

Synergistic antitumor effect of chemotactic-prostate tumor-associated antigen gene-modified tumor cell vaccine and anti-CTLA-4 mAb in murine tumor model

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Abstract

In this study, we demonstrate that an effective immune response against prostate tumors in mouse tumor model can be elicited using a strategy that combines CTLA-4 blockade and pSLC-3P-Fc-modified tumor cell vaccine (named B16F10-SLC-3P-Fc). Treatment of B16F10-3P-bearing mice resulted in a significant reduction in tumor incidence as assessed 2 months after treatment. In vivo Ab depletion confirmed that the antitumor effect was primarily CD8⁺ T cells and CD4⁺ T lymphocytes were required for the induction of CD8⁺ CTL response in B16F10-SLC-3P-Fc + anti-CTLA-4 mAb-immunized mice. Moreover, mice that were cured of an established tumor were protected against a rechallenge with the same tumor for at least 4 months, suggesting the generation of memory responses. Adoptive transfer experiments further indicate that antitumor reactivity can be transferred to naïve mice by splenocytes. These findings demonstrate that this combinatorial treatment can elicit a potent anti-tumor immune response and suggest potential of this approach for treatment of prostate cancer.

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1. Introduction

Prostate cancer is the most frequently diagnosed cancer and is the leading cause of cancer death in men in the U.S. with an estimated 234,460 new cases and 27,350 deaths in 2006 [1]. It has also become an increasingly important health problem in China. Although more men are diagnosed at the early stages of the disease, when radical treatment is feasible, one-third of them will recur. Prostate cancer, in general, is resistant to chemotherapy, which may be due to its intrinsic low proliferative index [2].

The development of new immunotherapeutic approaches that do not require a high proliferative rate of tumor cells has a strong rationale in the treatment of prostate cancer.

A number of prostate-specific and prostate cancer-specific gene products have been identified that contain epitopes, which can be recognized by CTL, thus fostering continued efforts to develop antigen-specific immunotherapy strategies for prostate cancer [3]. It has been shown that dendritic cells (DCs) pulsed with peptides specific for prostate-specific antigen (PSA) [4], prostate-specific membrane antigen (PSM) [5] or prostatic acid phosphatase (PAP) [6] are capable of stimulating potent CTL in vitro, suggesting their role as potential candidate antigens for prostate cancer immunotherapy. However, there is growing recognition that there may be significant advantages to inducing immune responses against a broad spectrum of antigens expressed by tumor cell rather than targeting single [7,8]. It

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has been reported that the emergence of antigen loss mutants frequently arising at metastatic sites may render a substantial proportion of tumors resistant to single antigen-specific immunotherapy [9]. Therefore, a vaccine containing peptides derived from several of these antigens may not only lessen the chances for clonal tumor escape but also may represent a more potent approach since CTL against the unique, patient-specific antigens are induced, which apparently represent the dominant rejection antigens in the antitumor response [10]. We have searched the entire sequence of human PSM, mouse PAP and human PSA 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) [11].

DCs are the most potent antigen presentation cells (APCs) for inducing differentiation of naive CD4⁺ and CD8⁺ T cells into helper and cytotoxic T cells, respectively, and for initiating primary and secondary immune responses [12,13]. To prime T cell responses, a promising approach is to link 3P to IgG Fc fragment to increase DNA vaccine potency. DCs express several receptors for the Fc portion of IgG (FcγR), which mediate internalization of antigen-IgG complexes and promote efficient major histocompatibility complex (MHC) class-restricted antigen presentation. Receptor-mediated internalization is 1000–10,000-fold more efficient than pinocytosis [14,15]. In addition, the occupancy of Fc to FcγRs activates DCs by up-regulating expression of surface molecules and cytokines secretion involved in antigen presentation [16]. Our previous study suggested the incorporation of the IgG Fc fragment into the tumor vaccine enhanced the immunogenicity [11].

Another promising strategy to improve the 3P-based vaccine potency is to link 3P with secondary lymphoid tissue chemokine (SLC). Accumulating data suggests that SLC play an integral role in the initiation of a specific immune response. SLC is a chemokine that originally was shown to be strongly expressed in secondary lymphoid organs, in particular in high endothelial venules and in the T cell zone of the lymph nodes [17,18]. It has been shown to be involved in the migration of DCs from the skin into the draining lymph nodes, suggesting that SLC would be active *in vivo* on DCs of an intermediate stage of maturity after antigen capture [13], on their way to secondary lymphoid organs. SLC is also a chemotactic factor for T and B cells [19], as well as NK cells [20]. 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. Our previous results demonstrated that human SLC also show chemotactic efficacy in mouse model [21].

T cell activation and proliferation is governed by a balance between positive and negative costimulatory signals that ultimately determine T cell effector function [22,23]. One is an antigen-specific signal that is provided when TCR is ligated by the antigenic peptide-MHC molecular complex. The other is a costimulatory signal via the B7-CD28 pathway. On the other hand, CTLA-4 is expressed on activated CD4⁺ and CD8⁺ T cells and binds to the B7 costimulatory ligands. Because of its higher avidity for B7, CTLA-4 efficiently competes for ligand binding with CD28. Following interaction with B7 on the

APC, CTLA-4 functions as a negative regulator of T cell activation [24,25]. Several studies have demonstrated that specifically blocking the interaction of CTLA-4 with B7 enhanced immune responses *in vitro* and *in vivo* [26,27]. It is apparent that anti-CTLA-4 administration could provide potent *in vivo* blockade of the B7-CTLA-4 pathway and might be a useful immune adjuvant to enhance T-cell mediated antitumor immune response.

We have 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 [11,21]. 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 prevent tumor growth. Here, 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).

In the current study, we examined the potential of CTLA-4 blockade plus B16F10-SLC-3P-Fc cell vaccines in the treatment of tumor-bearing mice. Our data indicated that B16F10-SLC-3P-Fc cell vaccines produce and secrete SLC-3P-Fc fusion proteins which can be efficiently captured and processed by DC via receptor-mediated endocytosis and presented to MHC class II and I (cross-priming), resulting in significant antitumor immunity. Our results also suggested the antitumor response is specific for PSM, PAP, and PSA antigens and CTLA-4 blockade resulted in enhancing the generation of proliferative and CTL responses to B16F10-SLC-3P-Fc cell vaccines. Finally, our findings demonstrate that the combination of CTLA-4 blockade and B16F10-SLC-3P-Fc cell vaccines is therapeutically effective against prostate cancer in a mechanism dependent on CD4⁺ and CD8⁺ cells. The combination of these vaccination strategies strongly enhances autoreactive cellular immunity leading to effective immunotherapy of prostate cancer.

2. Material and methods

2.1. Cell lines and mice

C57BL/6 melanoma cell line B16F10, 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) (hereafter, complete medium).

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

2.2. Plasmid constructs

hPSM, hPAP, mPAP, hPSA and human SLC were amplified by RT-PCR respectively as described in previous study. The 3P fusion gene and SLC-3P-Fc were constructed as described in previous study [11,21]. The 3P, hPSA, hPAP, hPSM, SLC,

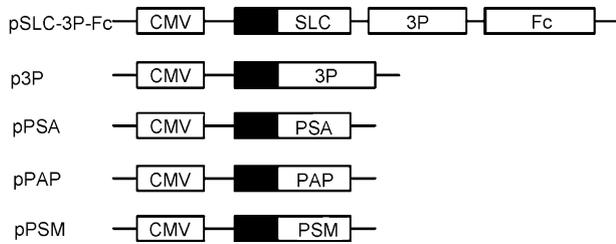


Fig. 1. Schematic representation of expression vectors. The SLC-3P-Fc fusion gene, 3P (secretory) gene, PSA gene, PAP gene, PSM gene was cloned into the pcDNA3.1 vector under the CMV promoter control. (■) Signal sequences.

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), and pcDNA3.1-SLC-3P-Fc (pSLC-3P-Fc), respectively (Fig. 1) [21]. 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 [28].

2.3. Cell transfection

pC, pcDNA3.1-3P, pPSA, pPAP, pPSM 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 $\mu\text{g}/\text{ml}$ 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-SLC-3P-Fc [11,21]. Total RNA extracted from pSLC-3P-Fc-, pC-transfected cells or target cells was used to verify the expression of SLC-3P-Fc, 3P, PSM, PAP, or PSA by RT-PCR. Secreted proteins and cellular proteins were analyzed by Western blotting. These results demonstrate that the constructs were expressed in the eukaryotic cells [11,21]. The cell proliferation was assayed by MTT assay and the results indicated that plasmid DNA had no effect on transfected target cell proliferation [11,21]. The Fc portion of human IgG1 can efficiently bind to human DC as well as to murine DC [29].

2.4. Histology

B16F10-SLC-3P-Fc or B16F10-pC cells (5×10^6 cells/ml) were pretreated with mitomycin-C (80 $\mu\text{g}/\text{ml}$) for 60 min and then washed with PBS for three times. The mice were intraperitoneally injected 100 μg of anti-CTLA-4 mAbs (kindly provided by Dr. L.P. Chen) or combined subcutaneously vaccinated at the left flank back with 1×10^6 of the pretreated B16F10-SLC-3P-Fc cells per mouse. And the mice were subcutaneously vaccinated at the left flank back with 1×10^6 of the pretreated B16F10-pC cells as a negative control. 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.

2.5. 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, 20. Anti-CTLA-4 mAb was given intraperitoneally on days 9,10 and 11. On day 30, mice were inoculated at the right flank back with 5×10^4 B16F10-3P cells/mouse.

For in vivo therapeutic experiment, 5×10^4 B16F10-3P cells/mouse were subcutaneously inoculated at the right flank back of male C57BL/6 mice on day 0, then immunized subcutaneously with 1×10^6 the modified tumor cells pretreated with mitomycin-C/mouse at the left flank back on days 4, 11 and 18. On days 4, 7 and 10, 100 μg of anti-CTLA-4 mAbs or control hamster IgG was given intraperitoneally in 100 μl of PBS.

Tumors were monitored every 2 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) [30].

2.6. Cytotoxic T-lymphocyte assay

Male C57BL/6 mice were subcutaneously challenged with 5×10^4 B16F10-3P cells/mouse in the right flank back on day 0, then subcutaneously immunized with anti-CTLA-4 mAbs plus 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, USA) according to the manufacturer's instructions. B16F10, B16F10-pC, B16F10-3P, B16F10-PSM, B16F10-PAP and B16F10-PSA 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 = $([E - \text{Se} - \text{St}] / ([\text{Mt} - \text{St}]) \times 100$, where *E* stands for 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*, the maximal release by target cells.

2.7. In Vivo depletion of CD4⁺ and CD8⁺ T Cells

To determine which subset of immune cells was contributing to the antitumor effect elicited by tumor cells combination with anti-CTLA-4 mAbs treatment, T cell subsets were depleted. Mice were subcutaneously vaccinated three times at 5-day intervals with B16F10-SLC-3P-Fc or B16F10-pC by subcutaneous injection, or injected intraperitoneally with 100 μg of anti-CTLA-4 mAbs on days 9,10 and 11, then 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.

To investigate which subset of immune cells was contributing to antitumor effect of CTLA-4 blockade, mice were challenged with 5×10^4 B16F10-3P cells/mouse on day 0, then treated with anti-CTLA-4 mAbs on days 9, 14 and 19. In vivo depletion was achieved by injection of anti-CD4 or anti-CD8 mAbs on day 9, 11 and 13, then twice per week before sacrificed.

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

2.8. 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 the modified tumor cells pretreated with mitomycin-C on days 4, 11 and 18. On days 4, 7 and 10, 100 μ g of anti-CTLA-4 mAbs was given intraperitoneally in 100 μ l of PBS. On day 28, splenocytes from the immunized mice were prepared as effector cells. 1×10^7 effector cells/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.

2.9. 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 the *p* was <0.05. Statistical analysis was performed using commercially available software (SPSS 11.0).

3. Results

3.1. Chemotactic activity of SLC-3P-Fc protein in vivo

As shown in Fig. 2, H&E staining of dermis tissue sections from anti-CTLA-4 mAbs treated mice showed infiltration of few lymphocytes into the immunization region. Tissues from

mice that received B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs showed a prominent infiltration of lymphocytes, which may be chemoattracted by SLC-3P-Fc.

Our previous observations [21], 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. Moreover, equivalent SLC concentration in culture supernatants of pSLC-3P-Fc- or pSLC-transfected B16F10 were detected in ELISA assay (data not shown).

3.2. Protective immune response induced by gene-modified tumor cells and blockade of CTLA-4

To investigate the protective antitumor immunity, we immunized mice with B16F10-pC, B16F10-SLC-3P-Fc, anti-CTLA-4 mAbs or B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs, then challenged mice with 5×10^4 B16F10-3P tumor cells/mouse. As shown in Fig. 3A, tumors grew progressively in B16F10-pC-immunized mice, but there was apparent protection from tumor growth in B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs-immunized mice. The results are expressed as mean tumor volume \pm S.E.M. The survival of the mice treated with B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs was also significantly greater than that of B16F10-pC-immunized mice (*p* < 0.01, by log-rank test) (Fig. 3B). In addition, our data indicated that B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs-immunized mice are capable of stimulating antitumor immune response that specifically recognized and lysed 3P-expressing tumor cells.

3.3. CTLA-4 blockade enhances the antitumor effect of gene-modified tumor cell vaccine

To evaluate whether the combination of modified tumor cell vaccine and blockade of CTLA-4 co-signaling pathway would result in a more effective anti-tumor response, mice were subcutaneously inoculated with B16F10-3P cells, then subcutaneously vaccinated with gene-modified tumor cells, intraperitoneal administrated anti-CTLA-4 mAbs or control IgG. As illustrated in Fig. 4A (one-way ANOVA, *p* < 0.001, data

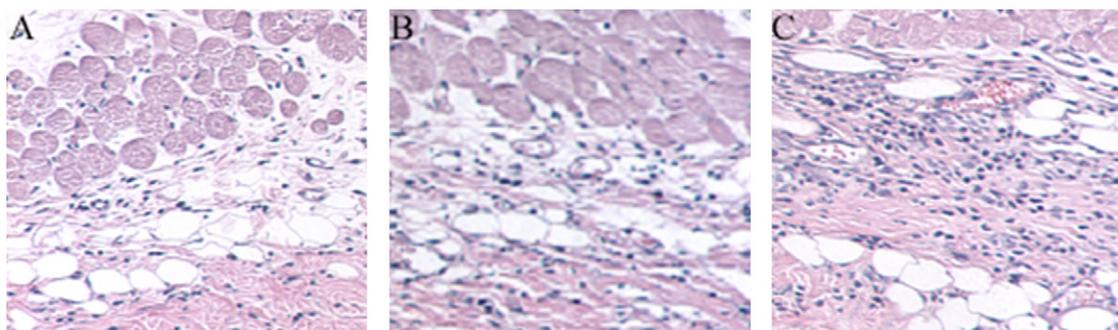


Fig. 2. B16F10-SLC-3P-Fc chemoattract lymphocytes to the immunization site. H&E staining of dermis tissue sections from C57BL/6 mice treated with B16F10-pC (A), treated with anti-CTLA-4 mAbs (B) or treated with B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs (C). Images are representative of multiple microscopic fields observed in at least three mice per group.

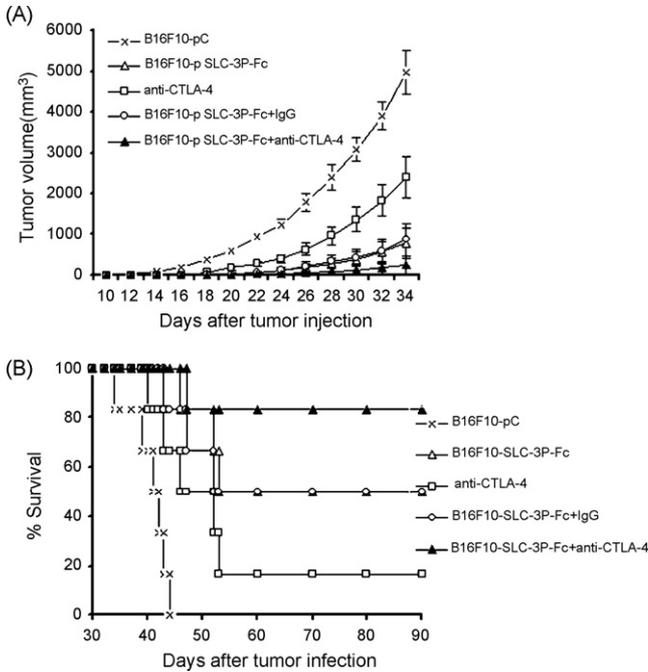


Fig. 3. Induction of the protective antitumor immunity. The mice were subcutaneously vaccinated with 1×10^6 gene-modified tumor cells pretreated with mitomycin-C/mouse at the left flank back on days 0, 10, 20. Anti-CTLA-4 mAbs was given with the second vaccine, on days 9, 10 and 11. On day 30, mice were inoculated at the right flank back with 5×10^4 B16F10-3P cells/mouse ($n=6$). (A) Tumor volume (mm³) was shown. (B) Survival of mice per treatment group. The survival of mouse per group that treated with B16F10-SLC-3P-Fc plus anti-CTLA-4 was 83% at day 90.

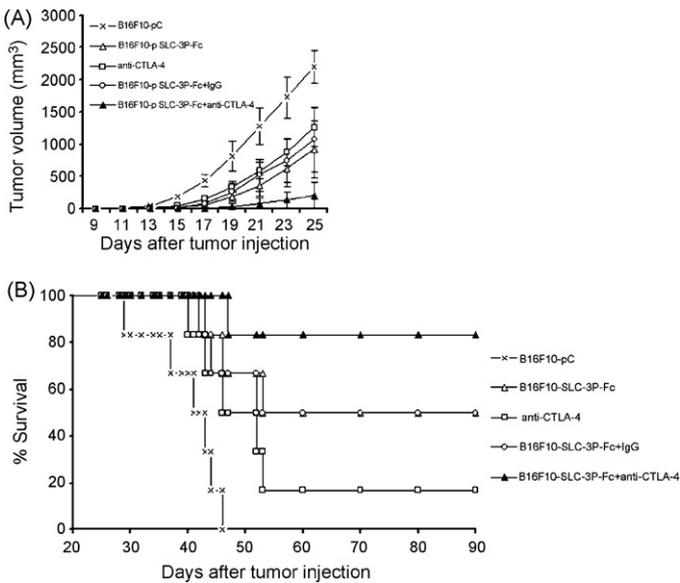


Fig. 4. Induction of the therapeutic antitumor immunity. C57BL/6 mice were inoculated subcutaneously with 5×10^4 B16F10-3P tumor cells/mouse on day 0, and the mice were treated with modified tumor cell vaccine on days 4, 11 and 18 followed by either hamster IgG or anti-CTLA-4 mAbs administration (intraperitoneal) on days 4, 7 and 10 ($n=6$). (A) Data were reported as the average tumor volumes (mm³) of six mice per group that vaccinated with B16F10-SLC-3P-Fc followed by IgG or anti-CTLA-4 treatment. (B) Survival of mice per treatment group. The survival of mouse per group that treated with B16F10-SLC-3P-Fc plus anti-CTLA-4 was 88% at day 90.

are expressed as mean tumor volume \pm S.E.M.), the combination of B16F10-SLC-3P-Fc and anti-CTLA-4 mAbs resulted in the greatest reduction of tumor growth and significant improvement of the mice survival as shown in Fig. 4B ($p < 0.001$, 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 + anti-CTLA-4 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 + anti-CTLA-4 mAbs treated mice compared to B16F10-pC-treated counterparts ($p < 0.01$; Fig. 5). 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 in B16F10-SLC-3P-Fc-immunized mice are specific for PSM, PAP, and PSA antigen.

For evaluation of the persistent protective effect of the combined therapy, the survivors with complete tumor regression in Fig. 6 and the naive control mice were subcutaneously rechallenge with 1×10^5 B16F10-3P tumor cells/mouse on day 120 after the initial tumor inoculation. No mice from the group treated with B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs developed tumors after an additional 60 days. In contrast, 33% of the mice from the group treated with B16F10-SLC-3P-Fc or B16F10-SLC-3P-Fc + control IgG developed tumor, 100% of the mice from the group treated with anti-CTLA-4 mAbs developed tumor (Fig. 6). The observation of long-term immunity implies that B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs may

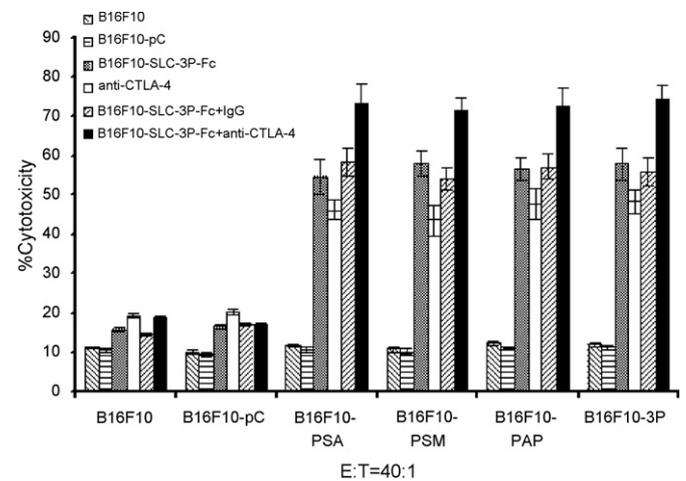


Fig. 5. CTL Induction by the B16F10-SLC-3P-Fc and anti-CTLA-4 mAbs combination therapy. C57BL/6 mice were inoculated subcutaneously with 5×10^4 B16F10-3P tumor cells/mouse on day 0 and were treated with modified tumor cell vaccine on days 4, 11 and 18 followed by either hamster IgG or anti-CTLA-4 mAbs administration (intraperitoneal) on days 4, 7 and 10 ($n=3$). Ten days after the last immunization, splenocytes from C57BL/6 mice, immunized with B16F10, B16F10-pC, B16F10-SLC-3P-Fc, anti-CTLA-4, B16F10-SLC-3P-Fc + control IgG or B16F10-SLC-3P-Fc + anti-CTLA-4 were tested for cytolytic activity against B16F10, B16F10-pC, B16F10-hPSA, B16F10-hPSM, B16F10-hPAP, and B16F10-3P cells. Data are expressed as mean \pm S.E.

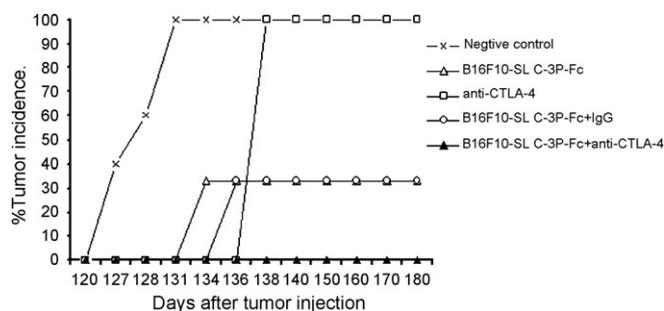


Fig. 6. Evaluation of persistent anti-tumor response. The tumor-free mice from B16F10-SLC-3P-Fc-treated group ($n=3$), anti-CTLA-4-treated group ($n=1$), B16F10-SLC-3P-Fc + IgG-treated group ($n=3$) or B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs-treated group ($n=5$) were subcutaneously rechallenged with 1×10^5 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.

successfully mediate the development of persistent memory immune response.

3.4. Role of T-cell subsets in B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs 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 + anti-CTLA-4 mAbs, B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs 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. 7D). In addition, B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs-immunized mice treated with anti-CD4 showed the anti-tumor activity against B16F10-3P, whereas the treatment with anti-CD8 partially abrogated the protective effects of B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs (Fig. 7C). Furthermore, if depletions were started before immunization with B16F10-SLC-3P-Fc + anti-CTLA-4 mAbs, *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. 7A). Mice depleted of CD4⁺ T lymphocytes or CD8⁺ T lymphocytes did not develop detectable CTL activity (Fig. 7B). 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 + anti-CTLA-4 mAbs vaccine.

To determine the relative contribution of T cell subsets on rejection of B16F10-3P tumor cells following CTLA-4 blockade, we depleted CD8⁺ or CD4⁺ T-cell subsets *in vivo* following CTLA-4 blockade. Nonspecific depletion of CD4⁺ T-cells cannot affect the antitumor immunity of anti-CTLA-4 mAbs, but the depletion of CD8⁺ lymphocytes showed partial abrogation of the antitumor activity *in vivo* (Fig. 7E and F).

3.5. 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 CTLA-4 co-signaling pathway mice, splenocytes from the immunized mice were used as effector cells and adoptively transferred into the tumor-bearing mice. As shown in Fig. 8A, B16F10-3P tumor growth can be significantly inhibited by inoculated with splenocytes from B16F10-SLC-3P-Fc plus anti-CTLA-4 mAbs immunized mice (one-way ANOVA, $p < 0.001$, data are expressed as mean tumor volume \pm S.E.M.). On day 90, 88% 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 from B16F10-pC immunized mice developed tumors and died within 50 days after tumor challenging (Fig. 8B).

4. Discussion

One of the major obstacles for achieving a tumor-specific immune response is overcoming peripheral T cell tolerance against tumor self-antigens and inducing CTLs that effectively eradicate disseminated tumor metastases and subsequently maintain a long-lasting immunological memory preventing tumor recurrence [31,32]. The vaccines described here are based on the premise that tumor cells will be destroyed by CD8⁺ T cells with help from CD4⁺ T cells. To better activate tumor-specific CD4⁺ and CD8⁺ T cells, we have designed cell-based vaccines that facilitate the presentation of MHC class II and class I-restricted tumor peptides to responding CD4⁺ and CD8⁺ T cells. After vaccination via subcutaneous injection, SLC-3P-Fc-modified tumor cells produce and secrete SLC-3P-Fc fusion proteins. The secreted fusion proteins can efficiently recruit T cells and DCs and cross-present tumor antigen to both MHC class II and class I by DCs in a cognate manner, leading to the activation of both antigen-specific Th and CTL responses [14–16,33,34]. Thus, this unifying antigen presentation strategy that can induce broad and potent antitumor immunity could be used to improve the efficacy of tumor vaccines and immunotherapies.

According to the strategy described above, we were here able to induce efficiently and specifically CTL to lyse target cells expressing 3P, PSM, PAP, or PSA, but not B16F10 and B16F10-pC (Fig. 5). The data indicated that the antitumor activity of B16F10-SLC-3P-Fc vaccine may result from the induction of CTL-mediated killing of tumor cells. In the protective and therapeutic experiments, our findings demonstrated that vaccination with B16F10-SLC-3P-Fc shows considerable retardation in tumor growth and significantly prolonged the survival of the tumor-bearing mice. 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 a monoclonal response. Second, SLC-3P-Fc-gene modification increased the

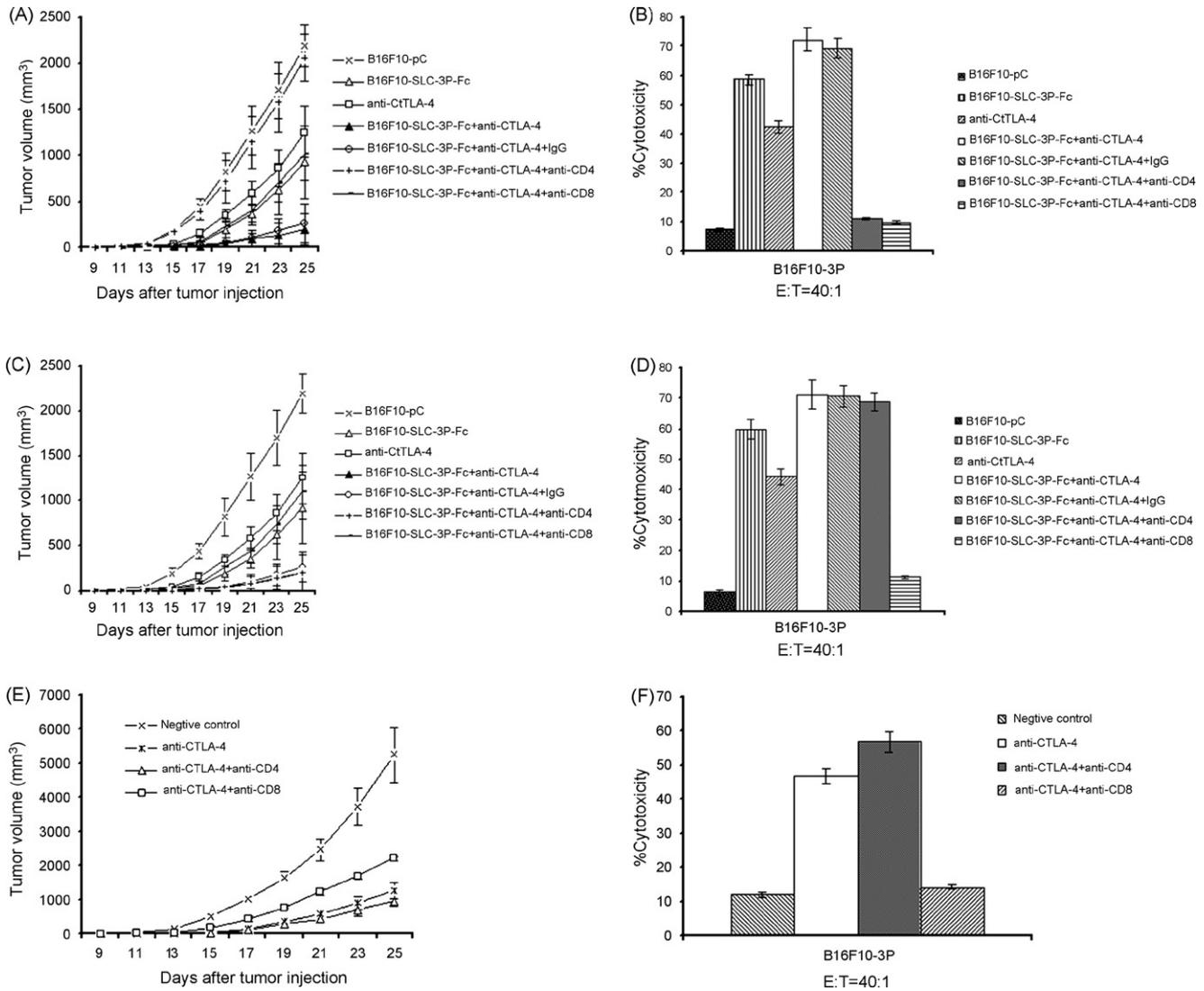


Fig. 7. Assessment of involvement of CD4⁺ and CD8⁺ T cells in anti-tumor effect. To determine which subset of immune cells was contributing to the antitumor effect elicited by tumor cells combination with anti-CTLA-4 mAbs treatment, T cell subsets were depleted (A–D). Before or after injected with mitomycin-C-treated B16F10-SLC-3P-Fc plus anti-CTLA-4 mAbs, 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 μ l/mouse) twice a week before killing ($n = 3$). (A) and (B) Abrogation of antitumor activity and of CTL-mediated cytotoxicity by in vivo depletion of the T-cell subsets before immunization with vaccine. (C) and (D) Abrogation of CTL-mediated cytotoxicity and of antitumor activity by in vivo depletion of the T-cell subsets after immunization with vaccine. To investigate which subset of immune cells was contributing to antitumor effect of CTLA-4 blockade, mice were challenged with 5×10^4 B16F10-3P cells/mouse on day 0, then treated with anti-CTLA-4 mAbs on days 9, 14 and 19. In vivo depletion was achieved by injection of anti-CD4 or anti-CD8 mAbs on day 9, 11 and 13, then twice per week before sacrificed. (E) Abrogation of antitumor activity by in vivo depletion of the T-cell subsets. (F) Abrogation of CTL-mediated cytotoxicity by in vivo depletion of the T-cell subsets.

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 autologous cellular vaccine.

Combining B16F10-SLC-3P-Fc cell vaccine with CTLA-4 blockade represents a novel approach to the immunotherapy of 3P-expressing tumor. CTLA-4 plays a significant role in reg-

ulating peripheral T cell tolerance by interfering with T cell activation through both passive and active mechanisms [22]. In vitro, CTLA-4 mAb blockade lowers the T cell activation threshold and removes the attenuating effects of CTLA-4. Administration of Abs that block the interaction between CTLA-4 and B7 in vivo results in the rejection of a number of transplantable tumors in mice including colon carcinoma, fibrosarcoma, lymphoma, and renal carcinoma [22]. In this study, we show that, when combined with SLC-3P-Fc-producing tumor cell vaccines, CTLA-4 results in rejection of established poorly immunogenic B16F10-3P tumor (Fig. 4). This occurs through a process which involves breaking tolerance to tumor-associated autoantigen. Our discovery that CTLA-4 blockade

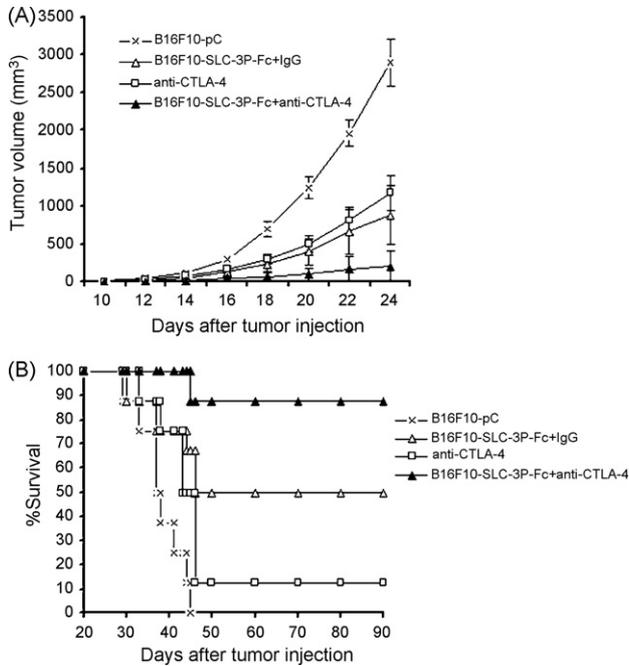


Fig. 8. Evaluation of therapeutic effect of adoptive therapy. Mice were immunized as described in Fig. 4, and splenocytes from the immunized mice were used as effector cells and adoptively transferred into the tumor-bearing mice which were subcutaneously inoculated with 5×10^4 B16F10-3P cells/mouse 4 days ago ($n=8$). (A) Tumor volume (mm^3) in mice treated with splenocytes from B16F10-SLC-3P-Fc plus anti-CTLA-4 mAbs immunized group and controls was shown. (B) Percentage survival of mice treated. The survival rate of B16F10-SLC-3P-Fc plus anti-CTLA-4 mAbs group was 88% at day 90.

enhances the effect of a potent SLC-3P-Fc-expressing tumor cell vaccine to cause rejection of established tumors extends our results on the enhancement of 3P-specific CTL responses by anti-CTLA-4 mAb treatment (Fig. 5). Rejection is accompanied by long-lived memory, as indicated by the fact that cured tumor-bearing mice reject rechallenge in the absence of treatment 4 months after the initial treatment (Fig. 6). Our data in this mouse tumor model also indicate a synergy between CTLA-4 blockade and SLC-3P-Fc modified tumor cell vaccine. B16F10-3P-bearing mice treated with either the vaccine or antibody alone had 50% or 33% reduction in tumor incidence or tumor size, whereas the combination of both resulted in 83% reduction in both criteria (Fig. 4). This suggests that an additional source of antigen from the cell-based vaccine contributes to T-cell priming, which is enhanced by blockade of CTLA-4/B7 interactions. In our view, there are at least two nonexclusive explanations for our observation that anti-CTLA-4 antibodies synergize with B16F10-SLC-3P-Fc cell vaccine to induce rejection: (a) CTLA-4 blockade greatly increases the burst size of T cells responding to the B16F10-SLC-3P-Fc vaccine, thus enhancing the mobilization of effector cells, and (b) CTLA-4 blockade lowers the threshold for T cell activation, thereby allowing the recruitment and activation of low-affinity autoreactive T cells that might have escaped central tolerance induction.

To explore the role of immune cell subsets played in this antitumor activity, we depleted CD4^+ or CD8^+ T lymphocytes through injection of the corresponding antibodies. If depletions

were started after immunization but before tumor challenge, the 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. 7A and B), suggesting that the killing activity observed results from CD8^+ CTL activity. We also found that, if depletions were started before immunization, the lack of CTL activity by the depletion of CD4^+ T lymphocytes were found to be associated with the complete abrogation of the antitumor activity, whereas the CTL activity by the depletion of CD8^+ lymphocytes was associated with partial abrogation of the antitumor activity. It is known that CD4^+ T lymphocytes can steer and amplify immune responses through secretion of cytokines and expression of surface molecules [35,36]. CD4^+ T lymphocytes have been reported to be required for the induction of the anti-tumor immunity by DNA immunization against human gp75/tyrosinase-related protein-1 in melanoma models [37,38]. In addition, it has been reported that CD4^+ T lymphocytes play a prominent role in classic mouse models of autoimmunity, such as experimental autoimmune encephalitis, systemic lupus erythematosus and autoimmune gastritis [36,39,40]. These findings may help explain the requirement for CD4^+ T lymphocytes in the induction of antitumor activity by the combination of CTLA-4 blockade with B16F10-SLC-3P-Fc tumor cell vaccine. 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, $\text{CD4}^+\text{CD25}^+$ regulatory T cells (Th2 cells) restrict the strength of the response [25]. Thus, if depletions were started after immunization with B16F10-SLC-3P-Fc plus anti-CTLA-4 mAb, 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 (Fig. 7C and D). More importantly, the antitumor activity can be acquired with adoptive transfer of lymphocytes from B16F10-SLC-3P-Fc-immunized and CTLA-4 blockade mice (Fig. 8). 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 plus anti-CTLA-4 mAb. Our results also suggested that, during the effector phase of immune response, the antitumor effects seen after CTLA-4 blockade using an anti-CTLA-4 mAb are not due to inhibition or depletion of T regulatory cells but rather appear to act through direct activation of CD4^+ and CD8^+ effector cells (Fig. 7E and F).

In conclusion, our data strongly support the concept that the presence of anti-CTLA-4 mAb is a requirement in breaking peripheral tolerance and mounting a therapeutic immune response in tumor-bearing mouse toward a prostate tumor-associated antigen. The potency of the combination of the B16F10-SLC-3P-Fc cell vaccine and anti-CTLA-4 antibody can likely be attributed to enhanced cross-priming of T cells by host APCs by the vaccine, together with a highly potentiated T cell response as a result of the removal of the inhibitory effects of CTLA-4 by antibody blockade. This results in a synergistic enhancement of the T cell response to a level capable of eliminating the preexisting tumor cell mass.

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