T Cell-dependent Antitumor Immunity Mediated by Secondary Lymphoid Tissue Chemokine: Augmentation of Dendritic Cell-based Immunotherapy

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ABSTRACT

Secondary lymphoid tissue chemokine (SLC) is a CC chemokine that is selective in its recruitment of naïve T cells and dendritic cells (DCs). In the lymph node, SLC is believed to play an important role in the initiation of an immune response by colocalizing naïve T cells with DC-presenting antigens. Here, we used SLC as a treatment for tumors established from the poorly immunogenic B16 melanoma. Intratumoral injections of SLC inhibited tumor growth in a CD8+, T cell-dependent manner. SLC elicited a substantial infiltration of DCs and T cells into the tumor, coincident with the antitumor response. We next used SLC gene-modified DCs as a treatment of established tumors. Intratumoral injections of SLC-expressing DCs resulted in tumor growth inhibition that was significantly better than either control DCs or SLC alone. Distal site immunization of tumor-bearing mice with SLC gene-modified DCs pulsed with tumor lysate elicited an antitumor response whereas control DCs did not. We also found that s.c. injection of lysate-pulsed DCs expressing SLC promoted the migration of T cells to the immunization site. This report demonstrates that SLC can both induce antitumor responses and enhance the antitumor immunity elicited by DCs.

INTRODUCTION

To induce an immune response against an established tumor, T cells specific for TAAs must become activated, most likely by specialized cells presenting those antigens (APCs; Ref. 1). However, because most of the known TAAs are nonmutated self proteins, the T cells that can mediate tumor eradication are: (a) self reactive by definition; and (b) probably exposed to those antigens in the periphery long before any active immunotherapy is initiated (2). Therefore, the goal of a therapeutic cancer vaccine is the proper uptake and presentation of TAAs by APCs such that a specific antitumor response is initiated. Considerable effort has been made in the study of antigen types (e.g., peptides, irradiated tumor cells, and gene fragments), adjuvants (e.g., chemical adjuvants, cytokines, and dendritic cells), and modes of delivery (e.g., DNA, pulsed DCs, and peptide/antigen complexes) with the explicit intent of optimizing the priming of TAA-specific T cells (3).

Because of their potent ability to stimulate T cells, particularly naïve T cells, DCs are generally conceived as the most potent members of the class of APCs (4). Based in part on current protocols that enable the generation of large numbers of DCs from peripheral blood (5, 6), DCs have been proposed as the basis of cancer vaccines. Indeed, encouraging results from preclinical and clinical studies highlight the promise of DC-based cancer immunotherapy (7–9). However, in these early studies, complete regression of tumors is not seen in the majority of patients, suggesting that modification of DC-based vaccines is required before they become a widespread treatment modality (10–12).

Genetic modification of DCs to express either tumor antigens or immunomodulatory proteins has met with success in preclinical animal models of tumor treatment (13, 14). Conceivably, DCs that process and present TAAs as transgene products present those antigens for a longer time than those antigens present in vaccines is required before they become a widespread treatment modality (15–17). In another treatment model, in which DCs are injected unpulsed directly into the tumor, expression of IL-12 (18) or IL-7 (19) by the DCs improves their therapeutic efficacy.

It is becoming increasingly more evident that chemokines play an integral role in the initiation of a specific immune response (20). Chemokines are a family of small secreted molecules that mediate leukocyte migration (21). One such chemokine, SLC, is a CC chemokine found on high endothelial venules and within the T-cell zones of both spleen and lymph nodes (22–25). SLC is capable of recruiting both DCs and naïve T cells via the CCR7 receptor found on both cell types (26–29). Because of its expression pattern and that of its receptor, SLC has been postulated to play an important role in the priming of naïve T cells by DCs (30). Indeed, mice deficient in either SLC or CCR7 have lower steady-state levels of T cells in peripheral lymph nodes, reduced migration of hapten-primed DCs to draining nodes, and impaired immune responses to encountered antigens (31, 32).

Because both DCs and naïve T cells express CCR7, the ligand for SLC, we hypothesized that SLC could be used to initiate or enhance antitumor immunity in mice bearing established tumors. We used a mouse model of a poorly immunogeneic B16-BL6 melanoma to determine the effects of SLC on the initiation of an antitumor response. We used three distinct treatment models to assess the therapeutic efficacy of SLC: (a) direct intratumoral injections of recombinant SLC; (b) intratumoral injections of DCs genetically modified to express SLC; and (c) distal site immunization of DCs expressing SLC that were pulsed with whole tumor lysate (33, 34). We used an adenovirus vector encoding SLC to modify DCs to express high levels of this chemokine. Our results show that SLC can induce a strong antitumor response that results in significant infiltration of immune effector cells into treated tumors and that genetic modification of DCs...
to express SLC enhances their capacity to elicit tumor rejection in vivo.

**MATERIALS AND METHODS**

**Animals.** C57BL/6d (denoted B6) and BALB/c female mice, 6–8 weeks of age, were purchased from Harlan Laboratories (Indianapolis, IN) and housed at the Animal Maintenance Facility at the University of Michigan Medical Center for at least 1 week prior to use. Animals were 8 to 12 weeks of age before use in studies.

**Medium and Cytokines/Chemokines.** CM consisted of RPMI 1640 with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone, and 5 × 10⁻³ M 2-mercaptoethanol. Recombinant murine GM-CSF (specific activity, ≥ 5 × 10⁶ units/mg) was obtained from Immunex Corp. (Seattle, WA); recombinant murine IL-4 (2.8 × 10⁶ units/mg) was obtained from Schering-Plough Pharmaceutical Research Institute (Kennisworth, NJ); recombinant murine SLC was obtained from Chiron Corp. (Emeryville, CA); and recombinant murine RANTES was purchased from R&D Systems (Minneapolis, MN).

**Tumor Cell Lines.** B16-BL6 is derived from B6 mice and is a poorly immunogenic melanoma of spontaneous origin (35). MT-901 is a subline of the MT-7 tumor cell line derived from a dimethylbenzanthrene-induced mammary carcinoma in BALB/c mice (36). Tumors were cultured in vitro in CM and were used before the 10th passage.

**Microchemotaxis.** Splenocyte responder cells were generated by gently rubbing spleens between frosted glass slides and passing over a nylone mesh filter (70 μm). RBCs were lysed, and the splenocytes were resuspended in RPMI 1640 containing 5% FCS (RPMI-FCS) and subjected to two rounds of adherence to plastic at 37°C. Nonadherent cells were resuspended to 1 × 10⁶ cells/ml in RPMI-FCS prior to use in microchemotaxis assays. DC responders were obtained from 7-day bone marrow cultures as described below and were used at 2.5 × 10⁶ cells/ml in RPMI-FCS. Assays were performed in 24-well plate format with 6.5-mm diameter, 5 μm pore polycarbonate Transwell inserts (Costar, Cambridge, MA) in duplicate samples. SLC was added to the lower chambers at the indicated concentrations in a volume of 600 μl and incubated at 37°C for 30 min prior to addition of cells. One hundred μl of cell suspension were added to the top chamber, and the assay was carried out at 37°C in a humidified incubator with 5% CO₂. A 1:5 dilution of the cells was also directly added to the lower chamber of two wells for determination of the input amount. After 2 h, the assay was stopped by the removal of the insert, followed by the addition of 10³ poly styrene beads (15-μm diameter; Bangs Laboratories, Fishers, IN) to the lower chamber. Samples were stained with antibodies against CD4 and CD8 (splenocytes) or MHC II and CD86 (DCs) and counted on a FACScaliber (Becton Dickinson, San Jose, CA). In separate experiments, CD4 and CD8 cells were counterstained for expression of CD62L and CD86 (data not shown). CD4 and CD8 cells were enriched by density centrifugation over 14.5% sucrose and added to the lower chamber at the indicated concentrations. Six hundred μl of CM were added to the samples to achieve a concentration of 5 × 10⁶ beads/ml. Samples were stained for the presence of CD4 and CD8 with PE-conjugated antibodies (PharMingen). Samples were analyzed by FACS with counting of 50,000 lymphocyte-sized events (based on splenocyte controls). The number of infiltrating CD4 or CD8 cells/tumor was determined by the following equation: (number of PE events/number of bead events) × 5 × 10³ × 1 g cell sample volume. Because the tumors were of different sizes, the data were normalized to the tumor volume by dividing the total number of infiltrating CD4+ or (CD8+) cells by the tumor volume using the volume equation V (in mm³) = 0.4(ab²), where a is the long diameter and b is the short diameter.

**Preparation of Adenovector Vectors.** Ad2028816 (Ad-SLC) carries an SLC expression cassette in its E1 region. The cassette was excised as an SfiI-BspLU111 fragment from pCMVII-Amp-SLC, blunt-ended, and cloned into the BgII site of shuttle vector pD1954-BglII. The resulting plasmid contains adenoviral DNA from 0–1, 9.3–20.2, and 98.2–100 map units. This plasmid was digested with BspEI to separate the left and right ends of the adenoviral genome and recombined in BJS183 cells (38) with Hirt prep DNA (39) prepared from mammalian cells infected with an E1–, E3-deleted adenovirus. The intact Ad-SLC genome was released from the resulting plasmid (pD2028#16) by restriction digest and transfected into C7 cells to recover virus (40, 41). pAdEasy1-GFP, containing the Ad-GFP genome, was a gift from Dr. Bert Vogelstein (42). Viruses were propagated on C7 cell monolayers and purified on CsCl gradients according to a standard protocol (43). Purified virus was dialyzed against 20 mM HEPES (pH 7.4) containing 5% sucrose, aliquoted, and frozen in a dry ice/ethanol bath (44). A₅₇₀ was determined after particle disruption at 35°C for 10 min in 0.1% SDS, 10 mM Tris-Cl (pH 7.4), and 1 mM EDTA. Particle concentration was calculated using an extinction coefficient of 9.09 × 10⁻¹³ OD/ml/cm/virion (45). Plaque assays were also performed and yielded similar vector particle/infectious unit ratios for all preparations (mean, 84 ± 11).

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Generation of Bone Marrow-derived DCs. Erythroid-depleted bone marrow cells flushed from the femurs and tibias of B6 mice were cultured in 10 ng/ml GM-CSF and 10 ng/ml IL-4 at 1 × 10⁶ cells/ml in CM. At day 3, fresh cytokines were added, and nonadherent cells were harvested on days 4–7 by gentle pipetting. DCs were enriched by density centrifugation over 14.5% (w/v) matrigel (Sigma; Ref. 37). The low density population (buffy coat) was washed several times in RPMI 1640 + 2% FCS prior to use. The resulting DC population was > 80% positive for coexpression of MHC II, CD11c, CD40, CD80, and CD86 (data not shown).

**Tumor Harvest for Immunohistochemistry and FACS Analysis.** B6 mice received 2 × 10⁴ B16-BL6 cells s.c. in the right flank and were treated with daily intratumoral injections of 3 μg of SLC (or vehicle as control) from days 6 to 10. For immunohistochemical analysis of DCs, tumors were harvested. Tumor sections were sectioned with use of a 15-gauge needle (B. J. N.) for the presence of DCs with the DEC-205–specific antibody (Seron-tec, Raleigh, NC). DCs were counted in 10 high powered fields (×40) per section (two sections/tumor) in a blinded fashion. For analysis of T-cell infiltration, B16-BL6 tumors were measured, harvested, removed of extraneous tissue, and digested for 2 h at room temperature in 1 mg/ml type IV collagenase (Sigma) with constant stirring. Dusted tumors were passed over a 70 μm nylon mesh, washed once with HBSS, and resuspended in PBS + 3% BSA to approximately 1 × 10⁶ cells/ml. Polystyrene beads (15-μm diameter) were added to the samples to achieve a concentration of 5 × 10⁶ beads/ml. Samples were stained for the presence of CD4 and CD8 with PE-conjugated antibodies (PharMingen). Samples were analyzed by FACS with counting of 50,000 lymphocyte-sized events (based on splenocyte controls). The number of infiltrating CD4 or CD8 cells/tumor was determined by the following equation: (number of PE events/number of bead events) × 5 × 10³ × 1.0 g cell sample volume. Because the tumors were of different sizes, the data were normalized to the tumor volume by dividing the total number of infiltrating CD4+ or (CD8+) cells by the tumor volume using the volume equation V (in mm³) = 0.4(ab²), where a is the long diameter and b is the short diameter.
tumor cell:DC ratio for 18 h (33, 34). After pulsing, the DCs were collected, their cultured supernatants were harvested for microchemotaxis, washed several times in HBSS, resuspended to 5 × 10^6 cells/ml, and irradiated with 2000 rads prior to use.

Quantitation of SLC Production by Gene-modified DCs. Because there are no currently available monoclonal antibody pairs against SLC suitable for ELISA, a microchemotaxis-based bioassay was performed to determine the amount of functional protein produced by gene-modified DCs. Supernatants from DCs infected with either Ad-GFP or Ad-SLC were added to the bottom chamber of 24-well plates in duplicate to quadruplicate samples (in some cases, a 1:2 dilution was used), and a microchemotaxis assay with splenocyte responder cells was performed as described above. Concurrently, known amounts (10, 100, 500, 1000, and 5000 ng/ml) of recombinant SLC were also added to separate wells in duplicate to generate a standard curve of SLC activity. The equation of the standard curve was generated by nonlinear regression using GraphPad Prism software. We chose a one-site binding equation \( Y = \frac{B_{\text{max}} \times X}{K_d + X} \), where \( Y = \% \) input, \( B_{\text{max}} = \) maximum migration, \( K_d = \) chemokine concentration for half maximal migration, and \( X = \) chemokine concentration. Chemokine amounts presented as ng/10^6 cells in 18 or 24 h were determined from the equation derived from the standard curve for each microchemotaxis assay. The R^2 for each standard curve in nine of nine experiments was ≥0.92.

Treatment of Established B16-BL6 Tumors with Gene-modified DCs. B6 mice received injections s.c. of 5 × 10^7 B16 cells in the right flank. Treatment began on day 6 when palpable tumors of 29 mm^3 were present. DCs (5 × 10^7) were injected into tumors on days 6, 9, and 13. A cohort of mice were treated with daily intratumoral injections of recombinant SLC on days 6–10. As described above, tumor size was monitored twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters with Vernier calipers. Data are reported as the average tumor area ± SE, with five or more mice/group.

Analysis of T-Cell Migration in Vivo. B6 mice were injected intradermally with 1 × 10^7 gene-modified DCs that had been pulsed with B16-BL6 tumor lysate. Skin biopsies (1.5 × 1.5 cm) including and surrounding the injection site were harvested 3 days after injection. The tissue was minced and digested for 2 h at room temperature in HBSS plus 1 mg/ml collagenase (type IV), 1500 units/ml DNase I (type IV), and 1 mg/ml hyaluronidase (type V; all from Sigma) with constant agitation. Samples were passed through nylon mesh to remove particulate matter and resuspended to approximately 1 × 10^6 cells/ml. Polystyrene beads were added to achieve a final concentration of 5 × 10^5 beads/ml. Samples were stained for the presence of T lymphocytes using PE-conjugated antibodies against CD4 and CD8. The number of infiltrating CD4 or CD8 cells/tumor was determined by the following equation: (number of PE events/number of bead events) × 5 × 10^5 × cell sample volume.

Statistical Analysis. For comparisons of treatment groups, a one-way ANOVA (followed by a Newman-Keuls post hoc test) was performed using GraphPad Prism software. Statistical significance was achieved when P < 0.05.

RESULTS

SLC Is Chemotactic for DC, CD4, and CD8 T Cells in Vitro. Prior to initiation of tumor treatment, we analyzed the effect of SLC on the migration of bone marrow-derived DCs and T cells. As seen in Fig. 1, DCs were 10–100 times more sensitive to SLC, as measured by microchemotaxis, than freshly isolated splenic T cells, consistent with previous reports (28). Among the major T-cell subsets, significantly more CD4 T-cell migration was seen in response to SLC (P < 0.01). In the case of both CD4 and CD8 T cells, >95% of the migrating cells were of the naïve phenotype (as measured by CD62L expression; data not shown). The DCs used in these studies were generated from 7-day bone marrow cultures in the presence of GM-CSF and IL-4, but similar chemotactic capabilities were seen from 4-day bone marrow cultures and DCs generated in the absence of IL-4 (data not shown).

Treatment of Established Tumors with Intratumoral Injections of SLC. Because SLC was capable of attracting both DCs and naïve T cells in vitro, we addressed the question of whether SLC could promote an antitumor effect in vivo. To that end, we established s.c. tumors in B6 mice with an injection of 3 × 10^5 B16-BL6 melanoma cells. We began treatment 6 days after tumor challenge, at a point when palpable tumors were at least 9 mm^2. Mice were treated with daily injections of 3 μg of SLC on days 6–8, and tumor size was measured. Fig. 2A shows the results of one representative experiment of five performed. When SLC was administered intratumorally, B16-BL6 tumor growth was inhibited by at least 50% of that in mice treated with vehicle alone (P < 0.05). Intratumoral injection of SLC was necessary because tumor growth was not affected by s.c. injections of SLC in the opposite flank. To show that this antitumor effect of SLC was not strain or tumor type specific, we treated established breast tumors of the MT901 line in BALB/c mice. Again, we found that intratumoral injections of SLC (on days 7–9) inhibited growth of established MT901 tumors, whereas s.c. injection at a distant site had no significant antitumor effect (P < 0.01; Fig. 2B).

To address the dose dependence of the SLC-mediated antitumor effect, we treated 6-day established B16 tumors with three daily injections of SLC in amounts ranging from 0.1 to 25 μg. We found that 0.1 μg had little effect on tumor growth, whereas doses from 1 to 25 μg resulted in equivalent inhibition of tumor growth (P < 0.05; Fig. 3). In separate experiments, we found no difference between a 3-day and 5-day course of treatment or between 1 or 2 cycles of five daily injections that were separated by 2 days (data not shown). Addition of SLC to in vitro cultures of B16-BL6 and MT901 tumor cells had no effect on growth rates (data not shown).

To initially address the mechanism operative in the antitumor effect of SLC, we treated 6-day established B16-BL6 tumors in mice that had been depleted of either CD4 or CD8 cells 4 days prior to treatment. As seen in Fig. 4, depletion of CD8 but not CD4 cells completely eliminated the effect of SLC on tumor growth.

SLC Promotes the Recruitment of DC and T Cells to the Tumor in Vivo. Because SLC is chemotactic for both T cells and DCs, we hypothesized that intratumoral injections of SLC would elicit migration of these cell types to the tumor site. Mice bearing 6-day estab-

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**Fig. 1.** Chemotactic response of DCs and T-cell subsets toward SLC. DCs (2.5 × 10^7/sample) derived from 7-day bone marrow cultures or splenocytes (1 × 10^7/sample) from B6 mice were placed in the top chamber of 6.5-mm Transwell inserts (5-μm pore size) with recombinant SLC added to the bottom chambers in the indicated concentrations. After a 2-h incubation at 37°C, 10^5 polystyrene beads were added to each well, and the samples were stained for DC markers (MHC II and CD86) and for CD4 and CD8. The number of migrating cells in each sample was calculated as described in “Materials and Methods.” The migrating samples were compared with input samples that did not involve microchemotaxis, and the data are reported as the percentage of input migrating cells for DCs ( ), CD4 ( ), and CD8 ( ), P < 0.01 for CD4 versus CD8 by Student’s t test. Bars, SE.
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Established tumors were treated with intratumoral injections of SLC or PBS for five consecutive days. To determine the presence of infiltrating DCs, we stained frozen sections for the presence of DEC-205. Tumors from PBS-treated mice contained only rare and focal areas of tumor necrosis at 4 and 7 days after treatment; only isolated and scattered DCs were found (<1 DC/10 high-power fields). In contrast, tumors from SLC-treated mice contained more extensive and frequent zones of necrotic tumor cells (data not shown). Moreover, there were also significantly more infiltrating DCs in tumors from SLC-treated mice.

Sections of SLC-treated tumors harvested 4 and 7 days after treatment began contained 6.2 ± 2.9 and 5.9 ± 1.8 DCs/10 high-power fields, respectively.

To analyze tumor infiltration by T-cell subsets, we harvested tumors on days 2 and 4 of SLC or PBS treatment. Prior to harvest, the tumor diameters were measured. After excision, tumors were enzymatically disaggregated in collagenase to obtain a single-cell suspension. We analyzed the tumor samples for the presence of CD4 and CD8 T cells by FACS analysis. To quantify the number of infiltrating CD4 and CD8 T cells, we normalized the number of infiltrating cells to the tumor volume (Fig. 5). After 2 days of treatment, tumors from SLC-treated mice contained 3–5-fold more CD4 and CD8 T cells than those from PBS-treated animals (P < 0.05). Significantly more infiltrating CD4 and CD8 T cells were also seen after 4 days of treatment (P < 0.001 and P < 0.05, respectively).

Taken together, these data suggested that intratumoral injections of SLC could increase the number of DC and T cells within the infiltrate of s.c. tumors.

Genetic Modification of DCs to Produce SLC. Our data suggest that the presence of SLC in tumors promotes the migration of DC and T cells and inhibits tumor growth through a CD8+ T cell-dependent manner. We next determined whether alternate modes of delivery of SLC could improve its antitumor effect. In other tumor models, intratumoral injections of DCs have been shown to promote a T cell-dependent antitumor response (18, 19). We examined whether direct tumoral delivery of SLC via gene-modified DCs could improve the antitumor effect. To that end, we constructed an adenoviral vector containing the gene for SLC.

We used a microchemotaxis assay to determine the levels of SLC produced in the supernatant by gene-modified DCs. Supernatants produced in the supernatant by gene-modified DCs. Supernatants contained 3–5-fold more CD4 and CD8 T cells than those from PBS.
produce chemokines (46), including the CCR7 agonist ELC, these molecules did not appear to be present in large amounts in cultured supernatants.

**Intratumoral Injections of SLC-expressing DCs Promote a Potent Antitumor Effect.** We used SLC gene-modified DCs to treat 6-day established B16 tumors. Mice received three intratumoral injections of SLC (or GFP) gene-modified DCs on days 6, 9, and 13. Another cohort of mice also received daily injections of recombinant SLC for 5 consecutive days beginning on day 6. Mice receiving GFP gene-modified DCs showed some inhibition in tumor growth (Fig. 7), consistent with previous reports (18, 19). The antitumor effect of the GFP-expressing DCs was slightly less than that elicited by recombinant SLC. However, intratumoral injections of SLC gene-modified DCs elicited an antitumor effect that was significantly greater than that elicited by either the GFP-expressing DCs or SLC alone ($P < 0.01$; Fig. 7).

The amount of chemotactic activity in cultured supernatants from SLC gene-modified DCs was >10 fold more than that elicited by GFP-expressing DCs (Fig. 6). Cultured supernatants from GFP gene-modified DCs promoted microchemotaxis of T cells to the same extent as that of cultured supernatants from the D5 variant of the B16 cell line (data not shown). This finding suggests that although DCs from infected cells were used as the source of chemoattractant in microchemotaxis assays with splenic responder cells. We also performed microchemotaxis using a range (10–5000 ng/ml) of concentrations of recombinant SLC to generate a standard curve for T-cell chemotaxis. From the standard curve, we could determine the concentration of chemokine present in DC-cultured supernatants. The standard curve used in determining SLC concentrations was generated by analyzing the migration of CD4$^+$ cells in the splenocyte responders. However, similar values were obtained using a standard curve generated from migrating CD8$^+$ cells or by total migrating lymphocytes (data not shown). As shown in Fig. 6, which represents the data from nine separate experiments, ~750 ng of SLC were produced within 18 h by $1 \times 10^6$ SLC gene-modified DCs. SLC was detected in 24-h culture supernatants 3 days after infection, suggesting that gene expression endured for at least this period of time (data not shown). Genetic modification of DCs with adenoviral vectors resulted in a modest up-regulation of the T-cell costimulatory receptors CD80 and CD86 (data not shown).

The amount of chemotactic activity in cultured supernatants from SLC gene-modified DCs was >10 fold more than that elicited by GFP-expressing DCs (Fig. 6). Cultured supernatants from GFP gene-modified DCs promoted microchemotaxis of T cells to the same extent as that of cultured supernatants from the D5 variant of the B16 cell line (data not shown). This finding suggests that although DCs
Tumor Lysate-pulsed, SLC Gene-modified DCs Elicit Systemic Antitumor Immunity. We next determined whether treatment of established B16-BL6 tumors with tumor lysate-pulsed DCs could be improved by genetically modifying the DCs to express SLC. Pulsing of DCs with tumor lysate did not significantly affect the levels of SLC produced by gene-modified DCs (data not shown). We immunized mice bearing 6-day established tumors with DCs expressing SLC (or GFP) and pulsed with B16-BL6 tumor lysate contralaterally to the site of growing tumors. Fig. 8 shows that although GFP gene-modified DCs were ineffective in reducing the growth of s.c. tumors, SLC-expressing DCs were able to mediate a significant antitumor response. This response was dependent upon presentation of tumor antigen(s) by the DCs, because unpulsed SLC gene-modified DCs were unable to elicit an antitumor response when administered at a site distal from the tumor (Fig. 8).

One possible explanation for the enhanced effect of SLC gene modification on tumor lysate-pulsed DC immunizations is enhanced recruitment of host-derived T cells in vivo. Because the vast majority of DCs remain at the s.c. immunization site 24 h after injection (47, 48), it is possible that T cells would migrate to skin sites containing SLC-expressing DCs. To determine the influx of T cells into DC skin injection sites, we immunized mice s.c. with B16-BL6 lysate-pulsed DCs expressing either GFP or SLC and harvested skin samples 3 days later. After enzymatic disaggregation, we analyzed these samples for the presence of CD4+ and CD8+ cells by FACS. As shown in Fig. 9, we found that SLC-expressing DCs attracted ~2–3-fold more CD4 and CD8 T cells to the injection site at both time points tested (P < 0.05). These data suggest that the improved adjuvanticity of DCs resulting from expression of SLC may be attributable, in part, to increased migration of T cells to the site of immunization.

DISCUSSION

In this study, we tested the antitumor properties of SLC in three treatment regimens. Using direct tumoral administration of recombinant protein, we found that SLC could inhibit the growth of the B16 melanoma and the MT901 mammary adenocarcinoma. SLC caused a marked influx of CD, CD4+, and CD8+ T cells into the tumor mass. In vivo depletion of CD8+ T cells eradicated the antitumor effect of SLC. Using an adenoviral vector encoding SLC, we were able to generate SLC-expressing DCs derived from bone marrow progenitors. When given intratumorally, these SLC gene-modified DCs could elicit an antitumor effect that was significantly better than either the recombinant protein or GFP gene-modified DCs. Finally, we found that SLC gene modification substantially improved the adjuvanticity of tumor lysate-pulsed DCs against the poorly immunogenic B16-BL6 melanoma and could attract T cells to the s.c. immunization site. Because of its poor immunogenicity, tumors of B16-BL6 origin are refractory to treatments that would ordinarily lead to tumor regression of weakly immunogenic tumor lines (17, 18), particularly when tumor burden is high (e.g., 6-day s.c. tumors).

The receptor for SLC, CCR7, is expressed on both naïve T cells and DCs, suggesting that it plays an important role in T-cell activation in peripheral lymphoid organs where the chemokine is expressed (22–25). This is underscored by the fact that both SLC-deficient (plt) and CCR7−/− mice have reduced responses to antigenic stimulation (31, 32). Because we found evidence of both DC and T-cell migration in tumors treated with SLC, it is possible that the emigrated T cells are being primed in the tumor by infiltrating DCs that have taken up apoptotic/necrotic tumor cells (49). Furthermore, because mature DCs may be able to activate CD8+ T cells in the absence of CD4+ T-cell help (50), intratumoral priming of CTLs may explain why SLC treatment is efficacious, even in the absence of CD4+ cells (Fig. 4). However, we cannot rule out the possibility that mature DCs attracted to the tumor by the presence of SLC take up TAAs and migrate to the draining lymph node(s), where they activate CTLs. We are currently investigating the phenotype and function of the tumor-infiltrating T cells as well as the stimulatory capacity of emigrating DCs.

In a recent report, Sharma et al. (51) showed that recombinant SLC could inhibit the growth of 5-day established tumors in mice. In this particular study, SLC-mediated antitumor immunity was not elicited in either CD4+ or CD8-deficient mice. The dependence upon CD4+ T cells contrasts with our data using antibody depletion (Fig. 4). These disparate results could be explained, in part, by the experimental systems used to determine subset contributions (subset-deficient mice for Sharma et al. versus antibody depletion here) or by the tumor models used (3LL and LC12 lung cancers versus B16 melanoma). Sharma et al. (51) also reported an increase in tumor infiltration by DC and T cells as a result of SLC treatment, which is in agreement with our data (Fig. 5).

Direct tumoral administration of other recombinant chemokines, i.e., IP-10 and Mig, has been shown to inhibit tumor growth as well (52–54). In these cases, the antitumor response was mediated by the antiangiogenic properties of these chemokines. IP-10 and Mig can
mediate the antitumor effect of IL-12 (52, 55), which may explain, in part, the increased antitumor effect elicited by IL-12 gene-modified DCs (18). SLC has been shown to bind to CXCR3, the receptor for both IP-10 and Mig, and to exert an angiostatic activity in vivo (56). However, because the antitumor effect of SLC was eliminated in mice depleted of CD8+ T cells, it is unlikely that SLC mediated its antitumor effect via direct inhibition of angiogenesis. However, it remains a possibility that SLC can indirectly affect tumor vasculature via recruitment of DC and T cells that produce angiostatic agents such as IP-10 and Mig.

Antitumor therapies based on chemokine gene transfer and expression have used chemokine-transfected tumor cells, adenoviral gene delivery to tumors, and gene-modified DCs (16, 17, 57–61). Previously, we reported that tumor cells stably expressing the CXC chemokine RANTES failed to grow in immunocompetent hosts (57). Similarly to intratumoral injections of SLC, the antitumor effect elicited by RANTES-secreting tumor cells was dependent upon CD8+ T cells. However, we were unable to detect T-cell or DC migration in response to RANTES in vitro (data not shown). Furthermore, RANTES-secreting tumors were ineffective as a treatment against established tumors (57). More recently, it was reported that tumor cells stably expressing ELC, another ligand for CCR7, also failed to grow in immunocompetent hosts (61). In contrast to our work with SLC, the antitumor response for ELC reported by Braun et al. (61) was dependent upon natural killer and CD4+ cells but did not involve CD8+ cells. Lptn, a C chemokine, has been shown to enhance an antitumor effect in two gene therapy models (16, 17, 62). Immunization of tumor-bearing mice with irradiated tumors containing Lptn-secreting cells had little effect on tumor growth, but resulted in reduction of tumor growth when combined with IL-2-secreting cells (62). DCs genetically modified to express Lptn and pulsed with either peptides derived from tumor antigens or tumor RNA triggered a stronger antitumor response than control gene-modified DCs (16, 17). However, the receptor for Lptn is not expressed on naive T cells (63), suggesting that the effect of Lptn gene expression depends on already activated T cells. Because CCR7 is found on naive T cells, our results are consistent with a model in which SLC enhances the priming of naive T cells through APCs.

Cytokine and chemokine gene-modified DCs promote stronger antitumor responses than their control gene-modified counterparts, regardless of whether the DCs are delivered intratumorally or pulsed with tumor antigens and administered at a distal site (15–19, 64). Here, we show that SLC-expressing DCs are superior to GFP gene-modified DCs in both treatment regimens. Gene-modified DCs express substantial amounts of SLC (∼750 ng/1 × 10^6 cells/18 h), and the adenoviral vector has no detrimental effect on DC phenotype. To our knowledge, this is the first report of genetic modification of DCs to express a chemokine selective for naive T cells. Of note, unmodified and control-modified, DC-cultured supernatants resulted in minimal migration of DCs, to an extent equivalent to those from an unmodified tumor cell line. One interpretation of these data are that although DCs express the genes for several chemokines, including ELC, they do not secrete significant amounts of the protein (46). Another possibility is that DCs cultured in vitro remove the secreted ELC via CCR7 expressed on their surfaces. In this model, it is possible that DCs also bound and removed the secreted SLC, but because of high expression levels, detectable amounts remained in culture.

When given intratumorally, SLC-expressing DCs reduced tumor growth of established B16 melanoma tumors to a greater extent than either DCs alone or SLCLC alone. Because the addition of recombinant SLC resulted in the infiltration of CD4 and CD8+ T cells (along with DCs), it is likely that injection of SLC-expressing DCs also resulted in T-cell infiltration and possible activation of T cells by the injected DCs (or by endogenous DCs attracted to the tumor by SLC). It is also possible that SLC-expressing DCs acquired TAAAs and migrated to the draining lymph nodes to enhance T-cell priming. Our future studies will determine the migratory capacities of SLC gene-modified DCs in vivo. Another explanation for the enhanced effect of SLC-expressing DCs could be attributable to the bioavailability of the protein in vivo. Recombinant SLC was given intratumorally once daily for 5 days, whereas the DCs were given three times over the course of 7 days. Because DCs expressed high levels of SLC in vitro for at least 3 days, it is possible that a therapeutically effective dose of SLC in the tumor (i.e., >0.1 µg) was maintained longer by the addition of SLC-expressing DCs. However, the kinetics and levels of SLC gene expression in vivo by adenovirus-infected DCs have yet to be determined.

We were also able to achieve efficacious treatment of established tumors by immunization with lysate-pulsed, SLC-expressing DCs, whereas GFP gene-modified DCs were ineffective as an adjuvant in this tumor model. To our knowledge, this is the first report combining chemokine gene-modification of DCs with tumor lysate pulsing to generate a therapeutically effective cancer treatment. One possible mechanism by which SLC enhanced the immunogenicity of DC-based vaccines was by the recruitment of T cells to the immunization site. It has been shown in both mice and humans that the vast majority of DCs injected s.c. remain in the injection site and do not reach the draining lymph node (47, 48). Here we showed that SLC-expressing DCs could recruit T cells to the immunization site. It is possible that the tumor lysate-pulsed DCs activated TAA-specific T cells within the migratory population locally. If indeed some T cells had become activated, it is not likely that they remained in the s.c. area for extended periods. T-cell migration to the skin in response to D5 cells expressing ELC, which binds to CCR7, occurred at 48 and 72 h after immunization but were no longer present 4 days after injection. Furthermore, T cells have been shown to lose expression of CCR7 after activation (65), suggesting that, once activated, TAA-specific T cells would no longer be expected to be retained in the immunization site by the SLC-expressing DCs.

Although our results do not show unequivocally that antitumor immunity is triggered by the DCs residing in the injection site, they do suggest that SLC expression may increase the effective number of DCs (i.e., those that prime naive T cells) present in each immunization. Indeed, we have found that direct tumoral administration of SLC-secreting DCs results in tumor infiltration of large numbers of IP-10 and Mig. Recombinant SLC has been shown in both mice and humans that the vast majority of IFN-γ-secreting CD4+ and CD8+ T cells in the absence of a concomitant increase in draining lymph node cellularity. Future studies using mice lacking peripheral lymph nodes should address the question of whether SLC-expressing DCs can prime an immune response without migration to lymph nodes. We have found that SLC gene-modified DCs are preferentially retained in the tumor compared with their control gene-modified counterparts. SLC gene modification may obviate the need for intranodal delivery of DCs presently used in some clinical applications (12). Comparison of the route of delivery (e.g., s.c. versus i.v. or i.p.) of SLC gene-modified DCs will further address the mechanisms behind the enhanced adjuvanticity of these cells.

Although this study used a first-generation adenovirus, use of “gutted” adenoviral vectors (40), which can incorporate large amounts of cDNA, should allow for the gene transfer of multiple cytokine and/or chemokine genes within a single vector. These newer generation vectors are also believed to be less immunogenic than earlier versions (66), lessening the possibility of gene-modified DCs induc-
ing antiviral immunity (67, 68). Collectively, our data demonstrate that SLC may be used as a therapeutic agent for the treatment of established tumors as both a stand-alone biotherapeutic and a gene therapy in conjunction with DC-based treatments.

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