ T cells activated by CD3⁺/CD28⁺ *in vitro* (19), autologous whole-cell tumor vaccines may compensate for this disadvantage of IL-15 by inducing a marked proliferation of CD4⁺ T cells (19). Therefore, it was hypothesized that, although the efficacy of combined vaccination was mainly attributed to CD8⁺ T cells, CD4⁺ T cells were also involved. This was confirmed in the present study, which

observed a larger number of CD4⁺ T cells in tumor sections from prophylactic immunized mice through immunohistochemical analysis. However, more evidence is required to further elucidate the function of the CD4⁺ T cells affected by combined immunization.

An adoptive therapy study was also performed in the present study to confirm the role of the cellular response against the tumor in the combined vaccination *in vivo*. The results suggested that splenic lymphocytes from the combined immunized mice induced significant inhibition of established tumor growth when compared with the other groups.

Although no significant difference was identified in the generation of IL-4 between the three experimental groups, the ELISA result exhibited a notably higher humoral response in the combined immunized mice. Previous studies have reported that IL-15 is markedly correlated with NK cell proliferation, differentiation and maturation (10,11). NK cells are considered as one of the most indispensable aspects of host antitumor activity owing to the marked upregulation of antibody-dependent cell-mediated cytotoxicity (ADCC) when IL-15 was used in tumor-bearing animals as an antineoplastic drug alone. In addition, whole-tumor cell vaccine and IL-15 can induce B cell proliferation and induce the production of antibodies (20). Therefore, another possible explanation for the antitumor efficacy enhancement of combined immunization is that the increasing number of tumor-specific antibodies and functional NK cells may co-act on the upregulation of ADCC. Immunohistochemical analysis of tumor sections in the present study confirmed that CD24⁺ B cells and CD57⁺ NK cells were substantially concentrated in the combined immunized tumor tissues.

In the present study, preventive vaccination with the liposomal IL-15 gene alone was invaluable in improving the median survival time of tumor-bearing mice, while the average tumor volume and immune reaction suggested a significant antitumor activity *in vivo*. A possible explanation for this is that IL-15 has an additional potential for the promotion of angiogenesis, as vascular endothelial cells express soluble receptor- α , which has high affinity to IL-15 (21). Thus, an increasing rate of lung metastasis may be observed in IL-15-treated subcutaneous lung cancer models due to the growing number of tumor angiogenic blood vessels. This hypothesis may also explain the previous evidence of a correlation between poor outcome and a high concentration of IL-15 in lung cancer patients (22). However, it has been hypothesized that tumor angiogenic blood vessels may assist antitumor immune cells, including CTLs, in reaching the target cells, which may be the reason for certain studies demonstrating the potential for improving the outcome of tumor-bearing animals using IL-15 as the only therapeutic agent. Further investigation is required to confirm the correlation between angiogenesis caused by IL-15 and the final outcome in lung cancer models.

Through use of an LL2 subcutaneous tumor model, the present study demonstrated that the cationic liposome-carrying IL-15 gene combined with an autologous whole-cell tumor vaccine may stimulate the host innate and adaptive immune system to develop robust antitumor immunity. This antitumor immunity protected the mice from challenge with a parental tumor. Furthermore, the adoptive transferring lymphocytes in the combined immunized mice inhibited the growth of the pre-challenged tumor. In addition, the development of tumor immunity in response to combined vaccination was principally dependent upon the function of CD8⁺ T cells and the activation of ADCC.

Acknowledgements

Yang Wu and Yan Luo participated in designing the experiments, analyzing the data and revising the manuscript. Xi Chen participated in performing the cell culture, animal studies, analyzing the data and writing the manuscript. Jie Ni participated in performing the animal studies and in collecting samples from tumor-bearing mice. Hui Meng and Dandan Li participated in conducting the animal studies and the CTL assay. All authors read and approved the final manuscript. This study was supported by the Special Projects of the National Natural Sciences Foundation of China (no. 81123003) and the Project of the National Natural Sciences Foundation of China (no. 30901773).

References

- Pardoll DM: Cancer vaccines. *Nat Med* 4: 525-531, 1998.
- Van Der Bruggen P, Zhang Y, Chauv P, *et al*: Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol Rev* 188: 51-64, 2002.
- Ward S, Casey D, Labarthe MC, *et al*: Immunotherapeutic potential of whole tumour cells. *Cancer Immunol Immunother* 51: 351-357, 2002.
- Le DT, Pardoll DM and Jaffee EM: Cellular vaccine approaches. *Cancer J* 16: 304-310, 2010.
- Belardelli F, Ferrantini M, Parmiani G, Schlom J and Garaci E: International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? *Cancer Res* 64: 6827-6830, 2004.
- Grabstein KH, Eisenman J, Shanebeck K, *et al*: Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* 264: 965-968, 1994.
- Ku CC, Murakami M, Sakamoto A, Kappler J and Marrack P: Control of homeostasis of CD8⁺ memory T cells by opposing cytokines. *Science* 288: 675-678, 2000.
- Becker TC, Wherry EJ, Boone D, *et al*: Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med* 195: 1541-1548, 2002.
- Zhang X, Sun S, Hwang I, Tough DF and Sprent J: Potent and selective stimulation of memory-phenotype CD8⁺ T cells *in vivo* by IL-15. *Immunity* 8: 591-599, 1998.
- Prlc M, Blazar BR, Farrar MA and Jameson SC: *In vivo* survival and homeostatic proliferation of natural killer cells. *J Exp Med* 197: 967-976, 2003.
- Carson WE, Giri JG, Lindemann MJ, *et al*: Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med* 180: 1395-1403, 1994.
- Basak GW, Zapala L, Wysocki PJ, Mackiewicz A, Jakobisiak M and Lasek W: Interleukin 15 augments antitumor activity of cytokine gene-modified melanoma cell vaccines in a murine model. *Oncology Rep* 19: 1173-1179, 2008.
- Christensen D, Korsholm KS, Andersen P and Agger EM: Cationic liposomes as vaccine adjuvants. *Expert Rev Vaccines* 10: 513-521, 2011.
- Gursel M and Gregoriadis G: Interleukin-15 acts as an immunological co-adjuvant for liposomal antigen *in vivo*. *Immunol Lett* 55: 161-165, 1997.
- Ge W, Li Y, Li ZS, *et al*: The antitumor immune responses induced by nanoemulsion-encapsulated MAGE1-HSP70/SEA complex protein vaccine following peroral administration route. *Cancer Immunol Immunother* 58: 201-208, 2009.
- Yang YW, Wu CA and Morrow WJ: Cell death induced by vaccine adjuvants containing surfactants. *Vaccine* 22: 1524-1536, 2004.
- Lee JC, Cevallos AM, Naeem A, Lennard-Jones JE and Farthing MJ: Detection of anti-colon antibodies in inflammatory bowel disease using human cultured colonic cells. *Gut* 44: 196-202, 1999.
- Kelso A, Troutt AB, Maraskovsky E, *et al*: Heterogeneity in lymphokine profiles of CD4⁺ and CD8⁺ T cells and clones activated *in vivo* and *in vitro*. *Immunological Rev* 123: 85-114, 1991.
- Lin SJ, Cheng PJ and Hsiao SS: Effect of interleukin-15 on effector and regulatory function of anti-CD3/anti-CD28-stimulated CD4(+) T cells. *Bone Marrow Transplant* 37: 881-887, 2006.
- Armitage RJ, Macduff BM, Eisenman J, Paxton R and Grabstein KH: IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J Immunol* 154: 483-490, 1995.
- Angiolillo AL, Kanegane H, Sgadari C, Reaman GH and Tosato G: Interleukin-15 promotes angiogenesis *in vivo*. *Biochem Biophys Res Commun* 233: 231-237, 1997.
- Seike M, Yanaihara N, Bowman ED, *et al*: Use of a cytokine gene expression signature in lung adenocarcinoma and the surrounding tissue as a prognostic classifier. *J Natl Cancer Inst* 99: 1257-1269, 2007.