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Research Paper

Enhancement of DNA Vaccine Potency by Sandwiching Antigen-Coding Gene Between Secondary Lymphoid Tissue Chemokine (*SLC*) and IgG *Fc* Fragment Genes

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KEY WORDS

DNA vaccine, tumor, secondary lymphoid tissue chemokine, IgG Fc

ABBREVIATIONS

SLC	secondary lymphoid tissue chemokine
DCs	dendritic cells
Th	T helper cells
MHC	major histocompatibility complex
CTL	cytotoxic T lymphocyte

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ABSTRACT

DNA vaccine has become an attractive approach for generating antigen-specific immunity. Targeting antigens to FcRs for IgG (FcγRs) on dendritic cells (DCs) has been demonstrated to enhance antigen presentation. Secondary lymphoid tissue chemokine (SLC) has been shown to increase immune responses not only by promoting coclustering of T cells and DCs in the lymph nodes and spleen but also by regulating their immunogenic potential for the induction of T cell responses. In this study, using HPV 16 E7 as a model antigen, we constructed a chemotactic-antigen plasmid DNA vaccine (pSLC-E7-Fc) by linking *SLC* and *Fc* gene sequences to each end of *E7* and evaluated its potency of eliciting specific immune response. We found that immunization with pSLC-E7-Fc generated much stronger E7-specific lymphocyte proliferative and cytotoxic T lymphocyte (CTL) responses than control DNA. All the mice receiving pSLC-E7-Fc prophylactic vaccination remained tumor free upon subcutaneous inoculation of TC-1 cells, while those given control DNA all developed tumors. These tumor-free mice were also protected against TC-1 rechallenge. Complete tumor regression with long-term survival occurred in 72% of mice given pSLC-E7-Fc as therapeutic vaccination. In experimental lung metastasis model wherein TC-1 cells were intravenously injected, therapeutic vaccination with pSLC-E7-Fc significantly reduced the number of tumor nodules in the lung. In vivo depletion with antibodies against $CD4^+$ or $CD8^+$ T cells both resulted in complete abrogation of the pSLC-E7-Fc-induced immunotherapeutic effect. Our data indicate that the DNA vaccine constructed by the fusion of *SLC* and IgG *Fc* fragment genes to antigen-coding gene is an effective approach to induce potent anti-tumor immune response via both $CD4^+$ and $CD8^+$ T cells dependent pathways.

INTRODUCTION

DNA vaccine has become an attractive approach for generating antigen-specific immunity. However, one of the concerns about DNA vaccines is their limited potency. Several strategies have been used trying to increase the potency of DNA vaccines, such as increasing expression of the encoded proteins by optimizing codon usage of antigen genes;[1,2] co-delivery of plasmid DNA encoding costimulatory molecules, or cytokines.[3,4] A promising approach is to link antigens to *SLC* to increase DNA vaccine potency. SLC (also known as CCL21, 6Ckine, Exodus-2) is constitutively expressed on high endothelial venules and within T-cell zones of both spleen and lymph nodes. It chemo-attracts naïve T cells and antigen-presenting DCs to T-cell zones of secondary lymphoid organs resulting in induction of cognate T-cell activation.[5-10] Thus, SLC plays an integral role in the initiation of a specific immune response. Of note, functions other than chemotactic activity are attributed to SLC, such as inhibiting apoptosis of mature DCs and tumor angiogenesis, and inducing T helper cell (Th) type 1 responses.[9-12] Taken together, these findings may have important implications for rational vaccine design. Investigations have shown enhanced systemic anti-tumor immune response in mice given SLC.[6-8]

Another promising approach to increase DNA vaccine potency is to link antigens to IgG *Fc* fragment. DCs express IgG Fc receptors (FcγRs), which mediate internalization of antigen-IgG complex and promote efficient major histocompatibility complex (MHC) class II-restricted antigen presentation. Receptor-mediated internalization is 1,000–10,000-fold more efficient than pinocytosis.[13-15] FcγRs-mediated endocytosis can cross-present the internalized antigen to MHC class I antigen processing pathway as well. In addition, the occupancy of Fc to FcγRs activates DCs by upregulating expression of

surface molecules and cytokines secretion involved in antigen presentation.[13,14] Thus, FcγRs represent a privileged antigen internalization route for efficient MHC class I- and II-restricted antigen presentation by DCs. Vaccines targeting tumor antigen to FcγRs have been showed to elicit effective systemic anti-tumor Th and CTL responses.[16,17]

In this study, we explored whether *SLC* and IgG *Fc* could cooperatively enhance DNA vaccine potency. Such an attempt, to our knowledge, has not yet been investigated. The model antigen used in this study was the early antigen E7 of HPV type 16. We constructed a fused plasmid DNA pSLC-E7-Fc by linking *E7* gene to *SLC* at its N-terminus and to IgG Fc fragment at its C-terminus. The results demonstrated that *SLC* and *Fc* could synergistically enhance the immunogenicity of an E7-expressing plasmid DNA vaccine, resulting in dramatic augmentation of systemic anti-tumor immunity against E7-expressing tumors.

MATERAILS AND METHODS

Mice. Female C57BL/6 mice (6–8 weeks old) were purchased from the Animal Center of Chinese Academy of Medical Sciences (Beijing, China) and kept under specific pathogen-free conditions.

Cell lines. C57BL/6 melanoma cell line B16F10 was kindly provided by L. Chen (Department of Immunology, Mayo Graduate and Medical Schools, Mayo Clinic, Rochester, USA) and cultured in complete RPMI 1640 medium; Mouse TC-1 tumor cells, derived from primary epithelial cells of C57BL/6 mice cotransformed with HPV-16 E6 and E7, and c-Ha-ras oncogene was a kind gift from Dr. T.C. Wu (Johns Hopkins Medical Institutions, Baltimore, MD) and cultured in complete RPMI 1640 medium supplemented with 400 μg /ml G418.

Plasmid DNA Constructs and Preparation. PCR was performed with the following primers:

P1: 5'-GGATCCATGGCTCAGATGATG-3'
P2: 5'-GAATTCTATCCTCTTGAGGGC-3' (with stop codon)
P3: 5'-GAATTCACCACCACCTCCTCTTGAGGGCTG-3' (without stop codon)
P4: 5'-GAATTCGCAGCAGTCCTGAC-3' (without stop codon)
P5: 5'-CGAATTCATGCACGGAGATACACC-3'
P6: 5'-TGATATCTATGGTTTCCGAGAACAG -3' (with stop codon)
P7: 5'-TGATATCTGGTTTCCGAGAACAG-3' (without stop codon)
P8: 5'- GGGCCCTCATTTACCCGGAGAC-3'
P9: 5'-TTGTTACCAACTGGGACGACATGG-3'
P10: 5'-GATCTTGATCTTCATGGTGCTAGG-3'

pcDNA3.1 (Invitrogen) was altered at multiple cloning sites to create pcDNA3f (p3f) in order to facilitate the transfer of genes to pShuttle-CMV (pCMV, Quantum Biotechnologies) eukaryotic expression vector. The human IgG1 *Fc* fragment was inserted into p3f to generate p3f-Fc and then Fc fragment was inserted into pCMV to generate pCMV-Fc. The Fc portion of human IgG1 could efficiently bind to human as well as murine DCs.[18]

Mouse *SLC* with or without stop codon and the signal sequence of *SLC* (Sig) were amplified by RT-PCR using primers P1 and P2, P1 and P3, and P1 and P4, respectively. HPV 16 *E7* with or without stop codon was cloned with PBR322-HPV16 plasmid (provided by Jie Li, Department of Immunology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China) as template with primers P5 and P6, and primes P5 and P7, respectively.

SLC, *Sig* and *E7* fragment were inserted into p3f to produce p3f-SLC, p3f-SLC-E7 and p3f-Sig-E7. *SLC*, *SLC-E7*, *Sig-E7* and *Sig* fragments were inserted into pCMV and pCMV-Fc to generate pCMV-SLC (pSLC), pCMV-SLC-E7 (pSLC-E7), pCMV-Sig-E7 (pE7),pCMV-SLC-Fc (pSLC-Fc), pCMV-Sig-E7-Fc (pE7-Fc),pCMV-SLC-E7-Fc (pSLC-E7-Fc) and pCMV-Sig-Fc (pFc), respectively (Fig. 1). pCMV plasmid was used as vector control. All the recombinant constructs were confirmed by DNA sequencing. Plasmids for DNA vaccination were purified from a large-scale culture by alkaline lysis and polyethylene glycol precipitation.[19]

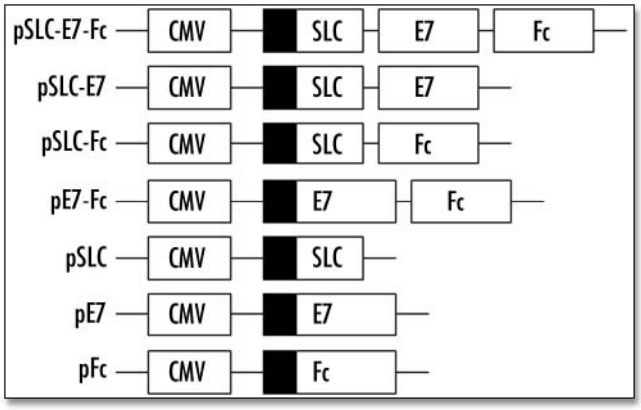

Figure 1. Schematic presentation of expression vectors. The *SLC-E7-Fc* fusion gene, *SLC-E7* fusion gene, *SLC-Fc* fusion gene, *E7-Fc* (secretory) fusion gene, *SLC* gene, *E7* (secretory) gene, *Fc* DNA fragment with a signal sequence (secretory) were each cloned into pCMV vector under the CMV promoter control. ■, signal sequence.

DNA vaccination. Preparation of DNA-coated gold particles was performed according to a previously described protocol.[20] DNA-coated gold particles (1.25 μg of DNA/bullet) were delivered to mouse shaved abdominal region using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 p.s.i.

RT-PCR and Western blot analysis. B16F10 melanoma cells were transfected with pCMV, pSLC, pFc, pE7, pSLC-Fc, pSLC-E7, pE7-Fc, and pSLC-E7-Fc respectively using Fugene6 Transfection reagent (Roche) according to the manufacturer's instructions. Cells were harvested after 48 hours. For RT-PCR analysis, total RNA was isolated, treated with DNase I (Promega) and cDNA was synthesized. PCR was performed with the following primers: P1 and P2 for *SLC*; P1 and P8 for *Fc*, *SLC-Fc*, *E7-Fc* and *SLC-E7-Fc*; P1 and P6 for *E7* and *SLC-E7*; P9 and P10 for β-actin (internal control).

For Western blot analysis, the culture supernatants were harvested and the proteins were separated by 12.5% SDS-PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane were blocked by incubation in 5% nonfat dry milk, washed and incubated with a goat anti-human IgG Fc antibody (Santa Cruz, CA) or goat anti-mouse SLC antibody (R & D Systems), followed by incubation with an horseradish peroxidase labeled rabbit anti-goat IgG (Sigma).The membrane was visualized using the ECL Western blotting system (Amersham).

ELISA for SLC. B16F10 cells were transfected with pSLC-E7-Fc, pSLC, pE7-Fc and pCMV respectively. The culture supernatants were collected 48 hours later and assayed for SLC concentrations using the mouse SLC/CCL21/6Ckine (SLC also known as CCL21, 6Ckine[10]) ELISA kit according to the manufacture's instructions (R&D Systems, Cat # DY457). Briefly, 96-well plates were coated overnight with 4 μg/ml of rat anti-mouse SLC. Plates were then washed three times with 0.05% Tween-20 in PBS and blocked with 1% BSA in PBS for 1 hour. After three washes, plates were incubated with mouse SLC standards or with sample supernatants in duplicate for 2 hours. Plates were then washed three times and incubated with 50 ng/ml of biotinylated goat anti-mouse SLC for 2 hours. After three washes, plates were incubated with streptavidin conjugated to horseradish-peroxidase for 20 min. Plates were then washed again and incubated with substrates for 20 minutes. Reactions were stopped with 2N H_2SO4 and plates were read at 450 nm with a correction wavelength of 570 nm. The levels of SLC detected in supernatants were extrapolated from a curve generated using recombinant SLC as standards.

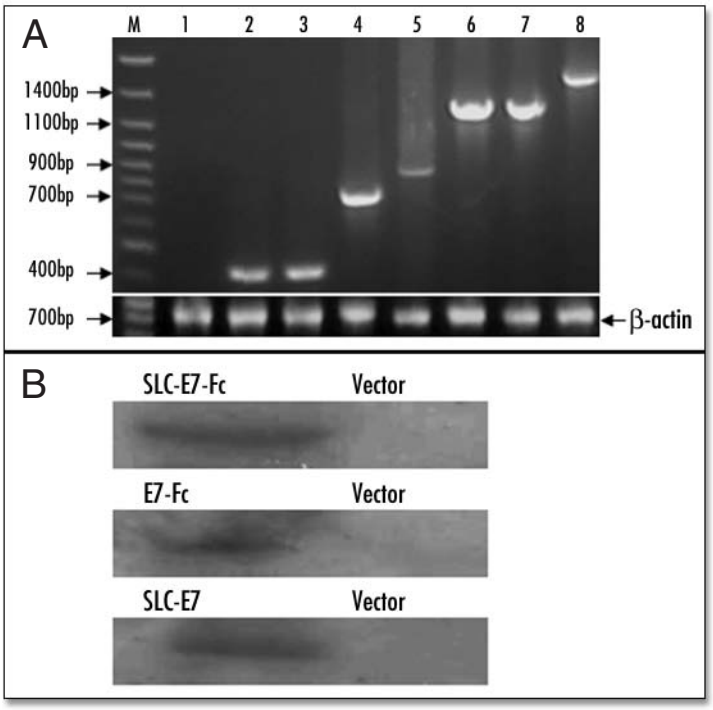

Figure 2. (A) RT-PCR detection of B16F10 cells transfected with various recombinant plasmids. DNA marker (M),Lane 1 (vector control), Lane 2 (*SLC* fragment), Lane 3 (*Sig-E7* fusion fragment), Lane 4 (*SLC-E7* fusion fragment), Lane 5 (*Sig-Fc* fusion fragment), Lane 6 (*SLC-Fc* fusion fragment), Lane 7 (*Sig-E7-Fc* fusion fragment), Lane 8 (*SLC-E7-Fc* fusion fragment). (B) Western blotting analysis of B16F10 transfectants. SLC-E7-Fc fusion protein (50kD), E7-Fc fusion protein (38kD),SLC-E7 fusion protein (22kD).

Chemotaxis assays. B16F10 cells were transfected with pSLC-E7-Fc, pSLC, pE7-Fc and pCMV respectively and culture supernatants were collected 48 hours later. The supernatants (plus anti-mouse SLC or control antibody, 2 μg/ml) were added to the lower wells of the chemotaxis microchamber (Boyden microchamber; Neuro Probe, Inc.). Mouse spleen cells 5 x 10^5/well in 50 μl medium was added to the upper wells that were separated from the lower wells by a 5-μm pore polyvinylpyrrolidone-free polycarbonate filter (Neuroprobe). After incubation for 4 hours at 37°C, cells that had not migrated into the membranes were wiped from the upper surfaces of membranes. Membranes were fixed and stained. Cells on the lower surface of membranes were counted in five randomly selected high-power fields (x400) per well in a blinded fashion. The results were expressed as the fold increase in cells migrating in response to chemoattractant vs. the medium control (chemotaxis index).

For chemotaxis assay in vivo, mice were given 1.25 μg of pSLC-E7-Fc, pSLC and pCMV respectively by means of gene gun. After 72 hours, mouse dermis at the injection sites was excised, fixed for 24 hours in 10% neutral buffered formalin, embedded in paraffin and sectioned. Sections were stained with H&E. H&E sections were observed and lymphocytes were counted in twenty randomly selected high-power fields (four fields per mouse) (x400) by several professional pathologists.

In vitro lymphocyte proliferation assay and cytotoxicity assay. Mice (three mice per group) were subcutaneously challenged with 5 x 10^4 TC-1 cells/mouse on the left flank (day 0). Mice were given a total of 2.5 μg of pSLC-E7-Fc and control DNA (including pCMV, pSLC, pFc, pSLC-Fc, pE7, pSLC-E7 and pE7-Fc) by gene gun on days 14 and 21. Mice were killed on day 31 and pooled spleen cells were used for specific lymphocyte proliferation and cytotoxicity assay.

For the lymphocyte proliferation assay, spleen cells (4 x 10^5 cells per well) and mitomycin-treated TC-1 or B16F10 cells (1 x 10^4 cell per well) were seeded in triplicates in 96-well culture plates and incubated for three days at 37°C in a humidified 5% CO_2 atmosphere. MTT assay was used to test the lymphocyte proliferation.[21,22] The colorimetrical absorbance was measured at 570 nm and the results were expressed as proliferation index: proliferation index = [(A - B)/C] x 100%, where A is the experimental absorbance from spleen cells plus tumor cells cocultures, B is the absorbance from tumor cells alone and C is the absorbance from spleen cells alone.

Table 1 **Equivalent SLC concentration in supernatants of B16F10 cell cultures 48 h after non-transfected or transfected with pSLC-E7-Fc, pSLC, pE7-Fc or pCMV**

Treatment	SLC Protein Concentration (pg/ml)
nontransfected	n.d.[a]
pCMV	n.d.[a]
pE7-Fc	n.d.[a]
pSLC	701.5
pSLC-E7-Fc	670.4

(n.d.[a], not detectable).

For cytotoxicity assay, spleen cells were used as effector cells and TC-1 or B16F10 cells served as target cells. Effector cells were mixed with target cells (10^4 per well) at various effector/target (E/T) ratios (10:1, 20:1 and 40:1). After a 4-hour incubation, the supernatant was collected to assess the amount of lactate dehydrogenase (LDH) in the cultured media according to a previously described protocol.[20]

Tumor prophylactic and treatment experiments. For the in vivo tumor prophylactic experiments, mice (five mice per group) were vaccinated with 1.25 μg of pSLC-E7-Fc and control DNA (including pCMV, pSLC, pFc, pSLC-Fc, pE7, pSLC-E7, pE7-Fc, and pSLC + pE7 + pFc). Mice receiving no vaccination served as negative control. Ten days later, mice were subcutaneously challenged with 5 x 10^4 TC-1 cells/mouse on the left flank and growth of tumor was monitored once every four days. On day 60 after TC-1 challenge, the tumor-free mice were rechallenged by injecting 1.5 x 10^5 TC-1 cells per mouse on the right flank and five naïve mice receiving the same amount of TC-1 cells served as control.

For the in vivo tumor treatment experiments, mice were subcutaneously injected with 5 x 10^4 TC-1 cells/mouse on the left flank (day 0). On day 14, mice with palpable tumors (2 mm x 2mm minimum) were selected and assigned into nine groups (seven mice per group). Mice were vaccinated with a total of 3.75 μg of pSLC-E7-Fc and control DNA (including pCMV, pSLC, pFc, pSLC-Fc, pE7, pSLC-E7 and pE7-Fc) on days 14, 21 and 28. Mice receiving no vaccination served as negative control. Tumor volumes were calculated according to the formula: tumor volume = 0.5 (width^2 x length).[23]

For the in vivo tumor treatment experiments using a lung metastasis model, mice (eight mice per group) were challenged with 1 x 10^5 TC-1 cells/mouse via tail vein (day 0). On day 7, mice were vaccinated with 1.25 μg of pSLC-E7-Fc, pE7-Fc and pCMV respectively and killed on day 21. The lungs were resected and fixed in Bouin's Solution (picric acid saturated aqueous solution: formalin: glacial acetic acid = 15:5:1) for 24 hours. After fixation, the lungs became yellow and the tumor nodules became white and then the number of pulmonary metastatic nodules was counted with a magnifier by experimenters blind to sample identity.

In vivo depletion of CD4$^+$ or CD8$^+$ T cells. Depletion studies were performed as previously described.[24,25] Mice (five mice per group) were vaccinated with 1.25 μg of pSLC-E7-Fc. Ten days later, mice were subcutaneously challenged with 5 x 10^4 TC-1 cells/mouse on the left flank. Anti-CD4 (clone GK1.5, rat IgG) and anti-CD8 (clone 2.43, rat IgG) ascites fluids were generated by injecting hybridoma cells (provided by L. Chen) into pristane-primed nude mice. Mice were depleted of CD4$^+$ or CD8$^+$ T cells by intraperitoneal injection of 100 μl of ascitic fluid on day -17, -15,

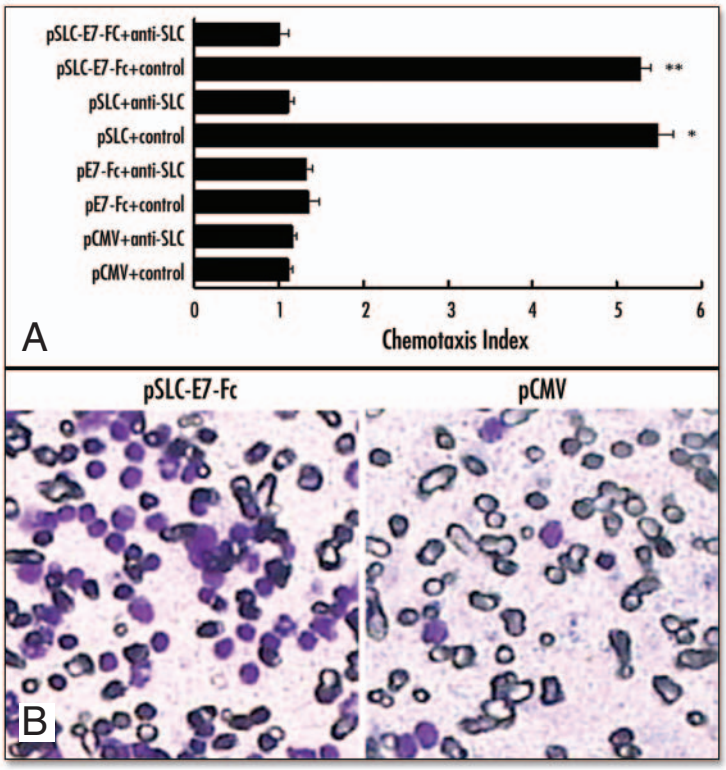

Figure 3. (A) Chemotactic activity of culture supernatants of B16F10 cells nontransfected or transfected with pSLC-E7-Fc, pSLC, pE7-Fc and pCMV (plus anti-SLC or control antibody), measured as chemotaxis index. *, indicate $p < 0.05$, pSLC compared with pE7-Fc or pCMV; **, indicate $p < 0.05$, pSLC-E7-Fc compared with pE7-Fc or pCMV. Data are presented as mean chemotaxis index ± SD of five samples per group in a representative experiment. Experiments were repeated two times with similar results. (B) Representative photographs of chemotactic activity assay (200x).

-13, -11, -7 and -4, -1 relative to TC-1 challenge (day 0). A group of mice received normal rat IgG as control. Depletions of $CD4^+$ or $CD8^+$ T cells were greater than 98% as validated by flow cytometry.

Statistical analysis. ANOVA was used to determine the levels of difference between groups (followed by the Student-Newman-Keuls test). Survival data were compared with 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).

RESULTS

Expression of DNA constructs. B16F10 cells were transiently transfected with various DNA constructs. Expression of *SLC*, *E7* and *Fc* gene at mRNA levels in each transfectant was confirmed by RT-PCR (Fig. 2A). After being treated with DNase I, RNA samples without reverse transcription were amplified by PCR and no PCR products were generated, which eliminated the possibility that RT-PCR products were generated from the contaminated transfected plasmids in RNA samples. The secreted SLC-E7 fusion proteins in culture supernatants of B16F10 cells transfected with pSLC-E7 were successfully detected by goat anti-mouse SLC antibody. The secreted SLC-E7-Fc or E7-Fc fusion proteins in culture supernatants of B16F10 cells transfected with pSLC-E7-Fc or pE7-Fc were screened by goat anti-human Fc antibody (Fig. 2B). Taken together, these results demonstrated expression of the constructs in eukaryotic cells.

Chemotactic activity of SLC-E7-Fc protein in vitro and in vivo. Equivalent SLC concentration in supernatants of B16F10 cell transfected with pSLC-E7-Fc or pSLC was 670.4pg/ml or 701.5pg/ml, whereas in supernatants of B16F10 cells nontransfected or transfected with pE7-Fc or pCMV, no SLC was detectable (Table 1). As shown in Figure 3A and B, supernatants from pSLC-E7-Fc- or pSLC-transfected cells attracted more spleen lymphocytes compared with those from pE7-Fc-, pCMV-transfected cells ($p < 0.05$). No statistical difference was observed in chemotactic response from lymphocytes between supernatants of pSLC- or pSLC-E7-Fc-transfected cells. The chemotactic activity was abrogated by the addition of a neutralizing anti-SLC antibody. These results showed that the enhancement in chemotaxis of lymphocytes was due to SLC and the secreted SLC-E7-Fc protein had the same chemotactic activity as SLC protein. As shown in Figure 4, the infiltrating lymphocytes in dermis from pSLC-E7-Fc or pSLC-immunized mice were much more in number than that from pCMV-treated mice ($p < 0.05$).

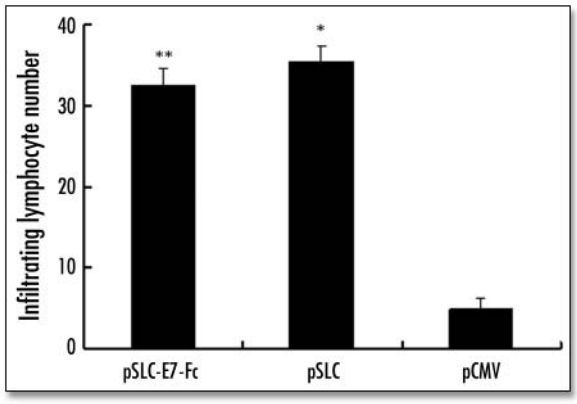

Figure 4. Infiltrating lymphocyte numbers at the immunization sites of dermis. Sections of H&E staining were observed and lymphocytes were counted by professional pathologists. *, indicate $p < 0.05$, pSLC compared with pCMV; **, indicate $p < 0.05$, pSLC-E7-Fc compared with pCMV. Data are presented as mean infiltrating lymphocyte numbers ± SD of five mice per group in a representative experiment. Experiments were repeated two times with similar results.

Vaccination with pSLC-E7-Fc DNA generates the strongest E7-specific lymphocyte proliferation responses. As shown in Figure 5, spleen cells from pSLC-E7-Fc-vaccinated mice stimulated with TC-1 cells proliferated much more vigorously than those from control DNA-vaccinated mice. In addition, the proliferation was responsive to TC-1 cells but not to B16F10 cells that expressed irrelevant antigens.

Vaccination with pSLC-E7-Fc DNA generates the highest E7-specific CTL responses. As shown in Figure 6, spleen cells from mice vaccinated with pSLC-E7-Fc showed the highest CTL activities compared with those from mice immunized with control DNA when TC-1 cells were used as target cells. The specificity of killing was further demonstrated by the inability of the spleen cells of pSLC-E7-Fc-immunized mice to kill B16F10 cells that expressed irrelevant antigens. In addition, there was only a tiny increase in NK mediated lysis against NK sensitive YAC-1 cells in spleen cells from pSLC-E7-Fc-treated mice compared to control DNA-treated counterparts (data not shown), which indicated that NK mediated lysis made a limited contribution to the enhanced splenic cytolytic activity.

Vaccination of mice with pSLC-E7-Fc DNA induces strong protection against the growth of E7-expressing tumors upon tumor initial challenge and rechallenge. To determine whether the observed enhancement in E7-specific lymphocyte proliferation and CTL responses could be translated to a significant E7-specific anti-tumor effect, in vivo tumor protection experiments were performed. As shown in Figure 7A, 100% of mice receiving pSLC-E7-Fc remained tumor-free for 60 days after TC-1 challenge, whereas all the mice immunized with control DNA and nonimmunized developed tumor within 30 days after tumor challenge. These results also indicate that fusion of *SLC*, *Fc* and *E7* in one DNA construct was required for the enhanced anti-tumor effect, since a mixture of pSLC, pFc and pE7 did not enhance protection of mice against TC-1 cells challenge. The anti-tumor immunity was long-lasting and systemic as no tumor grew until eight weeks after

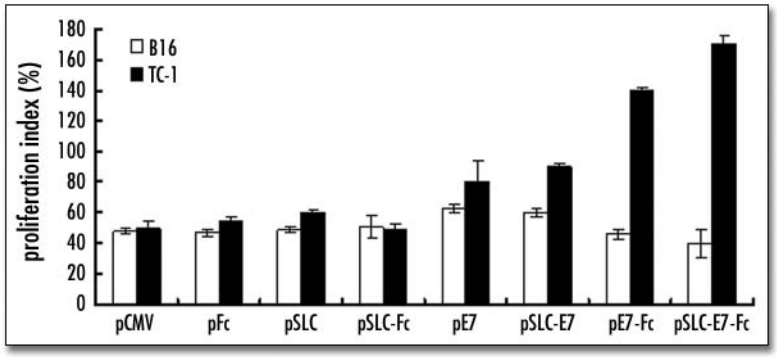

Figure 5. Lymphocyte proliferation in mice (n = 3) immunized with pSLC-E7-Fc and control DNA on days 14 and 21 after TC-1 cells challenge (day 0). Pooled spleen cells were harvested on day 31 and MTT assay was performed. Data are presented as mean percent proliferation index ± SD of triplicate wells of a pooled sample per group in a representative experiment. Experiments were repeated three times with similar results.

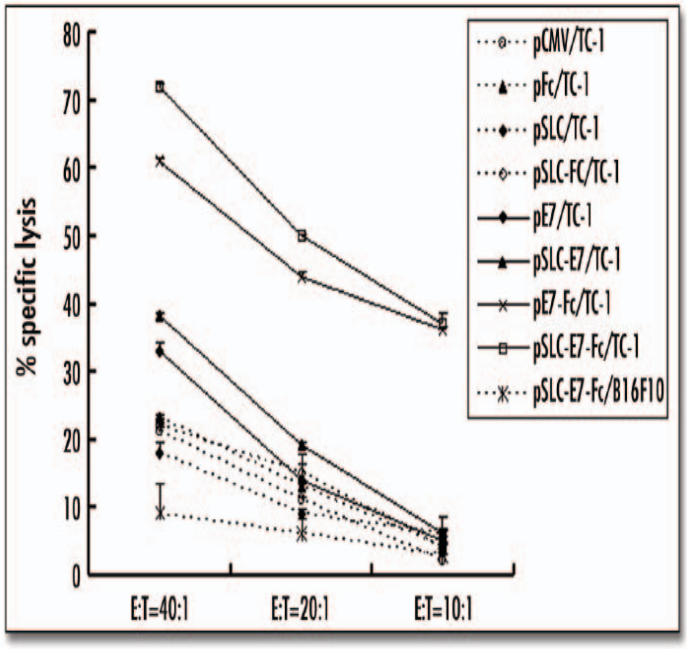

Figure 6. CTL responses in mice (n = 3) immunized with pSLC-E7-Fc and control DNA on days 14 and 21 after TC-1 cells challenge (day 0). Pooled spleen cells were harvested on day 31 and a standard LDH assay was performed. Data are presented as mean percent specific lysis ± SD of triplicate wells of a pooled sample per group in a representative experiment. Experiments were repeated three times with similar results.

reinoculation of even a larger dose of TC-1 cells to the opposite flank of the tumor-free mice. To verify the tumorigenicity of TC-1 cells used for rechallenging, a group of naïve mice was challenged; all of them developed tumors within seven days (Fig. 7B).

Vaccination with pSLC-E7-Fc DNA leads to regression of established TC-1 tumor and long-term survival. To test the efficacy of DNA vaccines in eradicating established TC-1 tumors, in vivo tumor treatment experiments were performed. DNA immunization was initiated on day 14 after TC-1 cells inoculation when the tumor was palpable (2 mm x 2 mm minimum). Within the 48-day observation period, the tumors progressively grew in 72% of the pE7-Fc-immunized mice and in 100% of the other control DNA immunized mice, whereas in 72% of the pSLC-E7-Fc-immunized mice the tumor completely regressed (Fig. 8A).

For comparison, the mean tumor volumes from various groups of mice were shown in Figure 8B, mice immunized with pSLC-E7-Fc and pE7-Fc demonstrated the lowest average tumor volumes than mice immunized with other control DNA ($p < 0.05$). No statistical difference in mean tumor volumes was observed in mice treated with pE7-Fc and pSLC-E7-Fc.

To determine the effect of pSLC-E7-Fc vaccination on long-term survival, these animals were followed for a period of 250 days. As shown in Figure 8C, the survival rate of pSLC-E7-Fc-immunized mice was 72% over this 8-month period and that of pE7-Fc-immunized mice was only 28%. In contrast, all mice vaccinated with control DNA died within 100 days after TC-1 challenge. The survival of mice treated with pSLC-E7-Fc was significantly longer than that of pSLC-E7-, pSLC-Fc-, pE7-Fc-, pSLC-, pE7-, pFc-, pCMV-immunized or nonimmunized mice ($p < 0.05$, by log-rank test).

Treatment with pSLC-E7-Fc DNA leads to significant reduction of pulmonary tumor nodules in mice. As shown in Figure 9A and B, the mean number of lung tumor nodules in mice vaccinated with pSLC-E7-Fc (12.4 ± 4.1) was much lower than that in mice vaccinated with pE7-Fc (64.3 ± 15), or pCMV (202 ± 50.8; $p < 0.05$).

Both $CD4^+$ and $CD8^+$ T Cells are Involved in the Anti-Tumor Effect Generated by pSLC-E7-Fc DNA Vaccine. As shown in Figure 10, all naive mice and mice depleted of $CD4^+$ or $CD8^+$ T cells grew tumors within 16 days after TC-1 challenge. Furthermore, $CD8^+$ T cell-depleted mice showed a bit delayed but complete loss of protection against tumor compared with naive mice. In contrast, all nondepleted mice and control IgG-treated mice remained tumor-free 60 days after tumor challenge. The data suggest that both $CD4^+$ and $CD8^+$ T cells are essential for the anti-tumor immunity generated by pSLC-E7-Fc vaccine.

DISCUSSION

DCs are critical for initiating and modulating immune responses elicited by DNA vaccination. For DNA vaccination to be successful, antigens must be effectively presented by DCs and antigen-loaded DCs must be efficiently carried back into the secondary lymphoid tissue to facilitate the interaction with T cells. Therefore, strategies that can enhance the antigen presentation of DCs and promote coclustering of DCs and T cells in the lymphoid tissue should be able to markedly enhance the immunogenicity of DNA vaccines. One such novel chemotactic-antigen DNA vaccine strategy is described in this report. Specifically, we constructed a chemotactic-antigen plasmid DNA vaccine pSLC-E7-Fc and evaluated whether its potency could be enhanced by the synergistic effect of SLC and Fc on antigen presentation and activation of T cells.

Our results demonstrated that immunization with pSLC-E7-Fc elicited the strongest E7-specific cellular immune responses and generated the most robust preventive and therapeutic effects against growth of E7-expressing tumors compared with immunization with control DNA. Thus, this strategy that combined linkage of SLC and Fc to antigen in the form of chimeric DNA cooperatively enhances the potency of antigen-expressing DNA vaccines.

Our data also showed that the anti-tumor effect so generated was dependent on both $CD4^+$ and $CD8^+$ T cells. It has been reported that $CD4^+$ T cells are required for the generation of cytolytic $CD8^+$ T cells during priming phase of anti-tumor immunity and may also contribute to anti-tumor immunity during effector phase via macrophage activation, cytokine production and/or direct killing of MHC class II-positive tumors.[26,27] Thus, the depletion of $CD4^+$ T cells before pSLC-E7-Fc vaccination might result in a total failure in induction of cell-mediated immunity and hence lead to complete abrogation of anti-tumor effect. In contrast, although the depletion

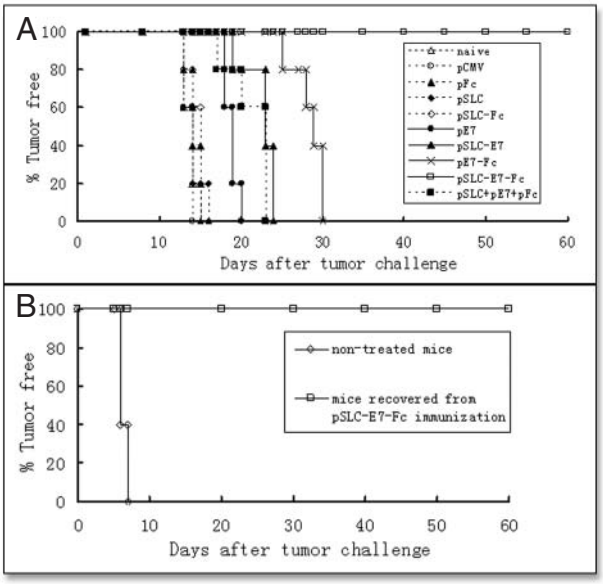

Figure 7. Prophylactic immunization with pSLC-E7-Fc protects mice against TC-1 initial challenge and rechallenge. (A) Mice (n = 5) were immunized with pSLC-E7-Fc and control DNA. Ten days later, mice were challenged subcutaneously with TC-1 cells (day 0) and observed for 60 days. (B) On day 60, tumor-free mice were rechallenged with a larger dose of TC-1 cells on the opposite flank and observed for an additional 60 days. In addition, a group of naive mice were challenged to verify the tumorigenicity of TC-1 inoculation. Results shown are from one representative experiment. Experiments were repeated three times with similar results.

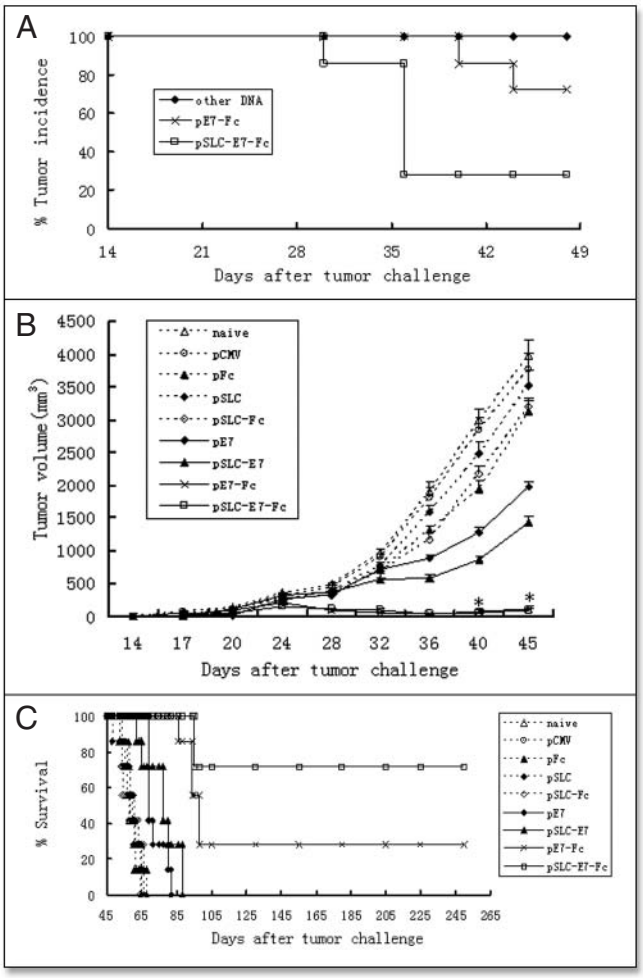

Figure 8. Therapeutic immunization with pSLC-E7-Fc induces TC-1 tumor regression and long-term survival. Mice were injected subcutaneously with TC-1 cells (day 0). On day 14, mice with palpable tumors (2 mm × 2 mm minimum) were selected, assigned into nine groups (n = 7), and vaccinated with pSLC-E7-Fc and control DNA respectively on days 14, 21 and 28. Tumor incidence, tumor volumes and survival rate are shown. (A) Percent tumor incidence in mice. Mice were scored for the presence of tumor nodules for 48 days after TC-1 injection. In this figure, "other DNA" represents any one of the pCMV, pSLC, pFc, pE7, pSLC-Fc, pSLC-E7-immunized and nonimmunized mice. (B) Tumor volumes were monitored for 45 days until the control mice began to die. Data are presented as mean tumor volumes ± SD of seven mice per group in a representative experiment. *, indicate $p < 0.05$, pSLC-E7-Fc compared with control DNA (except for pE7-Fc). (C) Long-term survival in mice. Mice were monitored for survival over a 250-day period after TC-1 inoculation. The survival of mice treated with pSLC-E7-Fc was significantly longer than that of pSLC-E7-, pSLC-Fc-, pE7-Fc-, pSLC-, pE7-, pFc-, pCMV-immunized or nonimmunized mice ($p < 0.05$, by log-rank test). Results shown are from one representative experiment. Experiments were repeated two times with similar results.

of $CD8^+$ CTL might lose the most essential anti-tumor component,[26,27] other response elements such as effector $CD4^+$ T cells still remained functioning and hence culminated in complete but a bit delayed abrogation of anti-tumor effect.

The possible mechanism responsible for the efficient E7 specific immune responses generated by immunization with pSLC-E7-Fc is illustrated as follows. After gene gun transfection, pSLC-E7-Fc constructs are taken up by skin cells, which express and secret SLC-E7-Fc fusion proteins (Fig. 2).[28] The secreted SLC-E7-Fc fusion proteins chemotactically recruit a large number of DCs and T cells at the vaccination site as SLC does, which may facilitate E7 antigen presentation (Fig. 3 and Fig. 4).[5-8] In addition, the Fc fragment in the secreted SLC-E7-Fc fusion proteins helps DCs capture, process and present the fusion protein by Fc receptor-mediated endocytosis.[13-17] SLC-E7-Fc fusion proteins may further augment E7 antigen presentation by directly activating DCs and extending the life of DCs as SLC does.[9,11] The E7 antigen-loaded DCs may migrate to the T cell zones in the draining lymph nodes where to induce efficient E7-specific T cell responses (Fig. 5 and 6). The E7 antigen-loaded DCs may also remain at the vaccination site and activate those lymphocytes attracted by SLC-E7-Fc fusion proteins (Fig. 3 and 4).[12,29] In addition, SLC-E7-Fc fusion proteins may promote the generation of effector and memory cellular immune responses as SLC does.[30,31] At last, the activated E7 specific immune cells within or outside the lymphoid tissue migrate to TC-1 tumor site to exert specific tumoricidal effect. Further studies would be necessary to ascertain this possible mechanism.

The novel strategy developed has several unique features and advantages. (A) The capacity of SLC to attract DCs increases the number of DCs to the antigen production site, thus facilitating antigen presentation by DCs.[5-8] (B) The competency of SLC to directly induce terminal maturation of antigen-loaded DCs augments antigen presentation, as optimal DCs antigen presentation requires maturation signal.[11] (C) SLC is capable of extending survival of mature DCs, which may increase the probability of antigen presentation and stimulation of antigen-specific T cells.[9] (D) SLC helps cocluster DCs and T cells in lymphoid tissues where DCs-T cells interaction takes place.[5-8] (E) SLC is able to directly activate T cells as a costimulatory molecule.[12] (F) SLC could enhance memory immune responses.[30] (G) With the help of the signal sequence in the

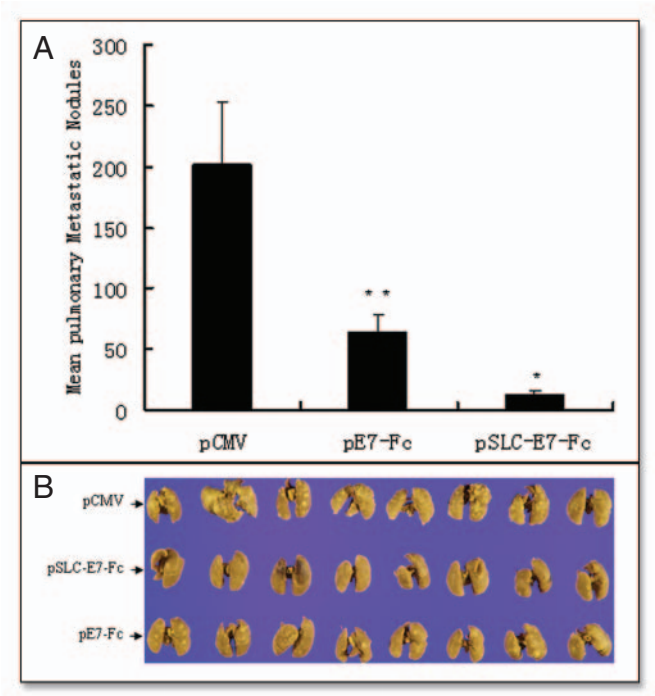

Figure 9. (A) Treatment with pSLC-E7-Fc leads to significant reduction of pulmonary tumor nodules in mice. Mice (n=8) were challenged with TC-1 cells via tail vein. Seven days after, mice received pCMV, pE7-Fc and pSLC-E7-Fc respectively. Mice were killed on day 21. Data are presented as mean number of pulmonary tumor nodules ± SD of eight mice per group in a representative experiment. *, indicate $p < 0.05$, pSLC-E7-Fc compared with pE7-Fc or pCMV; **, indicate $p < 0.05$, pE7-Fc compared with pCMV. (B) Gross picture of pulmonary tumors in each vaccinated group. Experiments were repeated two times with similar results.

recombinant DNA, the encoded protein can be secreted and efficiently taken up by DCs via Fc receptor-mediated internalization.[13-17] (H) Fc receptor-mediated antigen internalization allows DCs to process and present antigens via the MHC class II and MHC class I pathways in a cognate manner.[13-17] (I) Taking these advantages, when *SLC* and *Fc* genes are linked to antigen-coding gene (*E7* gene in the present study) in the form of chimeric DNA vaccine, as reported in this paper, dramatic enhancement of cell-mediated anti-tumor response was elicited. (J) This innovative approach is applicable for the design of DNA vaccines specific for any intracellular antigen. In fact, we have recently developed a similar recombinant DNA vaccine by sandwiching several prostate-associated antigens between *SLC* and *Fc* with high potency to elicit specific immune response against prostate cancer (data not shown). Thus, this novel strategy with the ability to efficiently induce cellular immunity against any intracellular antigen may provide a generic and powerful means for the development of more potent vaccines against tumors and other pathogens.

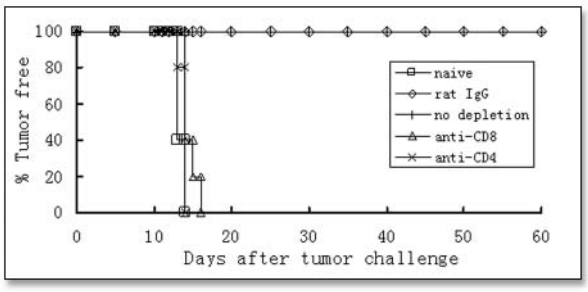

Figure 10. In vivo antibody depletion experiments. Antibody-depleted or nondepleted mice were immunized with pSLC-E7-Fc. Ten days later, mice (n = 5) were subcutaneously challenged with TC-1 cells (day 0). The mice were monitored for 60 days after TC-1 challenge. Data shown are from one representative experiment. Experiments were repeated two times with similar results.

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