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Potent Systemic Antitumor Immunity Induced by Vaccination with Chemotactic-Prostate Tumor Associated Antigen Gene-Modified Tumor Cell and Blockade of B7-H1

NING LI,[1] HANJUN QIN,[2] XIAOZHU LI,[3] CHUNXIA ZHOU,[1] DONGMEI WANG,[1] WENBO MA,[1] CHEN LIN,[4] YOUHUI ZHANG,[1] SHENGDIAN WANG,[3] and SHUREN ZHANG[1,5]

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We previously reported that several DNA fragments from human prostate-specific membrane antigen (hPSM), mouse prostatic acid phosphatase (mPAP), and human prostate-specific antigen (hPSA) genes were selected and fused to create a novel hPSM-mPAP-hPSA fusion gene (named 3P gene), and human secondary lymphoid tissue chemokine (SLC), 3P, and human IgG Fc genes were inserted into pcDNA3.1 to construct a DNA vaccine, designated pSLC-3P-Fc. In this report, to establish a more efficient treatment for immunotherapy against prostate cancer, the construct was transfected into B16F10 to generate gene-modified tumor cell vaccine (named B16F10-SLC-3P-Fc). In poorly immunogenic B16F10 mouse melanoma model, the immunization with B16F10-SLC-3P-Fc resulted in a strong antitumor response and 50% of tumor-bearing mice achieved long-term survival (>120 days). *In vivo* depletion of lymphocytes indicated that $CD8^+$ T cells were involved in the direct tumor killing, whereas $CD4^+$ T lymphocytes were required for the induction of $CD8^+$ CTL response in B16F10-SLC-3P-Fc-immunized mice. Splenocytes from B16F10-SLC-3P-Fc-immunized mice specifically recognized and lysed PSM, PAP, PSA, and 3P expressing tumor cells. The combined therapy of B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs further enhanced the immune response. Rechallenge experiment showed that a persistent memory response was successfully induced by the combined therapy. These observations suggest pSLC-3P-Fc-modified tumor cells could serve as a vaccine against prostate cancer, and the therapy combined with anti-B7-H1 MAbs further enhanced the antitumor immune response.

KEY WORDS: Prostate antigen; SLC; Fc; Cell vaccine; B7-H1 .

INTRODUCTION

Prostate cancer is one of the most common cancers among males in the United States. It was estimated that 232,090 men would be diagnosed with prostate cancer and 30,350 would die from the disease during 2005 (1). Surgery and radiation are the treatments of choice only for the early (localized) stage prostate cancer. There is yet no effective treatment for patients who develop recurrences or those who have metastatic disease at the time of diagnosis. Nowadays, immunotherapy may represent a valid noninvasive treatment for prostate cancer, which could be used to prevent metastatic spread or delay recurrences (2–4).

Prostate tissue and prostate cancer express several potential target antigens, including prostate-specific antigen (PSA), prostate-specific membrane antigen (PSM), and prostatic acid phosphatase (PAP). Single tumor antigen-derived peptides or gene products have been administered in many of the previous studies. However, it is evidenced that a vaccine containing peptides derived from several of these antigens could be more effective than is a vaccine directed against a single antigen (2, 3). There are two main reasons for it. Firstly, prostate tumor cells can express multiple tumor antigens and the expression of each of these can vary within the tumor. Secondly, elicitation of immune responses directed against several antigens can

[1]Department of Immunology, Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100021, China.
[2]Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Aurora, CO 80010, USA.
[3]Center of infection and immunity, National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China.
[4]State Key Laboratory of Molecular Oncology, Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100021, China.
[5]To whom correspondence should be addressed at Department of Immunology, Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100021, China; e-mail: biotherapy@caca.sina.net.

minimize the risk of selecting antigen loss tumor variants. Therefore, it is generally accepted that vaccination with multiple epitopes will be advantageous over single epitope-based vaccines (5).

We have searched the entire sequence of hPSM, mPAP, and hPSA genes in our previous study. DNA fragments which encode multiple CTL and Th cell epitopes were selected from them and fused to create a novel hPSM-mPAP-hPSA fusion gene (named 3P gene). We have also constructed a fused plasmid DNA pSLC-3P-Fc by linking 3P gene to SLC at its 5′ end and to IgG Fc fragment at its 3′ end (2, 3). Vaccination with pSLC-3P-Fc by gene gun inoculation induced strong antitumor response in a mouse tumor model, which significantly inhibited tumor growth and prolonged survival time of the tumor-bearing mice. However, the response was not sufficient to retard tumor growth.

In this study, to develop a more efficient gene-modified tumor cell vaccine, plasmid DNA pSLC-3P-Fc was transfected into B16F10 to generate gene-modified tumor cell vaccine (named B16F10-SLC-3P-Fc). There is a growing evidence that chemokines play an important role in the initiation of specific immune response, so one of the strategies to improve the 3P-based vaccine potency is to link 3P with chemokine. The secondary lymphoid tissue chemokine (SLC) is a CC chemokine expressed by high endothelial venules in lymph nodes and in T cell zones of spleen and lymph nodes that strongly attracts T cells and DCs (6, 7). It may increase the probability of the co-localization of an antigen presenting DC with its cognate T cells, and facilitate activation and priming of immune responses. Furthermore, it has the function of costimulating expansion of $CD4^+$ and $CD8^+$ T cells and inducing Th1 polarization and could increase the number of infiltrating T cells and mediate delayed tumor growth in animal models (8, 9).

Another promising strategy to improve the vaccine potency is to link 3P with IgG Fc. DCs express the receptors for the Fc portion of IgG (FcγRs), which mediates the internalization of antigen–IgG complexes and promotes efficient MHC class II-restricted antigen presentation, and is 1000- to 10,000-fold more efficient than is the fluid phase pinocytosis (10, 11). FcγR-mediated endocytosis can also cross-present the internalized antigen to MHC class I molecules (11, 12). Thus, FcγRs represent a privileged antigen internalization route for efficient MHC class I- and class II-restricted antigen presentation by DC. Our previous study suggested the incorporation of the IgG Fc fragment into the tumor vaccine enhanced the immunogenicity (11).

The outcome of the immune response is, in part, dependent on the balance between activating and inhibitory signals that accompany TCR engagement by antigen (13). PD-1 is the newest member of the CD28 family and express on activated T and B cells and a subset of thymocytes (14, 15). The ligands for PD-1 have been identified as PD-L1 (B7-H1), which is constitutively expressed on freshly isolated splenic T cells, B cells, macrophages, and dendritic cells, and is further up-regulated upon activation (16). Studies have shown that the engagement of PD-1 by B7-H1 may induce a negative regulatory signal and inhibit T-cell responses (17–20). Mutants of B7-H1, which have lost their ability to bind PD-1, costimulate normal responses to T cells from both PD-$1^{+/+}$ and PD-$1^{-/-}$ mice *in vitro* (21). It has been reported that blockade of B7-H1 on dendritic cells enhance T cell proliferation and cytokine production (22). Therefore, selective blocking of B7-H1 may represent a new opportunity for the improvement of cancer immunotherapy.

In this study, after vaccination via subcutaneous injection, SLC-3P-Fc-modified tumor cell produce and secrete SLC-3P-Fc fusion proteins. The secreted fusion proteins can be efficiently captured and processed by DC via a receptor-mediated endocytosis and presented to MHC classes II and I (cross-priming), resulting in a significant antitumor immunity. Given the potential benefit of anti-B7-H1 MAbs in enhancing the immune response, we also tested the effect of B7-H1 blockade in combination with the modified tumor cell vaccine. It was found that an additive effect from combining modified tumor cell vaccine with anti-B7-H1 MAbs, which resulted in significant tumor delay in animal model.

MATERIAL AND METHODS

Cell Lines and Mice

C57BL/6 melanoma cell line B16F10, mouse T lymphoma cell line YAC-1, human prostate cancer cell line LNCaP (HLA-A0201$^+$, hPSA$^+$, hPAP$^+$, and hPSM$^+$) (23), GK1.5 and 2.43 were kindly provided by L. Chen (Department of Immunology, Mayo Graduate and Medical Schools, Mayo Clinic, Rochester, USA). All cells were cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) (heat-inactivated, complete medium).

Male C57BL/6 mice (8–10 weeks old) were purchased from the Experimental Animal Institute of Peking Union Medical College.

Plasmid DNA Constructs and Preparation

hPSM, hPAP, mPAP, hPSA, and human SLC were amplified by RT-PCR, respectively, as described in a

previous study. The 3P fusion gene and SLC-3P-Fc were constructed as described in a previous study (2, 3). The 3P, hPSA, hPAP, hPSM, SLC, 3P-Fc, or SLC-3P-Fc were inserted into pcDNA3.1 to construct the expression vectors of pcDNA3.1-3P, pcDNA3.1-hPSA (pPSA), pcDNA3.1-hPAP (pPAP), pcDNA3.1-hPSM (pPSM), pcDNA3.1-SLC (pSLC), pcDNA3.1-3P-Fc (p3P-Fc), and pcDNA3.1-SLC-3P-Fc (pSLC-3P-Fc), respectively (3). The empty pcDNA3.1 plasmid was used as a control (pC). All the recombinant constructs were confirmed by sequence analysis and purified from a large-scale culture by alkaline lysis and polyethylene glycol precipitation (24).

Cell Transfection

pC, pcDNA3.1-3P, pPSA, pPAP, pPSM, p3P-Fc, and pSLC-3P-Fc were transfected into B16F10 cell lines by using Lipofectamine Plus reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The transfected cells were selected with 600 μg/ml G418 and cloned by limiting dilutions. Positive clones were screened by RT-PCR and the resulting cells were B16F10-pC, B16F10-3P, B16F10-PSA, B16F10-PAP, B16F10-PSM, B16F10-3P-Fc, and B16F10-SLC-3P-Fc. The cell proliferation was determined by MTT assay (2, 3). Reverse transcription PCR and Western blots were used to confirm the expression of the transgenes (2, 3).

ELISA for SLC

The culture supernatants of B16F10-SLC, B16F10-pC, B16F10-3P-Fc, B16F10- SLC-3P-Fc were collected 48 h later and assayed for SLC concentrations using the SLC/CCL21/6Ckine (SLC also known as CCL21, 6Ckine[10]) ELISA kit according to the manufacture's instructions (R&D Systems, Cat #D6C00). Plates were read at 450 nm with a correction wavelength of 570 nm.

Histology

B16F10-SLC-3P-Fc or B16F10-pC cells (5×10^6 cells/mL) were pretreated with mitomycin-C (80 μg/mL) for 60 min and then washed with PBS for three times. The mice were subcutaneously vaccinated at the left flank back with 1×10^6 of the pretreated tumor cells per mouse. After 72 h, mouse dermis tissues of the injection sites were surgically excised, fixed for 12–24 h in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5-μm thickness, and stained with hematoxylin and eosin (H&E) for histological examination.

Tumor Protection and Therapy Assay

For *in vivo* tumor-prevention experiments, the mice were subcutaneously vaccinated with 1×10^6 gene-modified tumor cells pretreated with mitomycin-C /mouse at the left flank back on days 0, 10, and 20. On day 30, mice were inoculated at the right flank back with 5×10^4 B16F10-3P cells or B16F10 cells/mouse.

For *in vivo* therapeutic experiment, 5×10^4 B16F10-3P cells cells/mouse were subcutaneously inoculated at the right flank back of male C57BL/6 mice on day 0, and the mice were subcutaneously vaccinated with 1×10^6 modified tumor cells pretreated with mitomycin-C at the left flank back on days 4, 14, and 24.

To study the therapeutic effect of the combination treatment, mice were subcutaneously inoculated with 5×10^4 B16F10-3P cells or B16F10 cells/mouse at the right flank on day 0, then immunized subcutaneously with 1×10^6 modified tumor cells pretreated with mitomycin-C on days 4, 11, and 18. On days 5, 8, 11, and 14, 100 μg of anti-B7-H1 MAbs (clone 10B5, kindly provided by Dr. L. P. Chen) or control hamster IgG was given intraperitoneally in 100 μl of PBS.

Tumors were monitored every two days, and tumor dimensions were determined by measuring with calipers (length $\times$ width $\times$ height), and the values were inserted into the formula: tumor volume (mm^3) $=$ 0.52 (length $\times$ width $\times$ height) (25).

In Vitro Cytotoxicity Assay

Male C57BL/6 mice were subcutaneously challenged with 5×10^4 B16F10-3P cells in the right flank back on day 0, then subcutaneously immunized with the gene-modified tumor cells pretreated with mitomycin-C. Ten days after the last booster, splenocytes from the immunized mice were prepared as effector cells. Cytotoxicity assays were performed using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. B16F10, B16F10-pC, B16F10-3P, B16F10-PSM, B16F10-PAP, B16F10-PSA, and YAC-1 cells were respectively used as target cells. Effector cells were added to target cells at a ratio of 40:1 (tested in triplicate). Specific lysis was calculated according to the formula: cytotoxicity (%) $=$ ([E $-$ Se $-$ St])/([Mt $-$ St]) $\times$ 100, where E is the experimental LDH release in effector plus target cell cocultures, Se the spontaneous release by effector cells alone, St the spontaneous release by target cells alone, and Mt is the maximal release by target cells.

Depletion of T Cell Subsets In Vivo

T cell subsets were depleted as described (2, 24, 25). Briefly, mice were subcutaneously vaccinated three times at 5-day intervals with B16F10-SLC-3P-Fc or B16F10-pC by subcutaneous injection, or nonimmunized and challenged with 5×10^4 B16F10-3P cells/mouse on day 5 after the third immunization. The mice were injected intraperitoneally with 500 μg of either the anti-CD4 (clone GK 1.5, rat IgG), anti-CD8 (clone 2.43, rat IgG), or isotype controls (normal rat IgG) mAbs 1 day before or 8 days after the first immunization and then twice per week for 3 weeks.

The mice were sacrificed on day 21 after tumor cell injection. Splenocytes from immunized mice were used in cytotoxicity assay as described earlier. The depletion of $CD4^+$ and $CD8^+$ T cells was consistently greater than 95% as determined by flow cytometry (Becton Dickinson).

Adoptive Therapy

Male C57BL/6 mice were subcutaneously inoculated with 5×10^4 B16F10-3P cells/mouse at the right flank on day 0, then immunized subcutaneously with 1×10^6 modified tumor cells pretreated with mitomycin-C on days 4, 11, and 18. On days 5, 8, 11, and 14, 100 μg of anti-B7-H1 MAbs (clone 10B5, kindly provided by Dr. L. P. Chen) was given intraperitoneal in 100 μl of PBS. On day 28, splenocytes from the immunized mice were prepared as effector cells. A total of 1×10^7 effector cells per mouse were adoptively transferred via the tail vein into tumor-bearing mice which were subcutaneously inoculated with 5×10^4 B16F10-3P cells/mouse on day 24.

Statistical analysis

For comparison of individual time points, ANOVA was used for the comparisons among three or more groups. Student's *t*-test was used to compare means between the two groups. Survival curves were compared by the log-rank test. Differences were considered significant when $p < 0.05$. Statistical analysis was performed using commercially available software (SPSS 11.0).

RESULTS

Construction of DNA Plasmids and Characterization of the Genetically Modified Tumor Cells

The Fc portion of human IgG1 can efficiently bind to human DC as well as to murine DC (24).

Total RNA extracted from pSLC-3P-Fc-, p3P-Fc-, pSLC-, or pC-transfected cells and target cells was used to verify the expression of SLC-3P-Fc, 3P-Fc, SLC, 3P, PSM, PAP, or PSA by RT-PCR (2, 3).

Secreted proteins and cellular proteins were analyzed by Western blotting (2, 3). These results demonstrate that the constructs were expressed in the eukaryotic cells.

The cell proliferation was assayed by MTT assay and the results indicated that plasmid DNA had no effect on transfected target cell proliferation (2, 3).

Chemotactic Activity of SLC-3P-Fc Protein In Vitro and In Vivo

Equivalent SLC concentration in culture supernatants of pSLC-3P-Fc- or pSLC-transfected B16F10 cells was 688 or 681 pg/mL, whereas no SLC was detectable in supernatants of p3P-Fc- or pC-transfected or nontransfected B16F10 cells (Table I).

As shown in Fig. 1, H&E staining of dermis tissue sections from B16F10-pC treated mice showed infiltration of few lymphocytes into the immunization region. Tissues from mice that received B16F10-SLC-3P-Fc showed a prominent infiltration of lymphocytes, which may be chemoattracted by SLC-3P-Fc.

Our previous observations (3), showing that supernatants from pSLC- or pSLC-3P-Fc-transfected cells attracted lymphocytes above control levels, no statistical difference was observed in chemotactic response between supernatants of pSLC- and pSLC-3P-Fc-transfected cells and human SLC also show chemotactic efficacy in mouse model.

Protective Immune Response of Genetically Modified Tumor Cells

To investigate the protective antitumor immunity, we immunized mice with B16F10-SLC-3P-Fc, B16F10-3P-Fc, or B16F10-Pc, and then challenged mice with 5×10^4 B16F10-3P or B16F10 tumor cells/mouse. As shown in Fig. 2A, tumors grew progressively in

Table I. Equivalent SLC Concentration in Supernatants of B16F10 Cell Cultures 48 h After Nontransfected or Transfected with pSLC-3P-Fc, pSLC, p3P-Fc, or pC

Treatment	SLC protein concentration (pg/mL)
pSLC-3P-Fc	688
pSLC	681
p3P-Fc	n.d.[a]
pC	n.d.[a]
Nontransfected	n.d.[a]

[a]n.d.: not detectable.

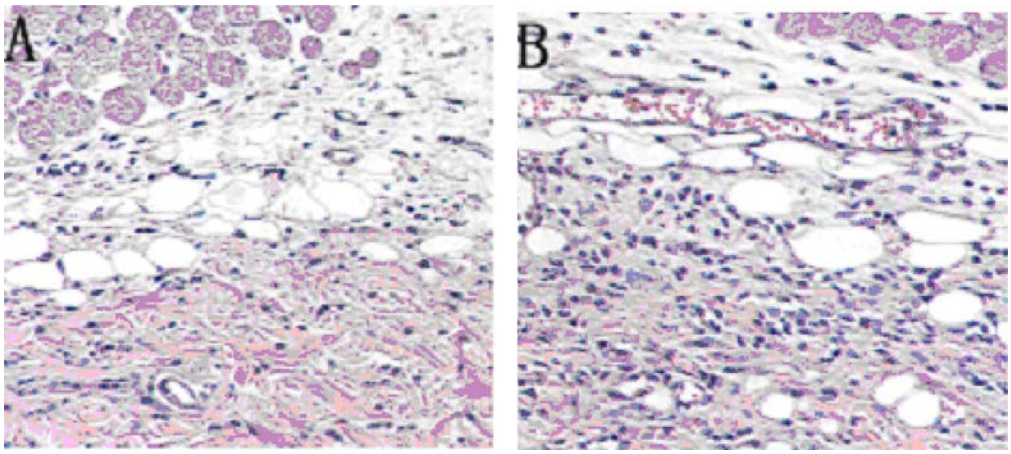

Fig. 1. B16F10-SLC-3P-Fc chemoattract lymphocytes to the immunization site. H&E staining of dermis tissue sections from C57BL/6 mice treated with (A) B16F10-pC and (B) B16F10-SLC-3P-Fc. Images are representative of multiple microscopic fields observed in at least three mice per group.

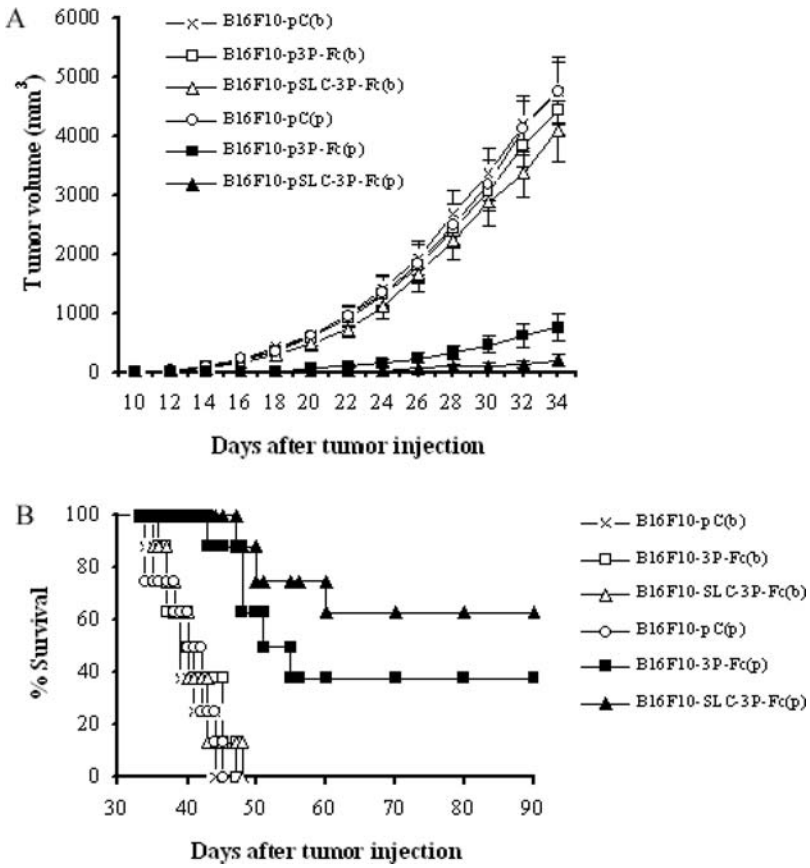

Fig. 2. Induction of the protective antitumor immunity. The mice were subcutaneously vaccinated with 1×10^6 gene-modified tumor cells pretreated with mitomycin-C per mouse at the left flank back on days 0, 10, and 20. On day 30, mice were inoculated at the right flank back with 5×10^4 B16F10-3P (p) cells or B16F10 (b) cells/mouse ($n = 8$). (A) Tumor volume (mm^3) treated with B16F10-SLC-3P-Fc and controls was shown. (B) Survival of mice per treatment group. The survival rate of the B16F10-SLC-3P-Fc-treated mice was 63% at day 90 for B16F10-3P melanoma cells.

B16F10-pC-immunized mice, but there was apparent protection from tumor growth in B16F10-SLC-3P-Fc-immunized mice. The results are expressed as mean tumor volume ± SEM. The survival of the mice treated with B16F10-SLC-3P-Fc was also significantly greater than that of B16F10-3P-Fc- or B16F10-pC-immunized mice ($p < 0.01$, by log-rank test). In addition, our data indicated that B16F10-SLC-3P-Fc-immunized mice are capable of stimulating antitumor immune response that specifically recognized and lysed 3P-expressing tumor cells.

Antitumor Activity Induced by Genetically Modified Tumor Cells

Although SLC and Fc synergistically enhanced the immunogenicity of 3P-based DNA vaccine, no mice had a complete tumor regression (2, 3). In order to achieve more effective antitumor response, the tumor-bearing mice were subcutaneously vaccinated with gene-modified tumor cells pretreated with mitomycin C on days 4, 14, and 24. As shown in Fig. 3A, mice vaccinated with B16F10-SLC-3P-Fc demonstrated the lowest average tumor volume on day 34 compared with mice vaccinated with B16F10-3P-Fc or B16F10-pC (one-way ANOVA, $p < 0.001$, data are expressed as mean tumor volume ± SEM). On day 90, 50% of B16F10-SLC-3P-Fc-immunized mice achieved long-term survival with complete tumor regression (Fig. 3B). The survival of the mice treated with B16F10-SLC-3P-Fc was also significantly longer than that of B16F10-3P-Fc- or B16F10-pC-immunized mice ($p < 0.001$, by log-rank test; Fig. 3B). Furthermore, B16F10-pC-immunized mice developed tumors and died within 50 days after tumor challenging.

Role of T Cell Subsets in B16F10-SLC-3P-Fc Induced Antitumor Activity

To determine the subset of lymphocytes that are important for the rejection of B16F10-3P, we depleted $CD8^+$ or $CD4^+$ T-cell subsets *in vivo* before tumor challenge. As observed previously, if depletions were started after immunization with B16F10-SLC-3P-Fc, B16F10-SLC-3P-Fc-induced cytolytic activity against B16F10-3P cells could be blocked by anti-CD8, but not by anti-CD4, control IgG, or PBS in a cytotoxicity assay (Fig. 4B). In addition, B16F10-SLC-3P-Fc-immunized mice treated with anti-CD4 showed the antitumor activity against B16F10-3P, whereas the treatment with anti-CD8 partially abrogated the protective effects of pSLC-3P-Fc (Fig. 4A). Furthermore, if depletions were started before immunization with B16F10-SLC-3P-Fc, *in vivo* depletion of $CD4^+$ T lymphocytes could completely abrogate the antitumor activity, whereas the depletion of $CD8^+$ lymphocytes showed partial abrogation of the antitumor activity *in vivo*. In addition, the treatment with rat IgG, or PBS showed no effect (Fig. 4C). Mice depleted of $CD4^+$ T lymphocytes or $CD8^+$ T lymphocytes did not develop detectable CTL activity (Fig. 4D). These data suggest that $CD8^+$ T cells are involved in a role of the direct tumor killing, whereas $CD4^+$ T lymphocytes are required for the induction of $CD8^+$ CTL response to the immunization with B16F10-SLC-3P-Fc vaccine.

Antitumor Immune Response Induced by Gene-Modified Tumor Cells and Blockade of B7-H1

To evaluate whether the combination of modified tumor cell vaccine and blockade of B7-H1 co-signaling pathway would result in a more effective antitumor response, mice were subcutaneously inoculated with B16F10-3P or B16F10 cells, then subcutaneously vaccinated with gene-modified tumor cells, and anti-B7-H1 MAbs or control hamster IgG was administrated by intraperitoneal injection. As illustrated in Fig. 5A (one-way ANOVA, $p < 0.001$, data are expressed as mean tumor volume ± SEM), the combination of B16F10-SLC-3P-Fc and anti-B7-H1 MAbs resulted in the greatest reduction of tumor growth and significant improvement of the mice survival as shown in Fig. 5B ($p < 0.001$, by log-rank test). On day 120, 83% of mice in this group (5/6 animals) achieved long-term survival with complete tumor regression.

To determine whether the antitumor response generated by vaccination of B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs is tumor-specific, splenocytes isolated from immunized mice were used in cytotoxicity assay. Results from this assay indicated a significant increase in tumor-specific lysis of B16F10-3P, B16F10-PSM, B16F10-PAP, or B16F10-PSA in splenocytes from B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs treated mice compared to B16F10-pC-treated counterparts ($p < 0.01$; Fig. 5C). In addition, this cytolytic activity appeared specific for B16F10-3P, B16F10-PSM, B16F10-PAP, or B16F10-PSA, not for B16F10, or B16F10-pC ($p < 0.01$). These results suggest that CTL responses from B16f10-SLC-3P-Fc-immunized mice are specific for PSM, PAP, and PSA antigen. In addition, there is no increase in NK activity against NK-sensitive YAC-1 target cells by the sensitized spleen cells.

To evaluate the persistent protective effect of the combined therapy, the survivors with complete tumor regression in Fig. 5 and the naive control mice were subcutaneously rechallenged with 1×10^5 B16F10-3P tumor cells on day 120 after the initial tumor inoculation. No mice from the group treated with B16F10-SLC-3P-Fc

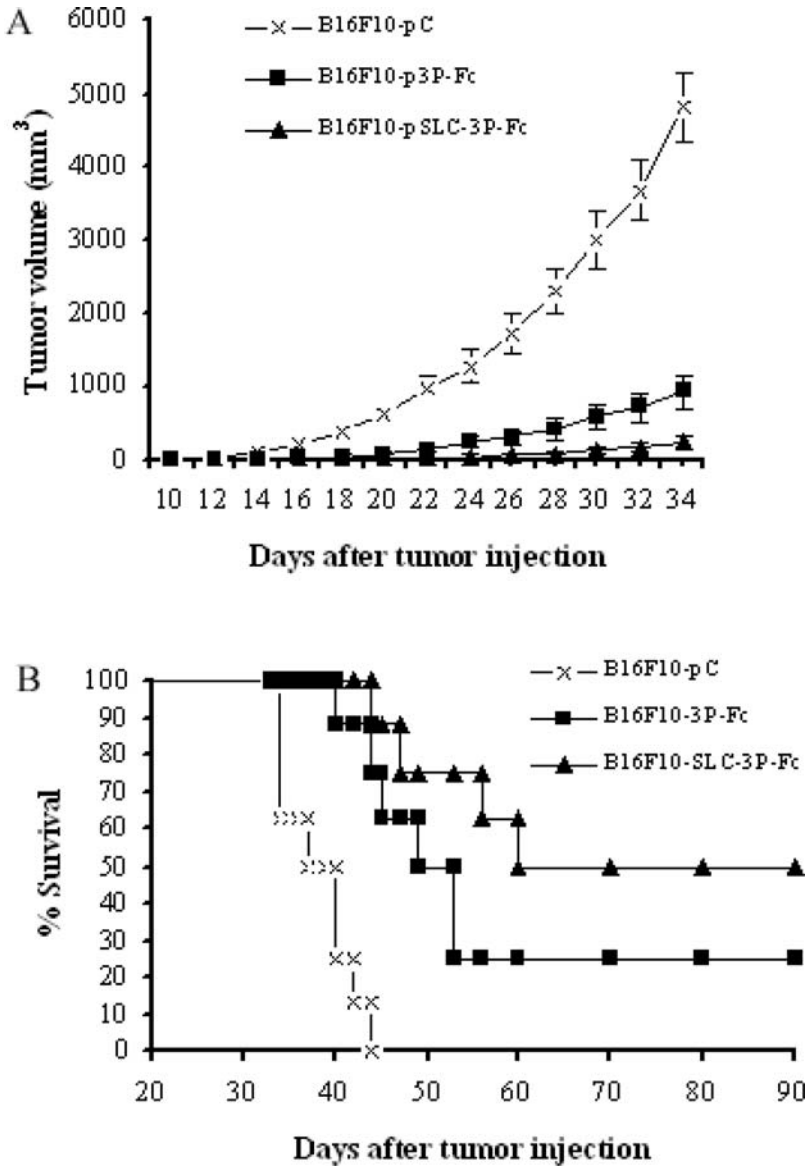

Fig. 3. Induction of the therapeutic antitumor immunity. A total of 5×10^4 B16F10-3P cells/mouse were subcutaneously inoculated at the right flank back of male C57BL/6 mice on day 0, and the mice were subcutaneously vaccinated with 1×10^6 modified tumor cells/mouse at the left flank back on days 4, 14, and 24 ($n = 8$). (A) Tumor volume (mm^3) in mice treated with B16F10-SLC-3P-Fc and controls was shown. (B) Survival of mice per treatment group. Percentage survival of mice treated. The survival rate of the B16F10-SLC-3P-Fc treated mice was 50% at day 90 for B16F10-3P melanoma cells.

plus anti-B7-H1 MAbs developed tumors after an additional 60 days. In contrast, 33% of the mice from the group treated with B16F10-SLC-3P-Fc plus control IgG developed tumor (Fig. 6). The observation of long-term immunity implies that B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs may successfully mediate the development of persistent memory immune response.

Adoptive Therapy

To assess whether the antitumor activity can be acquired by adoptive transfer of lymphocytes from B16F10-SLC-3P-Fc-immunized and blockade of B7-H1 co-signaling pathway mice, splenocytes from the immunized mice were used as effector cells and adoptively transfered into

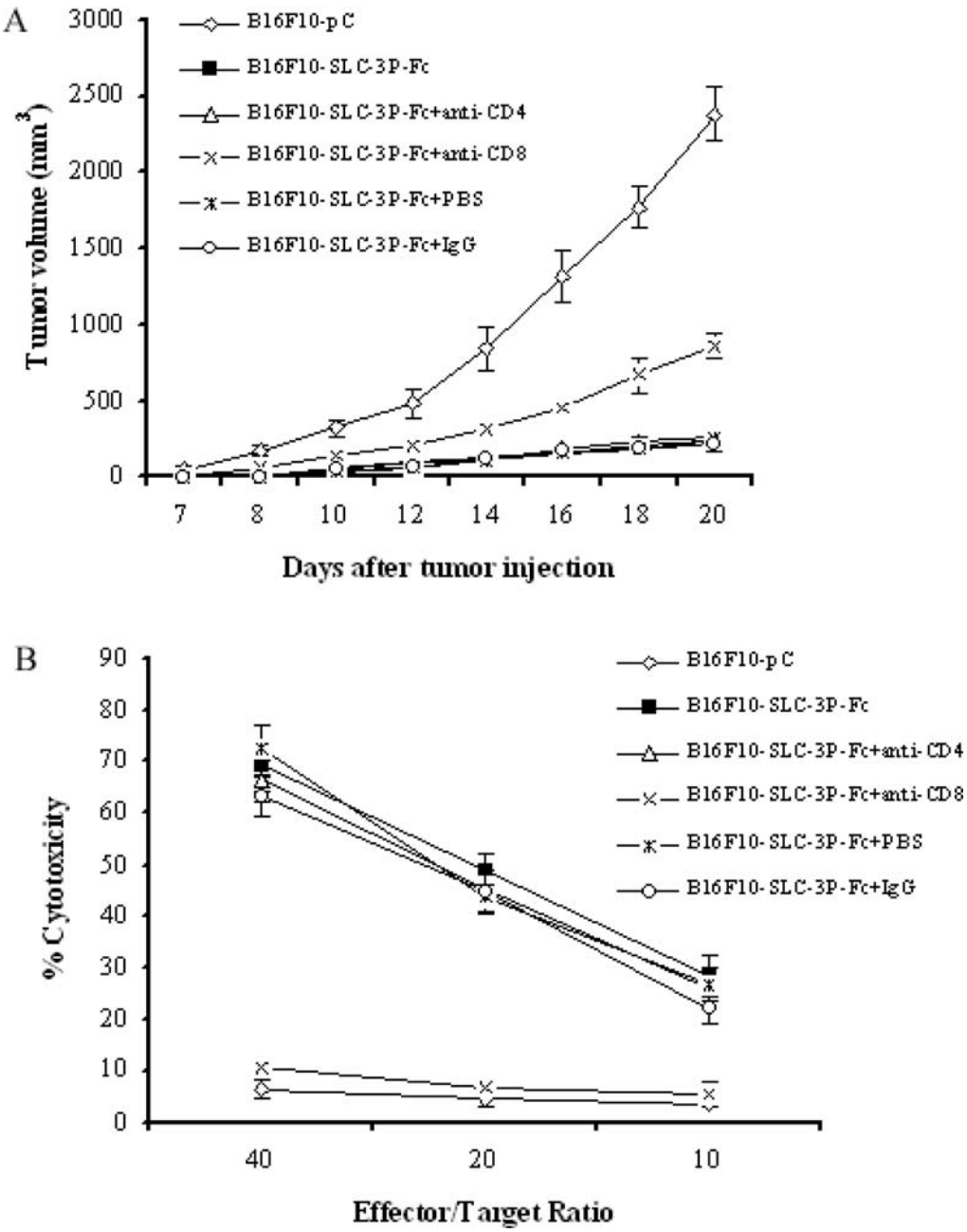

Fig. 4. Assessment of involvement of $CD4^+$ and $CD8^+$ T cells in antitumor effect. Before or after injected with mitomycin-C-treated B16F10-SLC-3P-Fc or B16F10-pC tumor cells, mice were subcutaneously challenged with 5×10^4 B16-3P cells/mouse on day 0. Cell subsets were depleted by the injection of GK1.5 (for $CD4^+$ T cells), 2.43 (for $CD8^+$ T cells) ascites fluids (100 μl per mouse) twice a week before killing ($n = 5$). (A) and (B) Abrogation of CTL-mediated cytotoxicity and of antitumor activity by *in vivo* depletion of the T-cell subsets after immunization with vaccine. (C) and (D) Abrogation of antitumor activity and of CTL-mediated cytotoxicity by *in vivo* depletion of the T-cell subsets before immunization with vaccine.

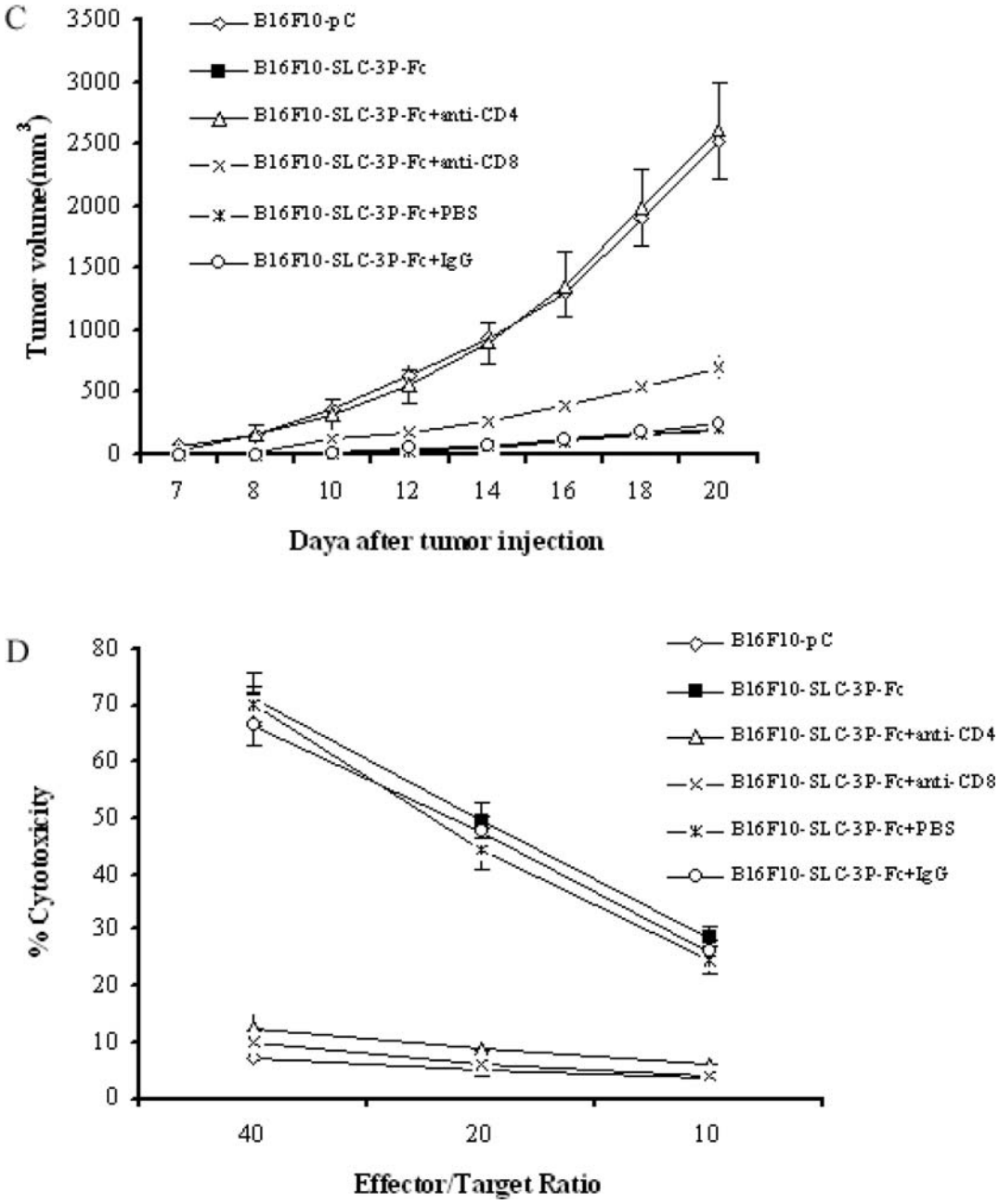

Fig. 4. Continued.

the tumor-bearing mice. As shown in Fig. 7A, B16F10-3P tumor growth can be significantly inhibited by inoculated with splenocytes form B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs immunized mice (one-way ANOVA, $p < 0.001$, data are expressed as mean tumor volume $\pm$ SEM). On day 90, 75% of mice in this group achieved long-term survival with complete tumor regression ($p < 0.001$, by log-rank test). Whereas the mice immunized with splenocytes form B16F10-pC immunized mice developed tumors and died within 50 days after tumor challenging (Fig. 7B).

DISCUSSION

The potential efficacy of immunotherapy for the treatment of cancer has led to the development of novel experimental strategies. Many of these immunotherapy

approaches focus on the activation of tumor-specific T lymphocytes because the cell-mediated arm of the immune system can be effective in destroying tumor cells and in providing long-term protection (memory) against the recurrence and/or outgrowth of primary and/or metastatic tumor cells (26–28). Because optimal activation of tumor-specific, cytotoxic $CD8^+$ T cells requires coactivation of $CD4^+$ Th lymphocytes, we are developing cell-based cancer vaccines that activate both $CD4^+$ and $CD8^+$ T cells. In this study, we demonstrated the feasibility of pSLC-3P-Fc-modified tumor cell vaccine (B16F10-SLC-3P-Fc). Our data indicated that B16F10-SLC-3P-Fc vaccination could induce a remarkable antitumor effect against 3P-expressing tumor.

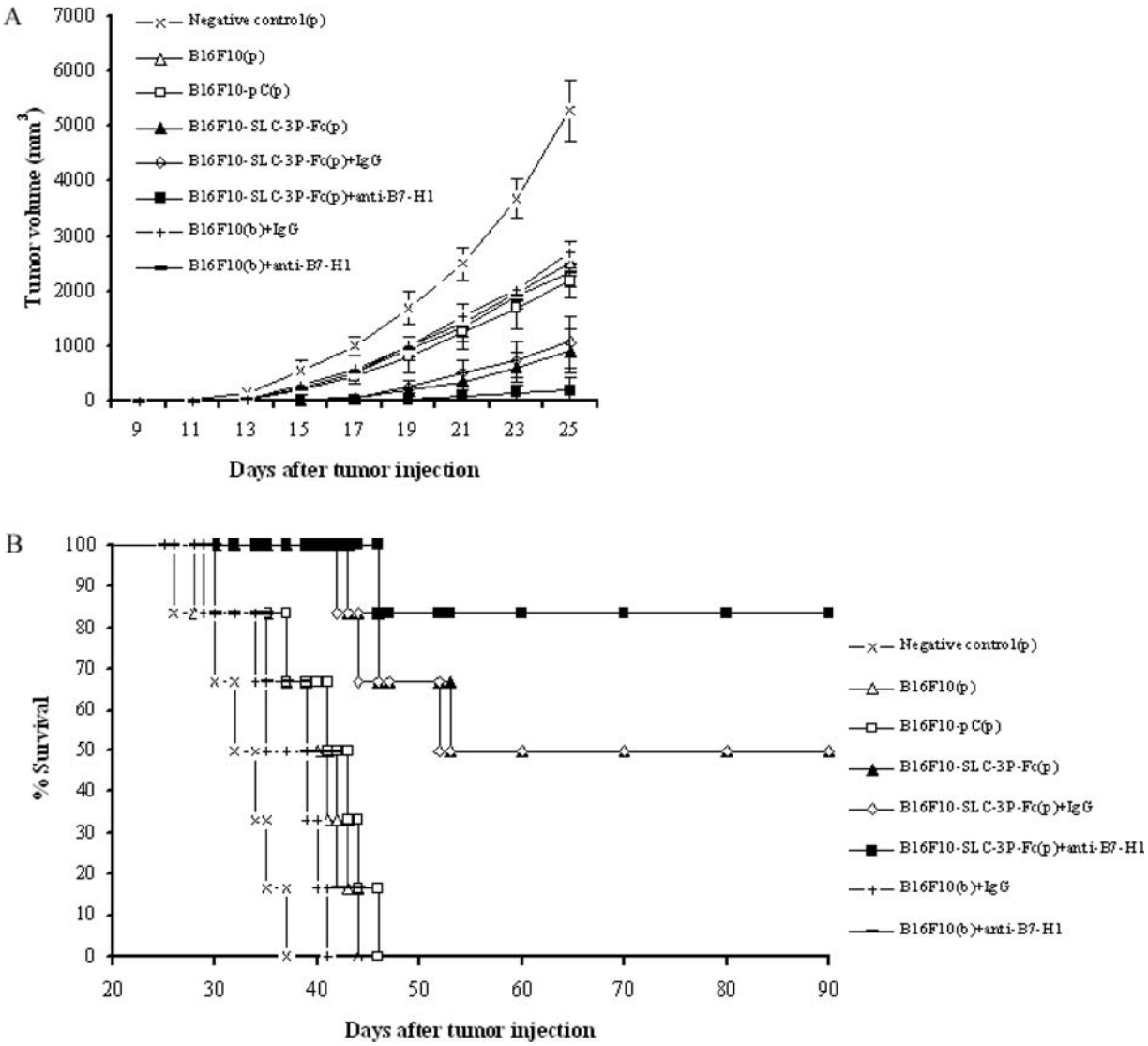

Fig. 5. Evaluation of the combination therapeutic effect on established tumor. C57BL/6 mice were inoculated subcutaneously with 5×10^4 B16F10-3P tumor cells/mouse (p) or 5×10^4 B16F10 cells/mouse (b) on day 0 and were treated with modified tumor cells immunization on days 4, 11, and 18 followed by either hamster IgG or anti-B7-H1 MAbs administration (intraperitoneal) on days 5, 8, 11, and 14 ($n = 6$). (A) Data were reported as the average tumor volumes (mm^3) of six mice per group that vaccinated with B16F10-SLC-3P-Fc followed by IgG or anti-B7-H1 treatment. (B) Survival of mice per treatment group. The survival of mouse per group that treated with B16F10-SLC-3P-Fc plus anti-B7-H1 was 83% at day 90. (C) CTL induced by the combination therapy ($n = 3$). Ten days after the last immunization, splenocytes from C57BL/6 mice, nonimmunized mice (A), immunized with B16F10 (B), B16F10-pC (C), B16F10-SLC-3P-Fc (D), B16F10-SLC-3P-Fc + control IgG (E), or B16F10-SLC-3P-Fc + anti-B7-H1 (F) were tested for cytolytic activity against YAC-1, B16F10, B16F10-pC, B16F10-hPSA, B16F10-hPSM, B16F10-hPAP, and B16F10-3P cells. Data are expressed as mean ± SE.

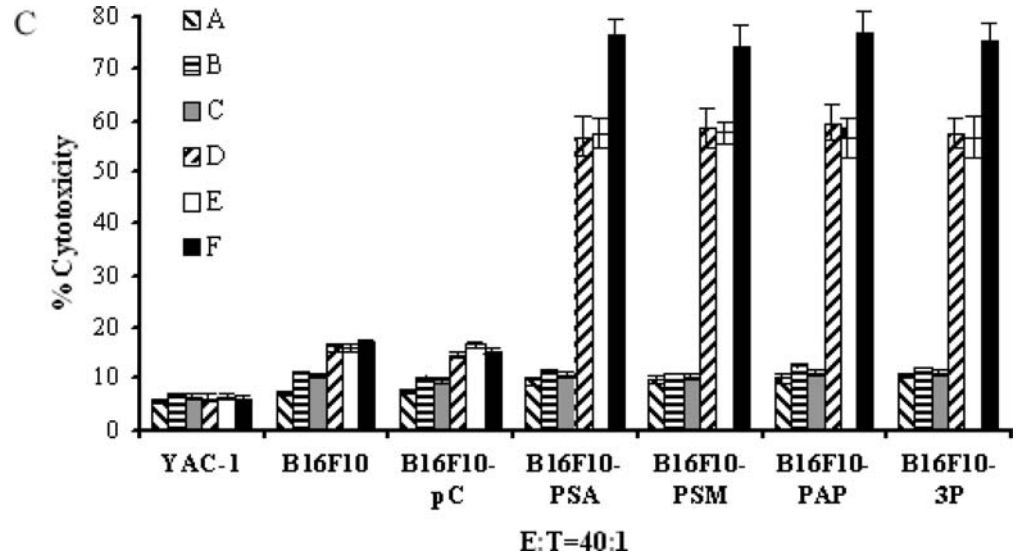

Fig. 5. Continued.

The novel approach we have developed has several unique and advantageous features: (a) the capacity of SLC to facilitate the colocalization of both DC and T cells may reverse tumor-mediated immune suppression and orchestrate effective cell-mediated immune responses; (b) this approach can efficiently allow DCs to cross-present antigens as exogenous to both class II via the endosomal class II pathway and class I via the cross-priming pathway in a cognate manner, leading to the generation of both Ag-specific Th and CTL responses; (c) the receptor-mediated antigen internalization activates DCs, which is important because an optimal DC antigen presentation requires the antigen processing and a maturation signal to DCs (23); (d) SLC acts in synergy with IgG1 Fc in SLC-3P-Fc fusion protein (3); (e) secreted tumor antigens can be efficiently captured by DCs in both autocrine

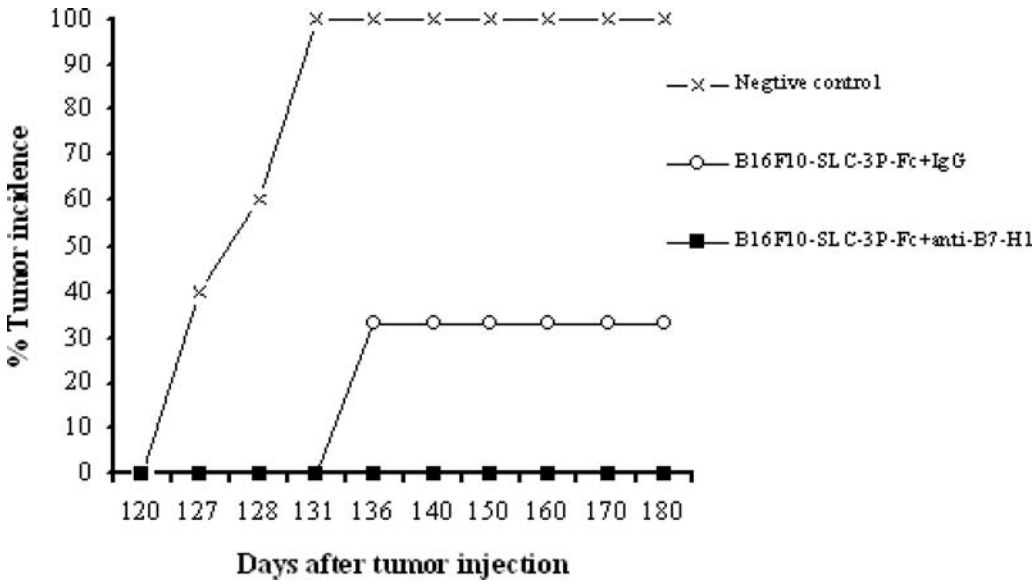

Fig. 6. Evaluation of persistent antitumor response. The tumor-free mice from B16F10-SLC-3P-Fc + IgG-treated group ($n = 3$) or B16F10-SLC-3P-Fc + anti-B7-H1-treated group ($n = 5$) were subcutaneously rechallenged with 10^5 B16F10-3P cells/mouse on day 120 after the initial tumor inoculation. As a control, naive mice were treated in the same way ($n = 5$). Tumor incidence was monitored over time after tumor inoculation.

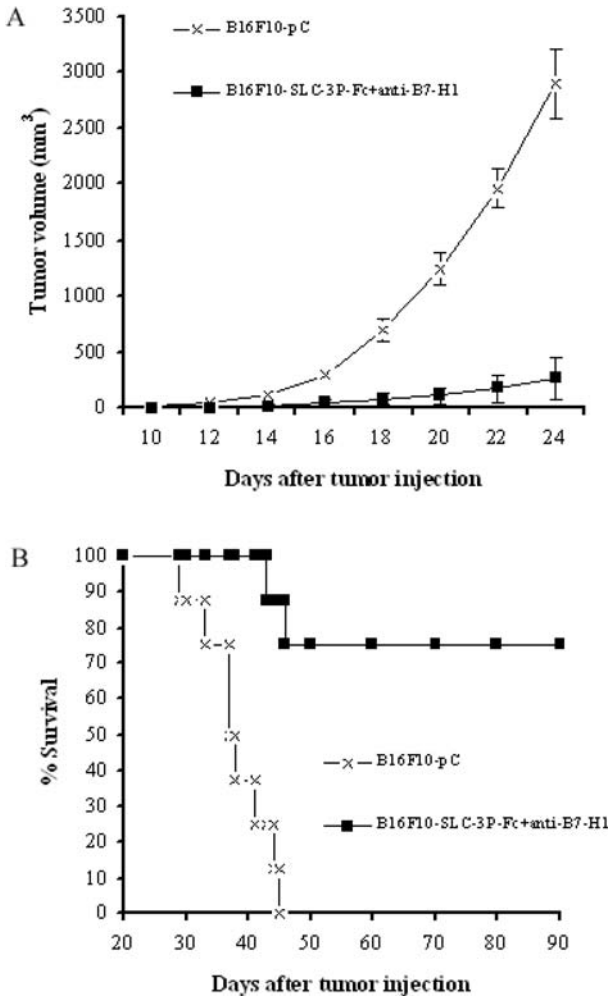

Fig. 7. Evaluation of therapeutic effect of adoptive therapy. Mice were immunized as described in Fig. 5, and splenocytes from the immunized mice were used as effector cells and adoptively transfered into the tumor-bearing mice which were subcutaneously inoculated with 5×10^4 B16F10-3P cells/mouse 4 days ago ($n = 8$). (A) Tumor volume (mm^3) in mice treated with splenocytes from B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs immunized group and controls was shown. (B) Percentage survival of mice treated. The survival rate of B16F10-SLC-3P-Fc plus anti-B7-H1 MAbs group was 75% at day 90.

and paracrine modes to further enhance Th and CTL responses.

The stringent procedures to facilitate the preferential generation of higher antitumor efficacy were made. One of the strategies was to modify tumor cells with pSLC-3P-Fc. Analysis of the antitumor response confirmed our hypothesis that higher effect was induced by the modified tumor cells vaccination (see Figs. 2 and 3). The responses induced by pSLC-3P-Fc-modified tumor cells vaccination might be attributed to several reasons. First, the antitumor response elicited by the modified tumor cells was directed against a mixture of tumor antigens encoded by the tumor cells and the corresponding oligo- or polyclonal responses could be more potent than is a monoclonal response. Second, SLC-3P-Fc-gene modification increased the immunogenicity of the tumor cells (data not shown). This modification might offer the possibility to trigger the recruitment of initiators or effectors of the immune response within the tumor, providing a microenvironment that favors innate and/or acquired immune mechanisms to prevent or reverse tumor development. Based on these results, future development of this approach may lead to practical use in treating tumors. For example, tumor removed from patients by surgery could be gene-modified with the pSLC-3P-Fc *in vitro*, then used as an autologous cellular vaccine.

Another strategy to improve the immunotherapeutic efficacy of B16F10-SLC-3P-Fc vaccine was focused on the combination with anti-B7-H1 MAbs. The antitumor effect induced by pSLC-3P-Fc-modified tumor cells vaccine plus anti-B7-H1 was evaluated. The combined therapies prolonged the survival of tumor-bearing mice. The treatment of B16F10 plus anti-B7-H1 had no significant effect against B16F10 tumor (Fig. 5). The result indicated that the mechanism of breaking CTL ignorance by immunization with a specific tumor antigen was necessary for anti-B7-H1 MAbs to induce a specific CTL response against poorly immunogenic tumors. *In vitro* studies have shown that the engagement of PD-1 by B7-H1 inhibited TCR-mediated T cell proliferation and cytokine production (IFN-, IL-10, IL-4, and IL-2) (11, 20). $CD4^+$ and $CD8^+$ T cell responses were markedly enhanced in B7-$H1^{-/-}$ mice compared with wild-type mice *in vitro* and *in vivo*. B7-$H1^{-/-}$ dendritic cells stimulated greater wild-type $CD4^+$ T cell responses than wild-type dendritic cells (29). These results demonstrate that B7-H1 on T cells and DC inhibits naive and effector T cell responses and plays a critical role in T cell tolerance. In the present study, blockade of B7-H1 by specific monoclonal antibodies could profoundly enhance therapeutic efficacy (see Fig. 5). Our findings thus support B7-H1 blockade as a viable strategy to enhance cancer treatment *in vivo*.

To test whether the antitumor response observed after B16F10-SLC-3P-Fc vaccination was a result of the CTL response elicited, we depleted $CD8^+$ or $CD4^+$ T-cell subsets *in vivo*. We found in the present study that, if depletions were started before immunization (Fig. 4C and D), *in vivo* depletion of $CD4^+$ T lymphocytes could completely abrogate the antitumor activity with the immunization of B16F10-SLC-3P-Fc. At the same time, mice depleted of $CD4^+$ T lymphocytes did not develop detectable CTL activity. These findings may suggest that

$CD4^+$ T lymphocytes are responsible for the antitumor activity by the vaccination of B16F10-SLC-3P-Fc vaccine. These suggestions were further supported by the important roles of $CD4^+$ T lymphocytes in the antitumor immunity (30–35). It has been reported that $CD4^+$ T cells are required for the generation and maintenance of cytolytic $CD8^+$ T cells (30, 36) and are generally believed to be essential for the generation of both a cellular and a humoral antitumor immune response (31–35). $CD4^+$ T cells regulate a $CD8^+$ T cell response in both directions. During primary responses, $CD4^+$ T cells promote the generation and accumulation of specific $CD8^+$ T cells, during memory responses, $CD4^+CD25^+$ regulatory T cells (Th2 cells) restrict the strength of the response (37). Thus, if depletions were started after immunization with B16F10-SLC-3P-Fc, depletion of $CD4^+$ T cells would deplete Th1 and Th2 cells where $CD8^+$ T cells remain intact and may function better against the B16F10-3P tumor cell in the absence of Th2 regulatory T cells (see Fig. 4A). More importantly, the antitumor activity can be acquired by adoptive transfer of lymphocytes from B16F10-SLC-3P-Fc-immunized and B7-H1 blockade mice (see Fig. 7). Taken together, these data indicated that $CD8^+$ T cells are involved in a role of the direct tumor killing, whereas $CD4^+$ T lymphocytes are required for the induction of $CD8^+$ CTL response to the immunization with B16F10-SLC-3P-Fc vaccine.

In conclusion, our data suggest that the chimeric construct of SLC and IgG Fc fragment with a specific antigen dramatically improved the antitumor effect, providing a novel approach to design tumor vaccine and the combined therapy of B16F10-SLC-3P-Fc vaccine with anti-B7-H1 significantly improved the antitumor effect. These findings are very encouraging for subsequent clinical development.

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