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Blocking Programmed Death-1 Ligand-PD-1 Interactions by Local Gene Therapy Results in Enhancement of Antitumor Effect of Secondary Lymphoid Tissue Chemokine

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The negative signal provided by interactions of programmed death-1 (PD-1) and its ligands, costimulatory molecules PD-L1 (also B7-H1) and PD-L2 (also B7-DC), is involved in the mechanisms of tumor immune evasion. In this study, we found that this negative signal was also involved in immune evasion in tumor immunotherapy. When we used different doses of a constructed eukaryotic expression plasmid, pSLC, which expresses functional murine secondary lymphoid tissue chemokine (SLC, CCL21), to treat BALB/c mice inoculated with H22 murine hepatoma cells, the inhibitory effect was enhanced along with the increase of pSLC dosage. Unexpectedly, however, the best complete inhibition rate of tumor was reached when pSLC was used at the dosage of 50 μg but not 100 or 200 μg. RT-PCR and real-time PCR revealed that both PD-L1 and PD-L2 genes were expressed in tumor and vicinal muscle tissues of tumor-bearing mice and the expression level was significantly increased if a higher dosage of pSLC was administered. We then constructed a eukaryotic expression plasmid (pPD-1A) that expresses the extracellular domain of murine PD-1 (sPD-1). sPD-1 could bind PD-1 ligands, block PD-Ls-PD-1 interactions, and enhance the cytotoxicity of tumor-specific CTL. Local gene transfer by injection of pPD-1A mediated antitumor effect and improved SLC-mediated antitumor immunity. The combined gene therapy with SLC plus sPD-1 did not induce remarkable autoimmune manifestations. Our findings provide a potent method of improving the antitumor effects of SLC and possibly other immunotherapeutic methods by local blockade of negative costimulatory molecules. The Journal of Immunology, 2004, 173: 4919–4928.

Effective antitumor responses require both APC and lymphocyte effectors (1). Given the chemotaxic properties of chemokines on the effector function of different immune cells in vitro and in vivo, numerous studies on the effects of chemokines in the antitumor responses have recently been initiated, including secondary lymphoid tissue chemokine (SLC; CCL21) (2).

SLC, also referred to as exodus 2, TCA4, or 6Ckine, is mainly expressed in lymphoid tissues (3–6) and is selective in its recruitment of the activated and naive T lymphocytes (7–10), and dendritic cells (DCs) (11–13). SLC is believed to play an important role in the initiation of an immune response. Injection of rSLC into the tumor or lymph node region evidenced T cell-dependent antitumor responses (14–16). The colon carcinoma tumor cell line transduced with a cDNA encoding mouse SLC showed reduced tumorigenicity in immunocompetent or nude mice (17). However, chemokines alone seem to show limited antitumor efficacy (2).

Ag-induced activation and proliferation of antitumor T cells are regulated by both positive and negative costimulatory molecules. The interactions of negative costimulatory molecules with their receptors, including CTLA-4 and programmed death-1 (PD-1), function to limit, terminate, and/or attenuate T cell responses, and appear to be particularly important for regulating T cell tolerance (18). CTLA-4 shares the B7 ligands with CD28, and plays a critical role in down-regulation of T cell responses (19). PD-1, in contrast, interacts with distinct ligands, PD-L1 (also B7-H1) and PD-L2 (also B7-DC), which are expressed on not only APCs but also other cell types in nonlymphoid tissues such as heart, lung, liver, kidney, and skeletal muscle (20–23). It is speculated that PD-Ls expressed in these tissues may function in part as “veto” molecules against the potentially autoreactive effector T cells (24, 25). Furthermore, PD-Ls are expressed on DCs (26, 27) and tumors including carcinomas of lung, ovary and colon, and melanomas (28), also squamous cell carcinomas of the head and neck (29), and glioma (30) and are involved in tumor immune evasion. It was reported that CTLA-4 blockade in vivo led to suppression or even rejection of a number of potentially lethal transplanted tumors (19). Growth of the myeloma cells in normal syngeneic mice was inhibited significantly albeit transiently by injection i.p. of anti-PD-L1 Ab (25). These results indicate that weak immune responses against the tumors can be converted into more potent ones by inhibiting the effects of negative immunoreceptors.

Because the antitumor effects of SLC mainly depend on its recruitment of T lymphocytes and DCs, and PD-Ls-PD-1 interactions are involved in the mechanisms of tumor immune evasion, we supposed that blockade of the PD-Ls-PD-1 interactions could
enhance the antitumor effects by augmentation of the immune responses against tumors when more immune cells were chemotactically recruited to tumor tissues by SLC. In this study, we constructed two recombinant eukaryotic expression plasmids (pSLC and pPD-1A) encoding murine SLC and the extracellular domain of murine PD-L1 (pPD-1), respectively. The expressed pSP-1 could block the PD-L1/PD-1 interaction, and local gene transfer of pSP-1 in tumor inoculation sites significantly improved the therapeutic effect of pSLC.

Materials and Methods

Mice

Female BALB/c (H-2b) mice, 6 to 8 wk old, were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) and maintained in our facilities under standard conditions. All studies involving mice were approved by the institute’s Animal Care and Use Committee.

Cells and Abs

The BHK cell line (baby hamster kidney cells) and the H22 cell line (murine hepatoma) were obtained from the China Type Culture Collection (Wuhan, China) and cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml streptomycin, and 100 mg/ml penicillin (complete medium). All cell culture reagents were obtained from Invitrogen Life Technologies (Grand Island, NY). The H22 cell line was also passaged in vivo with ascitic fluid. Spleen cells from BALB/c mice were treated with hypotonic buffer at room temperature for 2 min to remove erythrocytes and washed three times with the complete RPMI 1640 medium. For stimulation, the cells were cultured at the concentration of 2 × 10^6 cells/ml in the presence of 2 μg/ml Con A (Sigma-Aldrich, St. Louis, MO) in a 24-well culture plate for 2 days (31). For chemotaxis assay, T cells from spleens of mice were purified by plastic adherence and passage through nylon wool columns. The cells were cultured at 37°C in a humidified incubator with 5% CO₂. Rat anti-mouse SLC mAb (clone 59106) and goat anti-mouse PD-L1 polyclonal Ab were obtained from R&D Systems (Minneapolis, MN). Rat anti-mouse PD-L1 (MIH5) and PD-L2 (TY25) mAbs were obtained from eBioscience (San Diego, CA). Mouse anti-human CD3 (PC3/188A) mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Spleen cells stimulated by the HS7070 peptide complex

The heat shock protein 70 (HS7070)-peptide complex was prepared as described previously (32). For preparation of the peptide mixture, H22 tumor cells from the peritoneal cavity of tumor-bearing mice were washed with PBS and resuspended in PBS to a final concentration of 5 × 10^6/ml. After two freeze-and-thaw cycles, the cell suspension was mixed with a 2-fold volume of distilled water and vortexed for 30 s followed by centrifugation at 14000 × g for 15 min. Supernatants of cells transfected with pTracer and pPD-1B or different doses of pSLC were used for FACS or chemotaxis analysis, respectively. For stable transfection with pPD-1A, the transfected BHK cells were selected on the basis of G418 resistance (800 μg/ml) for 3 wk.

Chemotaxis assays

Mouse T lymphocytes obtained from spleen as described above were re-suspended in RPMI 1640 medium containing 10% FCS, incubated for 1 h at 37°C, and subjected to a chemotaxis assay. For the assay, 10^6 cells in 100 μl of medium were added to the top chamber of a 6.5-mm diameter, 5-μm pore polycarbonate Transwell insert (Costar, Cambridge, MA) and incubated with the supernatants of BHK cells nontransfected or transfected with pcDNA3.1 or different doses of pSLC (or plus Ab anti-mouse SLC, 2 μg/ml, as indicated) in the bottom chamber for 4 h. Cells migrating to the bottom chamber were counted with a FACS Calibur (BD Biosciences, San Jose, CA) for 50 s. The results were expressed as the fold increase in cells migrating in response to chemoattractant vs the medium control (chemotaxis index). All assays were done in triplicate.

Construction of expression plasmid vectors

All enzymes used for the construction of recombinant plasmids were products of Promega (Madison, WI). Total RNA was isolated from spleen cells using TRIzol reagent (Invitrogen Life Technologies) and reverse transcribed. The 857-bp cDNA of murine SLC (6kIncine-Ser) was produced by PCR using the primers sense 5’-GGGGATCCATGGAATCCTGTTCTGTTCGCTCAGATGCGCGCTTCGCTAC-3’ and antisense 5’-GGTTGAGAATTCTGGCTAGCAAAGGAGAAG-3’ and inserted into compatible enzyme restriction sites of pcDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA) to generate murine SLC eukaryotic expression plasmid (pSLC).

To construct the expression plasmid pSP-1A, carrying a cDNA encoding the extracellular domain of murine PD-L1 (pSP-1), specific primers were used as sense 5’-GGGATCCATGGAATCCTGTTCTGTTCGCTCAGATGCGCGCTTCGCTAC-3’ and antisense 5’-CCTGGTGAAATTCATGGAAGCGCGCTTCGCTAC-3’. The amplified fragment by RT-PCR was digested by restriction enzymes HindIII and EcoRI and inserted into compatible enzyme restriction sites of pcDNA3.1. Another plasmid, pSP-1B, carrying a cDNA encoding a fusion protein of pSP-1 (N-terminal) and a GIP fragment (C-terminal), was constructed using different primers except that the sense primer for PD-1 is the same as that used for pSP-1A. Another primer (antisense) was used 5’-GGGGATCCATGGAATCCTGTTCTGTTCGCTCAGATGCGCGCTTCGCTAC-3’, containing an EcoRI restriction site. To amplify the GIP fragment from the pTracer plasmid (Invitrogen Life Technologies), specific primers were used: sense 5’-GGTGGAGAATTCTGGCTAGCAAAGGAGAAG-3’ and antisense 5’-GGGATCCATGGAATCCTGTTCTGTTCGCTCAGATGCGCGCTTCGCTAC-3’, containing a HindIII restriction site. The two amplified fragments were digested by restriction enzymes HindIII and EcoRI, EcoRI and XhoI, respectively, and recombined with pcDNA3.1 digested with HindIII and XhoI. All cDNAs inserted into the constructed plasmids were sequenced and identical to the published sequences (4, 6, 34).

Cell transfection

The transfection of cells with plasmid was performed in a 24-well culture plate with 60–80% confluence of BHK cells using Dsper liposomal transfection reagent (Boehringer Mannheim, Mannheim, Germany) following the manufacturer’s instructions. The ratio of liposome to plasmid was 4 μl:1 μg. After transfection of 48 h, cells transfected with pTracer and pSP-1B were observed and images were obtained using an inverted fluorescence microscope (Olympus IMT-2; Olympus Optical, Tokyo, Japan), and supernatants of cells transfected with pSP-1B or different doses of pSLC were used for FACS or chemotaxis analysis, respectively. For stable transfection with pSP-1A, the transfected BHK cells were selected on the basis of G418 resistance (800 μg/ml) for 3 wk.

Gene therapy and tumor surveillance in vivo

Plasmid DNA used for gene therapy was prepared by the alkaline lysis method and purified by polyethylene glycol, followed by selective precipitation with spermine (Sigma-Aldrich, St. Louis, MO) as described (35). LPS concentration in the plasmid DNA preparation was determined by the Limulus amebocyte lysate (LAL) assay and was <1.5 endotoxin units/μg. All plasmid preparations for i.m. injections were resuspended in sterile 0.9% saline. Spectrophotometric analysis revealed 260/280 nm ratios ≥1.80. Purity and conformation of the prepared DNA were confirmed by agarose gel electrophoresis.

For experiments in vivo, BALB/c mice were inoculated with ascitic H22 hepatoma cells by the intraperitoneal route with 100 1 × 10^6 cells in 100 μl of PBS into the right hind thigh muscle. Two days after inoculation, the naked expression plasmids were injected, every other day for six times, into the inoculation site at the indicated doses in 100 μl of sterile saline. Mice of the control group received an equal volume of saline or an equal amount of pcDNA3.1 plasmid. Tumor size was measured using calipers fitted with a Vernier scale when tumor could be palpated. The tumor diameter was calculated using the formula (a + b)/2, with a as the larger diameter and b as the...
smaller diameter. Mice were sacrificed and tumors were dissected and weighed on the indicated days after inoculation or for ethical reasons when animals showed severe distress or when tumors exceeded 1.5 cm in diameter. The average inhibition rate percentage was calculated as (average tumor weight of pcDNA3.1 plasmid control group / average tumor weight of treated group) × 100%. The complete inhibition rate percentage was (mice without palpable tumor on indicated day/all treated mice in a group) × 100%.

Detection of protein expression in vivo

After plasmid injections, equal amounts of muscle tissues of injection site (6–8 mm diameter) were excised at the indicated time, minced, and cultured in a 12-well dish with 1 ml of medium (DMEM with 10% FCS and 2% chick embryo extract). After incubation in a CO2 incubator for 48 h, the supernatants were subjected to specific protein analysis. SLC protein was detected using an SLC-specific ELISA (R&D Systems) following the manufacturer’s guidelines. To detect sPD-1 protein expression, Western blot was used. Briefly, 48-h culture supernatants were concentrated using the Freeze Dry System (Labconco, Kansas, MO) and then separated by SDS-PAGE followed by transfer onto nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked in PBS containing 5% milk and 0.1% Tween 20 and probed with the anti-mouse PD-1 polyclonal Ab at a concentration of 0.2 μg/ml. After incubation with the secondary anti-goat Ab conjugated with HRP (Amersham Pharmacia Biotech, Piscataway, NJ), membranes were extensively washed and the reaction was visualized by ECL according to the manufacturer’s protocol (Amersham Pharmacia Biotech).

Analysis of gene expression by RT-PCR and real-time PCR

Total RNAs were isolated using TRIzol reagent (Invitrogen Life Technologies) from H22 cells or muscle tissues of normal mice, or half part tumor tissues together with half part vicinal muscle tissues of tumor-bearing mice after treatment with different doses of pSLC. After being treated with DNase I (Promega), equal amounts of RNAs were reverse-transcribed and the cDNAs were amplified by the PCR with gene-specific primers at 94°C for denaturing, 54°C for annealing, and 72°C for extension. β-Actin mRNA was used as an internal control and coamplified. Previous experiments determined 26 cycles to be optimal. Specific primers used were: PD-L1, sense 5'-TGAAGACCT GACCTTCTCC-3', antisense 5'-GACCTCTTGAGACAATTC-3'; PD-L2, sense 5'-CACGCCTTGTCCTGCG-3', antisense 5'-CCTGAA AGTCATTAGGC-3', β-actin, sense 5'-ATGGTACAGAGGAC TCCTATG-3', antisense 5'-ATCTCCGTGCTAGAATGAG-3'. The amplified products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Relative quantitative real-time PCR was performed using the TaqMan probe on the ABI Prism 7700 (Applied Biosystems, Foster City, CA) to confirm the results obtained using RT-PCR above, following the manufacturer’s instructions. The primer and probe sequences for β-actin were available commercially and others were as follows: PD-L1, sense 5'-GG AATTTGCTCAGAATGTC-3', antisense 5'-GATTTGCTCTGAGA AACGCT-3', and probe 5'-CAGCAACCTACTCTCCTCC-3', PD-L2, sense 5'-TCTGAGCTGTTTCTGCTCC-3', antisense 5'- CTTGAAATGCTATTAGGACC-3', and probe 5'-TCAAAGACT CTGGAACAGGACACCCAAT-3'. To detect sPD-1 expression in mouse muscle after the pPD-1A plasmid injections, the primer and probe sequences were: sense 5'-CCAGTGACCCTGGCTCTACT-3', antisense 5'- CCTGGAACAGGAGCTTCA-3', and probe 5'-TGGCTCTCAGT GAGCTGGCAATCA-3'. cDNA samples (100 ng each, except for β-actin (10 ng)) were reverse-transcribed from tissue RNA were mixed with primers and TaqMan Universal PCR Master Mix in a total volume of 50 μl. The PCR was conducted using the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Each gene was normalized to the housekeeping gene (β-actin) from the same sample before fold change was calculated (using crossing point values) to account for variations between different samples. The mRNA level in normal muscle tissue was used as the calibrator. All PCR assays were performed in duplicate and results are represented by the mean values.

FACS analysis

H22 cells or spleen cells stimulated with Con A for 48 h were washed with PBS and incubated with supernatants of BHK cells transfected with pTracer (control) or pPD-1B at 37°C for 1 h. In blocking experiment, PD-Ls on spleen cells or H22 cells were blocked using mAbs anti-PD-L1 and anti-PD-L2 (each 3 μg/ml) before incubation with the supernatants. The cells were then washed twice and parameters were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Cytotoxicity assay

Standard ³¹Cr-release assays were performed. Briefly, target cells, either untreated, or treated with mAbs anti-PD-L1 and anti-PD-L2 (each 3 μg/ml) and/or the supernatants of BHK cells transfected with pPD-1A for 1 h, were labeled with Na¹¹⁰CrO₄ (0.1 μCi/10⁵ cells; Amersham Pharmacia Bio- tech) at 37°C for 1 h. After extensive washing, target cells were incubated with effectors at different E/T ratios in triplicate for 4 h at 37°C, and ³¹Cr release (cpm) into the supernatants was measured in a gamma counter to calculate percentage-specific release. The percent-specific lysis was calculated by the formula: percentage lysis = 100 × (experimental cpm – spontaneous release)/(maximum release – spontaneous release).

Histology and immunohistochemistry

Mouse muscle tissues of injection sites from treated mice were surgically excised, fixed for 12–24 h in 4% formamint, embedded in paraffin, and sectioned. Sections were stained with H&E. For indirect immunostaining, sections were incubated overnight at 4°C with a mouse anti-CD3 mAb diluted at 1/100. Biotinylated Abs to mouse IgG were used as secondary Abs, followed by peroxidase-conjugated streptavidin in the third step.

Data analysis

Data were expressed as mean ± SEM and results were interpreted using one-way ANOVA (followed by Newman-Keuls test). Differences were considered to be statistically significant when p < 0.05.

Results

Dose-chemotaxis relationship of pSLC transfection in vitro

Although the sequence of SLC cDNA in pSLC was identical to that published, whether SLC expressed by pSLC was functional was unknown. To this end, BHK cells were transfected at different doses and the culture supernatants were harvested 48 h later. Chemotactic activity for spleen T cells was assessed by quantifying T cell migration across 5-μm pore polycarbonate membranes in a Transwell cell culture chamber assay to measure the response to chemotactic stimuli placed into the lower chamber.

As shown in Fig. 1, supernatants from pSLC-transfected cells attracted spleen T cells above control levels. Such an activity was increased when cells were transfected at higher doses but decreased obviously at 0.75 μg. The chemotactic activity was abrogated by the addition of a neutralizing anti-SLC mAb and it was not present in the supernatants of pcDNA3.1-transfected BHK cells.

Dose-effect relationship of pSLC plasmid on inhibition of mouse H22 hepatoma in vivo

To explore whether the transfection with pSLC had a therapeutic effect against tumors, we first observed whether the product expressed by pSLC in vivo had chemotactic activity. Different doses

FIGURE 1. Chemotactic attraction of spleen T lymphocytes by pSLC-encoded SLC. Chemotactic activity of culture supernatants of BHK cells transfected with pcDNA3.1, pSLC at dose of 0.125, 0.25, 0.5 (with or without neutralizing Ab, 2 μg/ml), 0.75 μg, measured as the fold increase in spleen T cells migrating in response to chemotacticant vs the medium control (chemotaxis index). Cells migrating to the bottom chamber were counted with a FACSCalibur for 50 s. ** Significant differences at p < 0.01 according to one-way ANOVA.
of pSLC were injected into the hind thigh muscle, and the muscle tissue of the injection site was analyzed by immunohistochemistry. As shown in Fig. 2, A and B, in the detected range, along with the increase of plasmid dosage, SLC protein expression levels were increased and chemotactic response on T lymphocytes was enhanced correspondingly. This was different from the results obtained in vitro that the chemotactic response was decreased at a high dose (Fig. 1).

Because the transfection with a high dosage of pSLC could result in the enhanced chemotactic response of immune cells, we reasoned that the local transfection with pSLC would have an antitumor effect and the increased dosage of pSLC would result in

![Graph showing dose-effect relationship of pSLC plasmid on mouse H22 hepatoma in vivo.](image)

**FIGURE 2.** Dose-effect relationship of pSLC plasmid on mouse H22 hepatoma in vivo. A and B, Dose-chemotaxis relationship of pSLC transfection in vivo. BALB/c mice received 100 μg of pcDNA3.1 plasmid, or 25, 50, 100, 200 μg of pSLC plasmid (a, b, c, d, and e, respectively) by injection into the hind thigh muscle twice. A, SLC protein expression levels were determined by ELISA as described in Materials and Methods. B, Immunohistochemical staining was performed with anti-CD3 mAb of paraffin sections of muscle tissue from the injection site surgically removed 72 h after the second injection. Images are representative of multiple microscopic fields observed in three mice per group. C, Mice inoculated with $1 \times 10^3$ H22 ascitic hepatoma cells in the right hind thigh muscle were treated, starting from day 3 after inoculation, with i.m. injection of 25, 50, 100, 200 μg of naked pSLC or 100 μg of pcDNA3.1 or saline six times. Individual tumor weight on day 20 after tumor inoculation was measured and the mean tumor weight was significantly decreased along with the increase of pSLC dosage (left, $p < 0.01$). Two independent experiments represented by open or filled circles have been pooled. Also, the presence of palpable tumor was checked three times a week (right).
augmentation of antitumor efficacy. To study this issue, BALB/c mice were inoculated with H22 ascitic cells by injection of cells into the right hind thigh muscle. We inoculated animals with ascitic tumor cells because such cells had adapted to the environment in vivo. Mice received pSLC at different doses every other day for six times and were sacrificed 20 days after inoculation. As shown in Fig. 2C, transfection with pSLC induced significant inhibition of malignant tumors compared with saline- or pcDNA3.1-treated control mice and showed a dose-effect relationship. The tumor inhibition efficacy was enhanced along with the increase of pSLC dosage. Injection of 200 μg of pSLC reached the best tumor inhibition rate, 92.2%.

Transfection with a lower dosage of pSLC reached a higher complete inhibition rate of tumors

Transfection with 25 μg of pSLC achieved a minor average inhibition rate of tumors (55.4%), and also a minor complete inhibition rate (21.4%). When pSLC was administered at 50 μg, both the average and complete inhibition rates of tumors were increased (70.6 and 50%, respectively, Fig. 2C). The complete inhibition rate could be increased at a higher dosage of pSLC according to this tendency. Unexpectedly, however, it was decreased at the dosage of 100 μg (35.7%) or even lower at 200 μg (21.4%), the same as that at the dosage of 25 μg (Fig. 2C, right), suggesting that although more immune cells chemoattracted into tumor sites could result in stronger inhibition of tumor growth, the ability of immune cells to eliminate the tumor cells was declined. We supposed that the mechanisms of tumor escape from immune attack had been developed.

Up-regulated expression of PD-L1 and PD-L2 genes in residual pSLC-treated tumors

The negative costimulatory molecules such as PD-L1 play a critical role in tumor immune evasion (28, 30). To evaluate whether PD-Ls-PD-1 pathways were involved in the mechanism of tumor evasion in pSLC treatment, RT-PCR was performed to detect the expression level of PD-Ls genes in H22 cells and tumor tissues. Fig. 3A shows that PD-L1 but not PD-L2 mRNA was expressed in H22 hepatoma cells. We also detected PD-Ls mRNAs in muscle tissues of normal mice or mice bearing tumors after pSLC treatment. Both PD-L1 and PD-L2 mRNAs, as expected, were expressed in detected tissues of tumor-bearing mice to a higher level as compared with normal mice (Fig. 3A). The expression level was significantly increased along with the increase of the dosage of pSLC administered. The quantitative technique, real-time PCR, was used to confirm the results. Fig. 3B shows an analysis of these data normalized for the levels of a housekeeping gene (mouse β-actin). The data provided evidence that more PD-Ls mRNA was expressed in tumor and vicinal muscle tissues in the face of more immune cells chemoattracted to tumor sites, and H22 tumors could evade immune attack through PD-Ls-PD-1 pathways. We reasoned that blocking these pathways would enhance the immune response against tumors, and thus improve SLC-mediated antitumor effects. To this end, we constructed a plasmid vector, pPD-1A, carrying a cDNA encoding the extracellular domain of murine PD-1 (sPD-1).

Expression products of plasmids carrying the cDNA coding for the extracellular domain of murine PD-1 could bind to PD-1 ligands

In theory, sPD-1, the extracellular domain of PD-1, should be capable of binding to both PD-L1 and PD-L2, thereby blocking interactions of either PD-L1-PD-1 or PD-L2-PD-1. To verify whether sPD-1 could be secreted out from transfected cells, we constructed an expression plasmid pPD-1B, carrying a cDNA encoding the fusion protein sPD-1-GFP. BHK cells were transfected with either plasmid pPD-1B or pTracer under the same conditions and checked for expression 48 h later. As shown in Fig. 4A, sPD-1-GFP produced by pPD-1B in BHK cells was mostly secreted out as compared with GFP produced by pTracer, which was constructed based on the similar vector of pPD-1B. The binding of sPD-1-GFP to PD-Ls on either H22 cells or spleen cells stimulated by Con A was determined by FACS analysis. Fig. 4B shows that fluorescence intensity on the cells incubated with sPD-1-GFP was significantly increased as compared with control. When H22 and
spleen cells were pretreated with mAbs anti-PD-L1 and anti-PD-L2 before incubation with sPD-1-GFP, however, the fluorescence intensity decreased nearly to the control level (data not shown). These data indicated that sPD-1-GFP was specific and bound efficiently to PD-Ls.

Blocking PD-Ls-PD-1 interactions mediated the enhanced lysis of H22 cells by HSP70-peptide complex-stimulated spleen cells

To evaluate whether sPD-1 could block PD-Ls-PD-1 interactions, H22 cells, either untreated or pretreated with sPD-1, were incubated with HSP70-peptide complex-stimulated spleen cells at different E:T ratios. Fig. 4C shows that pretreatment with sPD-1 enhanced lysis of H22 cells, which was consistent with the result when H22 cells were pretreated with anti-PD-L1 and anti-PD-L2 mAbs. To determine whether the increased tumor lysis was mediated by blockade of PD-Ls-PD-1 interactions though sPD-1, a blocking assay was performed in which H22 cells were pretreated with sPD-1 plus anti-PD-L1 and anti-PD-L2 mAbs. Under such conditions, as expected, tumor lysis was not enhanced further (data not shown). These results further reinforced the hypothesis that sPD-1 was capable of binding to PD-Ls, thereby blocking PD-Ls-PD-1 pathways.

Local gene transfer of sPD-1 mediated antitumor effect

To further evaluate whether sPD-1 has antitumor activity, 2 days after inoculation of H22 hepatoma cells, mice received 100 μg of pPD-1A by local injection into the inoculation site six times. Real-time PCR revealed that the sPD-1 mRNA expression levels became relatively stable from day 12 after inoculation. Western blot confirmed the sPD-1 protein expression (Fig. 5A). Injection with pPD-1A significantly inhibited tumor growth in 24 days. The tumor growth in 36.4% (4 of 11) of the recipients was completely inhibited (Fig. 5B), similar to the inhibitory effect of i.p.-injected anti-PD-L1 mAb on tumor (25). Meanwhile, all mice (8 of 8) in the control groups developed tumors. These findings, taken together with the above results from experiments in vitro, suggest that local expression of sPD-1 expression plasmid in vivo provides an effective approach for blocking PD-Ls-PD-1 interactions and thereby enhancing antitumor immune responses.

sPD-1 improved SLC-mediated antitumor immunity

Local gene transfer of SLC and sPD-1 was conducted after tumor inoculation, and the individual diameter of the tumor nodules in 20
days as well as the survival rates were investigated. For comparison with the pSLC plus pPD-1A group, pcDNA3.1 was used for supplementation of the amount of plasmid DNA in each of the pSLC and pPD-A1 groups. As shown in Fig. 6, treatment by injection with either pSLC or pPD-1A showed mild inhibition on the average tumor growth, and the tumor growth in 36.4% of the recipients (4 of 11) in both groups was completely suppressed. Treatment by injection with pSLC plus pPD-1A reached much better efficacy: the tumor growth in 57.1% of mice (8 of 14) was completely suppressed, and the rest of the mice showed prolonged survival as compared with those that received pSLC or pPD-1A alone (Fig. 6B). Meanwhile, 100% of the mice (7 of 7) in control groups that received saline or pcDNA3.1 showed progressive tumor growth and died within 7 wk (Fig. 6B). These results proved a significant synergistic effect of local gene transfer of SLC in combination with sPD-1.

Local combinatorial gene transfer of SLC and sPD-1 did not induce remarkable autoimmune manifestations

Because blockade of CTLA-4 by i.v. administration of anti-CTLA-4 Ab induced various autoimmune manifestations (36), and no reports are currently available for the autoimmune reaction induced by anti-PD-Ls Abs, the possible autoimmune manifestations induced by local combinatorial gene transfer of SLC and sPD-1 were investigated.

FIGURE 5. Antitumor effect of injection of pPD-1A. BALB/c mice were inoculated i.m. with $1 \times 10^5$ H22 ascitic cells per mouse and treated, starting from day 2 after inoculation, by i.m. injection with pPD-1A (100 μg), pcDNA3.1 (100 μg), or saline as indicated. A, sPD-1 mRNA expression levels (left) and sPD-1 protein expression before and after pPD-1A injections (right) were detected by relative quantitative real-time PCR (three mice per point) or Western blot. B, Individual follow-up of local tumor growth was monitored.

FIGURE 6. Treatment with pSLC in combination with pPD-1A. Mice inoculated with $1 \times 10^5$ H22 ascitic hepatoma cells in the right hind thigh muscle were treated, starting from day 3 after tumor cell inoculation, with i.m. injection of 100 μg of the indicated plasmids and 100 μg of pcDNA3.1 or 200 μg of pcDNA3.1 or saline. In the indicated group, mice were also injected with pSLC plus pPD-1A, 100 μg of each. A, Individual tumor diameter on day 20 after tumor inoculation. Statistical significance ($p < 0.01$) was found when comparing mice treated with pSLC plus pPD-1A with each of other groups. B, Long-term survival follow-up of these groups.
Because pPD-1A was locally used in muscle tissues that express PD-Ls, histopathologic changes of muscle tissues where pPD-1A was injected were examined. As shown in Fig. 7, H&E staining of muscle tissue sections from pPD-1A- and pSLC plus pPD-1A-treated mice. Lower panels, Immunohistochemical studies with mAb to CD3 showed predominance of CD3⁺ cells in the infiltrating cells of the tissues. Images are representative of multiple microscopic fields observed in at least four mice per group.

Discussion
One hallmark of cancers is that they could not be eliminated by immune system and continue to grow. Cancer progression has been attributed to a variety of immune evasion strategies. These include down-regulation of cell surface MHC class I molecules, secretion of immunosuppressive factors (for example, TGF-β), lack of T cell costimulation, and, the programmed cell death (apoptosis) of T cells when encountering tumor (37, 38), for example, through PD-L1-PD-1 interaction (28). In this study, we found that the negative signal provided by PD-Ls-PD-1 interactions was also involved in the immune evasion in tumor immunotherapy.

In theory, any chemokine capable of inducing the migration of T, NK cells, DCs, and/or macrophages could promote the regression or even eradication of a tumor mass by boosting the immune response against the tumor (39). Various approaches have been used to deliver chemokines in vivo including chemokine-transfected tumors, DCs or stromal cells, protein injection, and adenoviral vectors (2, 39). When we used naked plasmid to deliver SLC into tumor inoculation sites, similar antitumor effects were obtained as compared with other methods used. However, plasmids have different advantages with regard to efficiency of gene transfer, ease of production, safety, and sustained expression (40). SLC might mediate antitumor activity through at least two possibly related mechanisms: 1) the recruitment of immune cells such as T lymphocytes and DCs into local tissue, and 2) inhibition of tumor angiogenesis.

In our study, SLC mediated significant inhibition of malignant tumors and when it was locally expressed in vivo by the injection of pSLC below the dose of 200 μg, the tumor inhibition efficacy was enhanced along with the increase of pSLC dosage. However, our results could not reach the conclusion that a better therapeutic effect would be obtained if a higher dosage of pSLC was used, because chemotaxis depends on the concentration gradient of chemokine and a high concentration of chemokine would lead to significantly decreased chemotactic response and toxic side effects (2). This could be supported by the experiment that transgenic overexpression of SLC disrupted T cell migration in mice (41). In contrast, although more immune cells chemoattracted to tumor sites by gene transfer of a high dosage of pSLC could result in higher average tumor inhibition rate, the complete inhibition rate was decreased as compared with that when a lower dosage of pSLC was used for local gene transfer, suggesting that immune
evasion of tumors was developed and would not be attenuated in the face of increased immune cells.

Then, why do tumors continue to grow in the face of seemingly potent cellular antitumor reactions? We suppose that the mechanisms that account for this can come from 1) the tumor itself, and 2) immune responses that occur during treatment, which may lead to more potent immune evasion regardless of increased immune cells. Altogether, this indicates that the tumor microenvironment favors the recruitment and survival of leukocyte subsets that induce anergy or tolerance toward tumor Ags (2).

Our results showed that PD-L1 was also expressed in mouse H22 hepatoma cells. More importantly, the increased expression of PD-Ls was observed in pSLC-treated residual tumors and vicinal muscle tissues, and the expression level was significantly increased if the higher dosage of pSLC was administered (Fig. 3). These results suggest that immune tolerance through PD-Ls-PD-1 pathways may serve as a potent mechanism for residual pSLC-treated tumors to escape from seemingly enhanced immune responses. This is supported by the results that the local gene transfer of SLC in combination with sPD-1, a product that could block PD-Ls-PD-1 interactions, induced marked antitumor synergy.

Although we did not observe that SLC directly induced PD-L1 or PD-L2 expression in H22 hepatoma cells in vitro (data not shown), some reasons may account for the increased expression of PD-Ls in vivo after the local gene transfer of SLC: 1) IFN-γ, which can up-regulate expression of PD-L1 on tumor cells (28, 30), as well as PD-L1 and PD-L2 on endothelial cells (42), resident macrophages (43), and DCs (44). IFN-γ can be released from many different types of immune cells including some cells that can be chemotrastricted to SLC. 2) T lymphocytes and DCs that are chemotrastricted to SLC express PD-Ls. The recruitment of more such cells into tissues would result in the increase of a total level of PD-Ls expression in the microenvironment. 3) The continuous growth of tumor by evasion from immune attack could result in the increase of an absolute number of tumor cells expressing PD-Ls so that a vicious cycle occurred.

Because PD-Ls are expressed widely in tumors and involved in peripheral tolerance, the PD-Ls-PD-1 pathways may be involved in immune evasion in most, if not all, tumors, especially in residual tumors after immunotherapy. We have found that the expression of PD-Ls was also up-regulated in tumors treated with the HSP70-peptide complex, and treatment by the HSP70-peptide complex in combination with sPD-1 also mediated marked antitumor synergy (X.-H. Wang, G.-M. Zhang, Y.-F. He, and Z.-H. Feng, unpublished observations). Therefore, it is important to consider the possibility that tumors evade from immune attack in immunotherapy through PD-Ls, which are expressed in tumor cells as well as other cells such as DCs, because PD-Ls themselves are expressed in most tumor cells and could be up-regulated in tumor cells and immune cells by such molecules as IFN-γ during immunotherapy. IFN-γ is a cytokine involved in the antitumor mechanisms of many other cytokines used for immunotherapy, such as IL-12 (45) and SLC (14). Taken together, immune evasion involving PD-Ls-PD-1 pathways may be the "side effect" of many cytokines used in tumor immunotherapy, and the use of such cytokines in combination with the blockade of PD-Ls-PD-1 pathways may improve the antitumor effect.

Our results showed that although the combinational use of sPD-1 and SLC produced marked antitumor synergy, there were yet a portion of tumors that could not be suppressed completely, suggesting that the blockade of PD-Ls-PD-1 interactions alone could not completely brake the tolerance to such tumors. In fact, there are other negative costimulatory receptors on T cells except for PD-1, such as CTLA-4 and B and T lymphocyte attenuator, a newly discovered inhibitory receptor with similarities to CTLA-4 and PD-1 (46). Whether these receptors are involved in immune evasion of tumors treated with pSLC plus pPD-1A, and whether blocking these inhibitory pathways could result in stronger antitumor immunity or even complete eradication of the tumors are two very interesting issues and worth further study.

Abrogation of inhibitory costimulatory activity, by administration of mAbs and soluble receptors (often called immunoadhesins) that neutralize costimulatory molecules, can result in the enhanced cellular and humoral immune responses. However, an important issue to consider is whether such blockade could induce the side effect of autoimmune reaction. This concern is heightened by experiments showing that CTLA-4 blockade allowed the breaking of peripheral T cell tolerance to a normal tissue-specific differentiation Ag (19) and a clinical trial showing that blockade of CTLA-4 induced grade III/IV autoimmune manifestations in 43% of patients (36). In contrast, PD-1, whose deficiency in mice causes autoimmune diseases including autoimmunedilated cardiomyopathy, is considered to play a crucial role in peripheral tolerance through negative immunoregulatory signals delivered by PD-Ls-PD-1 pathways (18, 24, 47). Fortunately, however, we did not find remarkable autoimmune manifestations in sites of local gene transfer as well as liver when we treated H22 hepatoma models by local gene transfer of sPD-1. This may be explained by the possibility that immune cells chemoattracted by SLC induced immune response mainly to tumor Ags, and sPD-1 was expressed mainly in transfection sites and thus blocked the PD-Ls-PD-1 pathways mainly concerning antitumor T cells. This is different from the methods using mAbs and immunoadhesins that may play a broad role. Together, although we did not detect histopathologic changes of other tissues except for liver and muscle, and the issue of whether sPD-1 used locally with a higher dose of expression plasmid could induce marked autoimmune manifestations needs to be further studied, the blockade of inhibitory costimulatory molecules in tumor sites is a worthwhile therapeutic method in tumor immunotherapy.

To conclude, the results of this study indicate that the expression of SLC and blockade of PD-Ls-PD-1 pathways by local gene transfer could result in marked antitumor synergy. The combination of chemokines with other immunostimulatory cytokines provides enhanced and long-term antitumor immunity and represents a way to reduce toxic side effects (2). Therefore, our findings provide a potent method of improving antitumor effects of SLC and possibly other immunotherapeutic methods by local blockade of negative costimulatory molecules.

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