

# B16 cell lysates plus polyinosinic-cytidylic acid effectively eradicate melanoma in a mouse model by acting as a prophylactic vaccine

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**Abstract.** Th1 antigen-specific T cells secrete interferon- $\gamma$ , which is able to kill antigen-specific cancer cells and is helpful for cancer vaccines. The aim of the present study was to explore whether B16 cell lysates plus polyinosinic-cytidylic acid (poly I:C) can effectively inhibit the progression of melanoma in an animal model. In the present study, C57BL/6 mice were divided into three groups, with each group containing more than six mice. The groups of mice were immunized twice with B16 cell lysates plus poly I:C, B16 cell lysates, or phosphate-buffered saline only, respectively. The *in vivo* results demonstrated that splenocytes from the mice immunized with B16 cell lysates plus poly I:C contained higher percentages of CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes and CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes, which were detected by a fluorescence-activated cell sorter, and produced higher levels of antigen-specific splenocyte proliferation activity, as detected by MTT assay. The splenocytes from the mice immunized with B16 cell lysates in combination with poly I:C produced higher levels of interferon- $\gamma$ , as detected by quantitative polymerase chain reaction and ELISA, as well as cytotoxic T lymphocyte activity when stimulated *in vitro* with B16 lysates. Additionally, subcutaneous immunization of the C57BL/6 mice with B16 cell lysates plus poly I:C conferred greater protection against tumor-forming B16 melanoma cells than that of the mice immunized with injection of B16 cell lysate alone. In conclusion, the cancer vaccine of B16 cell lysates plus poly I:C exerts potently protective effects that polarize responses toward Th1 and elicit antitumor immunity.

## Introduction

Malignant melanoma is a serious disease arising from melanocytes which threatens human health in China and worldwide (1,2). The incidence and mortality rate of malignant melanoma continues to increase at a higher rate than that of any other type of malignancy (3,4). Although melanoma is curable if detected at an early localized stage, metastatic malignant melanoma has already become a therapeutic challenge (5). Hundreds of patients with advanced stage III or IV melanoma, particularly those with metastatic disease, have participated in studies of immunological therapy having failed on chemotherapy (6). Thus, ways to prevent and treat malignant melanoma using immunological methods are urgently required. Vaccination has been used for centuries, causing mortality due to infectious disease in humans to profoundly decrease, but a number of serious global diseases with no effective vaccines remain, including acquired immunodeficiency syndrome, influenza, malaria and cancer. It is harder to produce effective immune responses when using vaccines as a treatment for cancer, compared with when using preventive cancer vaccines (7,8). The cancer vaccine for cervical tumors is the first vaccine to prevent human cancer. With the success of the cervical cancer vaccine, an increasing number of researchers have been working to identify novel and effective cancer vaccines. Thus, the aim of the present study is to identify novel vaccines that elicit stronger and more directed antitumor immune responses.

Cancer vaccine antigens include purified or recombinant proteins or peptides. They are frequently poorly immunogenic and require an effective adjuvant to help elicit protective immune responses based on antibodies or activated T cells (9-11). Polyinosinic-cytidylic acid (poly I:C) is a synthetic double-stranded RNA that has been used as an adjuvant (12). Poly I:C can act with distinct types of pathogen recognition receptors, which bind to toll-like receptor 3 (TLR3) or activate cytosolic RNA helicases, including retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (13,14). Therefore, activation of TLR3 and MDA5 could trigger effective inflammatory responses. Rapid innate immunity would be elicited and the magnitude and durability of type-1 T helper

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(Th1) cell immunity and CD8<sup>+</sup> T cell immunity would be optimized compared with either pathway alone (15-18). Therefore, poly I:C was selected as the adjuvant for cancer vaccines in the present study.

The use of animal models is important in the study of malignant melanoma (19). The metastatic B16 mouse melanoma cell line originates from C57BL/6 mice and has a high metastatic frequency that easily mimics clinical metastatic melanoma (20). Thus, C57BL/6 mice bearing B16 melanoma were used as a mouse model for malignant melanoma in the present study. B16 melanoma lysates were used as the antigen combined with effective Th1 response-related poly I:C as an adjuvant in the cancer vaccine, which could effectively elicit the innate and adaptive immune responses. The objective of this study was to explore the role and identify the effectiveness of *in vivo* vaccination with B16 cell lysates on tumors in the mouse model. The study may aid the development of a vaccine for malignant melanoma and provide novel therapeutic ideas for this currently untreatable disease.

## Materials and methods

**Cell line.** The B16 melanoma cell line was maintained in our laboratory at the Chinese PLA General Hospital (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Thermo Trace Ltd, Melbourne, Australia) at 37°C in an atmosphere of 95% air and 5% CO<sub>2</sub>.

**Mice.** Male 6-8-week-old C57BL/6 mice were purchased from Vital River Biotechnology Co., Ltd. (Beijing, China). Animals were maintained in micro-isolator cages in specific pathogen-free conditions. They were handled under aseptic conditions following a protocol approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital (Beijing, China). All studies were approved by the Animal Study Committee of the Chinese PLA General Hospital.

**Animal grouping and immunization.** The mice were randomly divided into three groups. Each group contained more than six mice. Animals were injected intraperitoneally twice on days 1 and 15, with 50 µg B16 cell lysate antigen or 50 µg B16 cell lysate plus 50 µg poly I:C, 1.5% Al(OH)<sub>3</sub> or with PBS. After the final immunization, the C57BL/6 mice were inoculated intraperitoneally with 1x10<sup>5</sup> melanoma cells suspended in 100 µl PBS.

**Tumor cell lysate.** B16 cells were collected and washed three times with phosphate-buffered saline (PBS) buffer. Eight snap freeze-thaw cycles between liquid N<sub>2</sub> and 37°C were conducted. The cells were centrifuged at 500 x g to obtain the lysate, which was then filtered with a 70-mm Falcon filter (BD Biosciences, Erembodegen, Belgium). Coomassie blue staining method was used to measure the amount of protein and was performed according to the manufacturer's instructions (Benda Biotechnology, Co., Shanghai, China). The lysate was separated and kept frozen in liquid N<sub>2</sub> until required.

**Splenocyte proliferation assay.** Cell proliferation levels were determined using the MTT assay (21). One week after the final immunization, single-cell suspensions from the mice in each group were prepared under sterile conditions. Red blood cells (RBCs) were lysed using lysis buffer containing 0.75% NH<sub>4</sub>Cl in Tris-buffer. Cell concentrations were adjusted to 3x10<sup>6</sup> cells/ml in DMEM supplemented with 10% FBS. Samples (100 µl) of the suspensions were dispensed into 96-well round-bottom culture plates (Costar, Tewksbury, MA, USA) and incubated with 10 µg/ml B16 melanoma cell lysate for 48 h at 37°C in a 5% CO<sub>2</sub> humid incubator.

**Quantitative PCR analysis of mRNA expression.** Splenocytes from the immunized mice were cultured in six-well plates for 24 h at 37°C in the presence of 5% CO<sub>2</sub>, with or without 10 µg/ml B16 cell lysate. Total RNA was extracted with an RNAPure kit (Biotek, Beijing, China) and retrotranscribed with murine leukemia virus (MLV) reverse transcriptase (RT) (Invitrogen Life Technologies). PCR amplifications were performed using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and each sample was tested in triplicate. Thermal cycling conditions were 40 cycles of 12 sec at 95°C and 1 min at 60°C using SYBR-Green (Invitrogen Life Technologies). β-actin was used as the internal reference gene. The primers used were as follows: Interferon-γ (IFN-γ), 5'-CAGCAACAGCAAGGCGAAA-3' and 5'-CTGGACCTGTGGGTTGTTGAC-3'; β-actin, 5'-AGAGGGAAATCGTGCGTGAC-3' and 5'-CAATAGTGTGACCTGGCCGT-3'.

**Fluorescence-activated cell sorter (FACS) analysis.** Seven days after the final immunization, single cell suspensions were performed for spleen T-cell subtype analysis. RBCs from 50 µl heparin-treated orbital blood were lysed with RBC lysis buffer (eBioscience, San Diego, CA, USA). Lymphocytes were stained with 100 µl PBS plus 1% bovine serum albumin and 0.1% NaN<sub>3</sub> together with 5 µl fluorescein isothiocyanate-conjugated anti-CD3 monoclonal antibody (mAb) followed by simultaneous staining with 5 µl phycoerythrin anti-CD4 or anti-CD8 mAb, and then incubated for 20 min at 4°C. Flow cytometry was performed using CellQuest software and a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), with FlowJo software (Tree Star Inc., Ashland, OR, USA) used for data analysis.

**Statistical analysis.** Survival curves of the animals treated with different protocols were plotted according to the Kaplan-Meier method. Statistical significance in different treatment groups was compared using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Antigen-specific splenocytes proliferate in mice immunized with B16 cell lysates plus poly I:C.** In order to detect whether the splenocytes of the mice immunized with B16 cell lysates plus poly I:C had an elevated antigen-specific proliferation rate, the cell numbers were measured by MTT assay. Ten

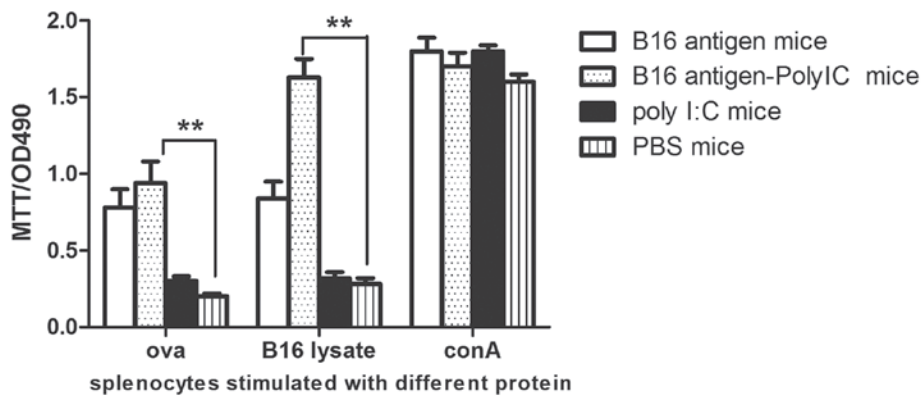


Figure 1. Splenocyte proliferation measured by MTT assay. Splenocyte numbers were measured in response to treatment with 10  $\mu\text{g/ml}$  ovalbumin, 50  $\mu\text{g/ml}$  B16 lysate or 10  $\mu\text{g/ml}$  final concentration of ConA for 24 h. Data are shown as the mean  $\pm$  standard error of the mean of at least three independent experiments on different individuals. \*\* $P < 0.01$  compared with B16 antigen group. Poly I:C, polyinosinic-cytidylic acid; PBS, phosphate-buffered saline; OD, optical density.

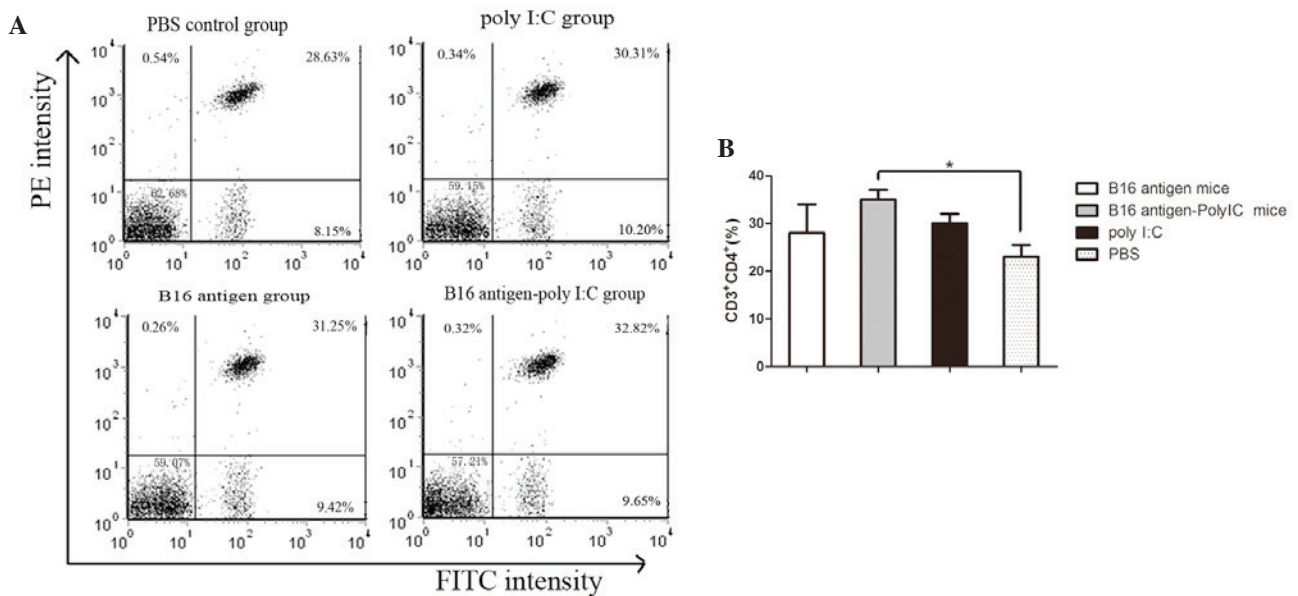


Figure 2.  $\text{CD4}^+\text{CD3}^+$  T cell subpopulations. Mice were immunized as described in Materials and methods. Seven days after the final immunization, leukocytes were collected from peripheral blood samples in the venous plexus of the eyes. Red blood cells were lysed with red blood cell lysis buffer, and the lymphocytes were stained for the T cell surface markers CD3 (fluorescein isothiocyanate-conjugated monoclonal antibody) and CD4 (phycoerythrin-conjugated monoclonal antibody). (A)  $\text{CD4}^+\text{CD3}^+$  T cell subpopulations was detected by FACS. (B) Histogram of  $\text{CD4}^+\text{CD3}^+$  T cell subpopulations. \* $P < 0.05$  compared with PBS group.  $n = 6$ ; values are the mean  $\pm$  standard error of the mean. Poly I:C, polyinosinic-cytidylic acid; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting.

days after the final immunization, spleens from mice in each group were removed and the rate of splenocyte proliferation was measured. After *in vitro* stimulation with B16 lysates for 24 h, the splenocytes from the mice immunized with B16 lysates in combination with poly I:C were significantly more numerous than those in either control group ( $P < 0.01$ ; Fig. 1). The cells treated with Concanavalin A (ConA) were used as positive controls and untreated cells were used as negative controls.

**Number of  $\text{CD4}^+\text{CD3}^+$  T lymphocytes and  $\text{CD8}^+\text{CD3}^+$  T lymphocytes increases in mice immunized with B16 cell lysate plus poly I:C.** T-cell subsets were also analyzed. The percentages of T helper cells ( $\text{CD4}^+\text{CD3}^+$  T lymphocytes) and cytotoxic T cells ( $\text{CD8}^+\text{CD3}^+$  T lymphocytes) were determined by flow cytometry. As shown in Fig. 2, the mice immu-

nized with B16 cell lysate plus poly I:C contained a higher percentage of  $\text{CD3}^+\text{CD4}^+$  T lymphocytes in their peripheral blood than that of the mice injected with PBS. In addition, the frequency of  $\text{CD8}^+\text{CD3}^+$  T lymphocytes in the peripheral blood appeared to increase in the mice immunized by B16 cell lysates plus poly I:C compared with that of the mice injected with PBS (Fig. 3).

**IFN- $\gamma$  expression determined by qPCR and cytokine secretion measured by ELISA.** C57BL/6 mice were immunized twice with B16 cell lysates in combination with poly I:C, the antigen alone, or PBS. Ten days after the final immunization, spleens were removed and splenocytes of single cell suspension were prepared. In order to compare the cell-mediated immune responses among the three groups, splenocytes from the immunized mice were *in vitro*-stimulated with B16

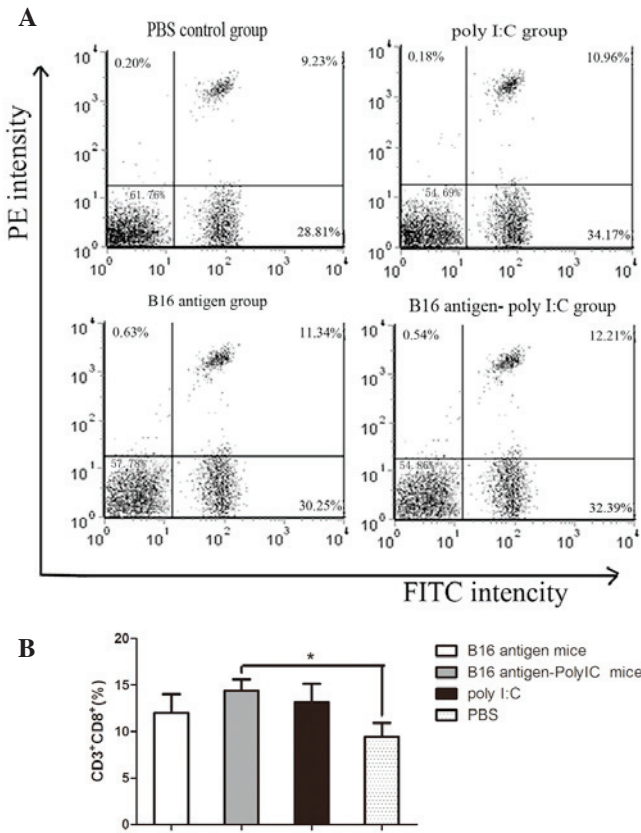


Figure 3. CD8<sup>+</sup>CD3<sup>+</sup> T cell subpopulations. Seven days after the final immunization, peripheral blood samples were collected from the venous plexus of the eyes. (A) Red blood cells were lysed with red blood cell lysis buffer and the lymphocytes were stained for the T cell surface markers CD3 (fluorescein isothiocyanate-conjugated monoclonal antibody) and CD8 (phycoerythrin-conjugated monoclonal antibody) using a FACS assay. (B) Histogram of CD4<sup>+</sup>CD8<sup>+</sup> T cell subpopulations. \*P<0.05 compared with PBS group. n=6; values are the mean ± standard error of the mean. Poly I:C, polyinosinic-cytidylic acid; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting.

antigen for 24 h and IFN- $\gamma$  mRNA levels were analyzed using qRT-PCR (amplification curve shown in Fig. 4A). The mean relative IFN- $\gamma$  mRNA expression in the mice immunized with B16 cell lysate plus poly I:C was significantly higher than that in the mice immunized with the B16 cell lysate only (P<0.01; Fig. 4B). IFN- $\gamma$  protein levels were also examined by ELISA. As expected, the mean IFN- $\gamma$  production in the mice immunized with B16 cell lysate was higher than that in the antigen-immunized group following *in vitro* stimulation with B16 cell lysates (Fig. 4C).

**Cytotoxic T lymphocyte (CTL) activity.** To demonstrate the cytotoxic activity of splenocytes from the immunized mice for B16 cells, the release of cytosolic lactate dehydrogenase (LDH) into the culture medium by a damaged B16 melanoma cell line was tested. Ten days after the final immunization, damage to the membranes of B16 melanoma cells was evaluated in a 24 h cytotoxicity assay by measuring LDH release. LDH release assays were performed with splenocytes as effector cells and B16 melanoma cells as target cells. The effector-to-target cell ratios were 5:1, 10:1 and 20:1. As shown in Fig. 5, the CTL response was significantly higher

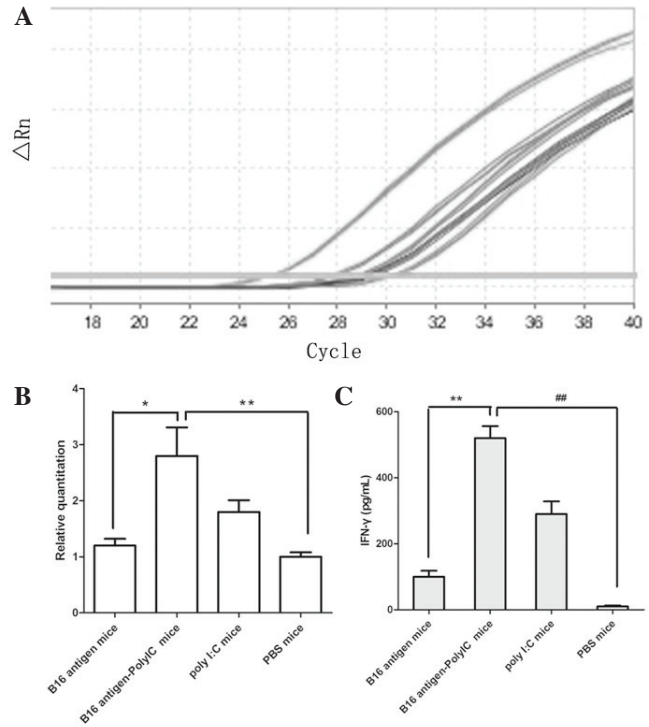


Figure 4. IFN- $\gamma$  gene expression and protein secretion in splenocytes harvested from immunized mice following *in vitro* restimulation. Splenocytes from the mice immunized with B16 antigen combined with or without poly I:C were isolated seven days after the final immunization and incubated *in vitro* with B16 lysate for 24 h. (A) Amplification curve of IFN- $\gamma$  in mice immunized by B16 antigen or B16 antigen with poly I:C. (B) IFN- $\gamma$  gene expression. (C) IFN- $\gamma$  production detected by ELISA. Data are the mean ± standard deviation of the relative quantities of cytokine mRNAs. \*P<0.05, \*\*P<0.01 and ##P<0.01. Poly I:C, polyinosinic-cytidylic acid; PBS, phosphate-buffered saline; IFN- $\gamma$ , interferon- $\gamma$ .

in the mice immunized with B16 lysate plus poly I:C than that in those immunized with B16 lysate alone (P<0.01) or PBS (P<0.01). B16 melanoma cells and splenocytes from the immunized mice did not release any LDH when measured at 24 h. These were cultured alone in DMEM medium and were used as negative controls.

**Improved antitumor effects in B16 lysate plus poly I:C vaccinated mice compared with those in mice immunized with B16 lysate or PBS only.** To assess *in vivo* antitumor responses in the immunized mice, the survival rates were evaluated in the immunized groups. Following the final immunization, all the mice received an intraperitoneal challenge of 1x10<sup>5</sup> B16 melanoma cells. The results revealed that subcutaneous immunization of C57BL/6 mice with B16 cell lysate plus poly I:C conferred improved protection against B16 melanoma cells than did immunization with B16 cell lysate or PBS alone. The survival rate of the B16 plus poly I:C group was significantly higher than that of the B16 lysate group and PBS group (P=0.029 vs. B16 lysate group, P=0.003 vs. PBS group), as shown in Fig. 6. Also the antitumor effects in the mice immunized by B16 antigen plus poly I:C were significantly greater than those in mice immunized by B16 antigen plus Al<sub>2</sub>(OH)<sub>3</sub>, which is used as the positive adjuvant in market.



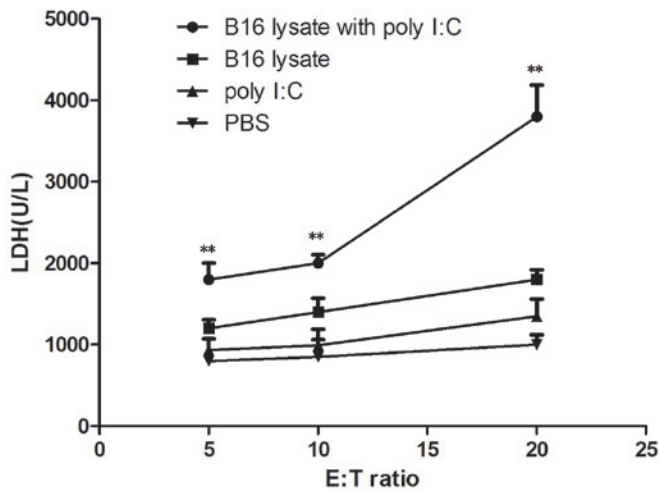


Figure 5. CTL assays. LDH release assays were performed with splenocytes as effector cells and B16 melanoma cells as target cells. The ratios of effector cell to target cell (E:T) were 5:1, 10:1 and 20:1. Specific lysis of target cells was calculated using the following formula: Specific lysis (%) = [(experimental LDH release) - (spontaneous LDH release)]/[(maximum LDH release) - (spontaneous LDH release)] x 100. All of the experiments were performed in triplicate and repeated 4 times and the results were calculated as the mean  $\pm$  standard deviation. ( $P < 0.01$ ). Poly I:C, polyinosinic-cytidylic acid; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; CTL, cytotoxic T lymphocyte.

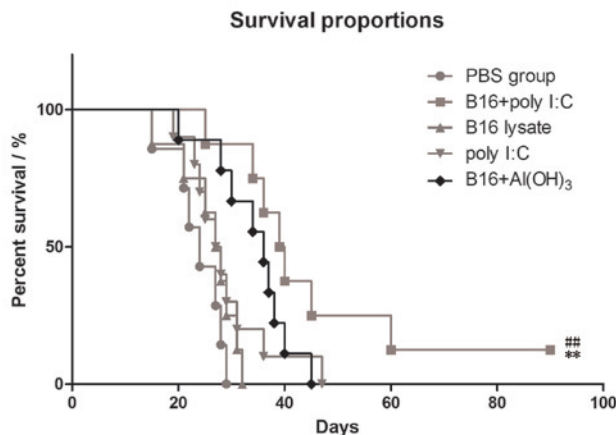


Figure 6. Survival proportions of immunized mice following lethal melanoma B16 challenge. C57BL/6 mice were immunized subcutaneously on the thigh with B16 in combination with poly I:C, Al(OH)<sub>3</sub> and B16 lysate, or PBS alone. One week after the final immunization, mice were challenged subcutaneously with  $2 \times 10^5$  B16 melanoma cells. Each group contained more than six mice. The survival rate was determined as 100 x (number of survivors)/(number of challenged mice). Poly I:C, polyinosinic-cytidylic acid; PBS, phosphate-buffered saline. \*\* $P = 0.029$  vs. B16 lysate group; \*\*\* $P = 0.003$  vs. PBS group.

## Discussion

Cancer vaccines have been studied for several decades and are intended either to prevent the development of cancer or to treat existing cancers (22-24). However, advances in this field have been slower than those in other forms of immunotherapy (24-26). In order to overcome the poor immunogenicity of tumors, administration of tumor

antigens with an effective adjuvant is theoretically a good strategy. The adjuvant could be a molecule that is able to activate dendritic cells (DCs) and induce potent antitumor T-cell immune responses (27). Ligands of toll-like receptors (TLRs) are the best candidates to activate DCs and can lead to DC maturation. Thus, with the aim of inducing potent antitumor T-cell responses, poly I:C, the ligand of TLR3, was selected as the adjuvant of the tumor antigen to strongly activate DCs and facilitate T-cell priming in the present study (14,26,28,29). The results of the study clearly demonstrate that poly I:C was an effective adjuvant for B16 cell lysates and successfully induced effective antitumor immune responses.

It is reported that antitumor activity requires the participation of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes (30,31). Th1 cells exhibit a critical role in cellular immunity by releasing cytokines that activate CD8<sup>+</sup> T cells. Thus, activation of CD4 T helper cells is an important step for the priming of memory CTL responses. CD8<sup>+</sup> T cells are the main effector cells with CTL activity, however the main cells producing cytokines are CD4<sup>+</sup> Th1 cells, including interleukin-2, IFN- $\gamma$  and tumor necrosis factor- $\alpha$ . In the present study, the antigen-specific Th1 responses and CTL response after the final immunization in different groups of mice were measured. Supporting the idea that the induction of IFN- $\gamma$  suggests polarization towards the Th1 response, the group of mice immunized with B16 cell lysate in combination with poly I:C produced increased levels of IFN- $\gamma$  and specific CTL activity when stimulated *in vitro* with B16 melanoma cell lysates. Increased levels of IFN- $\gamma$  and CTL activity contributed to the observed antitumor effect in the mice immunized with B16 cell lysate plus poly I:C. In addition, this was consistent with the potent antigen-specific antitumor immunity previously observed in the murine B16 melanoma challenge model (32-34). The survival rate of the mice immunized with B16 cell lysate in combination with poly I:C was significantly higher than that of the group immunized with B16 antigen or PBS only. It was found in previous studies that immunization with B16 plus poly I:C was able to fully protect mice in prophylactic vaccination experiments, not only in the short-term but also in the long-term (35-37).

Although the B16 melanoma cell lysate was used as a cancer antigen to assess the antitumor effects of the cancer vaccine in the current study, it is reasonable to hypothesize that poly I:C could confer adjuvant properties when used in combination with a variety of viral antigenic peptides or tumor-specific antigens. In addition, the findings of this study imply that the adjuvant poly I:C may be useful for eliciting immune responses or breaking immune tolerance in cases of spontaneous tumors as well as in cases of infections caused by the hepatitis B virus, the human papillomavirus, and the human immunodeficiency virus.

In conclusion, *in vivo* experiments with the mouse model in the present study demonstrated that the mice that received B16 cell lysate plus poly I:C exhibited enhanced antitumor prophylactic and therapeutic efficacy, which was associated with increased IFN- $\gamma$  production and induction of cytotoxic T lymphocyte activity. It is hypothesized that this strategy could be useful for the treatment of malignant tumors and metastasis.

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