

The Dynamics of the T-Cell Antitumor Response: Chemokine-secreting Dendritic Cells Can Prime Tumor-reactive T Cells Extranodally¹

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ABSTRACT

Direct administration of dendritic cells (DCs) genetically modified to express secondary lymphoid tissue chemokine (SLC) into growing B16 melanoma could result in a substantial, sustained influx of T cells within the mass with only a transient increase in T-cell numbers in the draining lymph node (DLN). DCs were retained at the tumor site with only a very small percentage trafficking to the DLN. The T cells infiltrating the tumor mass expressed the activation marker CD25 within 24 h and developed IFN- γ -secreting function within 7 days as tumor growth was inhibited. Similar results were obtained in lymphotoxin α -/- mice, which lacked peripheral lymph nodes. Our data demonstrate that effective T-cell priming can occur extranodally and result in measurable antitumor effects *in vivo*.

INTRODUCTION

The induction of an antigen-specific response requires the activation of naïve T-lymphocytes by APCs⁴ bearing cognate antigen. T-cell priming is thought to occur only in specialized compartments of the body, *i.e.*, secondary lymphoid organs such as the spleen and LNs. Within the LN, naïve T cells are activated by DCs bearing cognate antigen. DCs are sentinel APCs that reside in peripheral tissues or in circulation as phagocytic precursors (1). DCs acquire a mature phenotype upon uptake of antigen and response to stimuli such as inflammatory cytokines, necrotic cells, and microbial products, enabling them to migrate to LNs and Peyer's patches (2). Homing of both DCs and naïve T cells to lymphoid organs requires expression of the CCR7 chemokine receptor, which is bound by the chemokines CCL19 (ELC/MIP-3 β) and CCL21 (SLC/6Ckine; Refs. 3–5). SLC is deposited on the lumen of high endothelial venules in regions adjacent to T-cell-rich areas of the lymph node (6). Within the lymph node, ELC and SLC produced by the stromal cells provide a source of chemoattractant for antigen presenting DCs and naïve T cells (7). Mature DCs are also a source of ELC, which may enhance the colocalization of T cells and DCs (8).

The functional axis encompassing CCR7 and SLC/ELC makes up a key component in the initiation of the adaptive immune response. Mice deficient in either SLC (*plt*) or CCR7 have altered lymph node architecture, reduced migration of DCs and T cells to lymphoid organs, and a reduced response to peripherally encountered antigens

(9, 10). Recall response to contact antigens is inhibited in wild-type mice treated systemically with anti-SLC antibodies at the time of priming (11). Mice expressing SLC by a transgene controlled by the insulin promoter express lymphoid architecture, including organized T- and B-cell follicles and high endothelial venule-specific markers in pancreatic islets, suggesting that expression of SLC is sufficient for lymph node biogenesis (12). More recently, delayed but enhanced T-cell-specific responses in the spleens of *plt* mice were reported, suggesting that the presence of SLC and ELC regulates the kinetics and level of T-cell responses (13).

A primary goal of T-cell-mediated immunotherapeutic intervention against malignancy is the activation and clonal expansion of T cells reactive to antigens expressed by tumor cells. The majority of TAAs defined to date are nonmutated self antigens, raising the question of whether T cells present in the periphery are capable of differentiating into potent antitumor effectors because of peripheral tolerance (14). Alternatively, TAA-specific T cells in the periphery may represent those self-reactive T cells that escaped negative selection in the thymus as a result of bearing T-cell receptors with low affinity for TAAs, raising the possibility that these cells can never be primed to mediate an efficient antitumor response (15). Indeed, clinical trials involving peptide vaccination against defined CTL epitopes expressed by melanoma cells highlight the limitations of therapeutic strategies targeting known TAAs (16). However, it remains a possibility that T cells strongly reactive to as yet undefined TAAs are present in tumor-bearing animals and patients, representing a potentially effective precursor population of antitumor effector T cells (17). Treatment regimens involving immunization with whole tumor cell preparations or adjuvant therapies directly administered to a tumor mass seek to prime T-cell responses to all possible TAAs (18).

Ex vivo-derived DCs represent a potent adjuvant for use in tumor vaccination protocols. DCs are readily generated from monocyte precursors in peripheral blood or stem cell progenitors in rodent bone marrow after culture in the presence of GM-CSF and IL-4 (19–21). Once generated, DCs can be pulsed with tumor peptides, lysates, or RNA or fused with tumor cells prior to immunization of tumor-bearing animals or patients (22). When directly administered to a growing tumor mass, DCs can induce a potent antitumor response in the absence of *ex vivo* antigen pulsing, which is mediated by host-derived T cells (23, 24). Moreover, increasing the relative level of apoptotic cells within the tumor mass can enhance the antitumor effect of directly administered DCs (23). These data suggest that DCs are capable of engulfing dead or dying cells within the tumor mass, processing tumor antigens, and presenting those antigens to T-cells, presumably in the LN to which they migrate. However, it has been shown in both mice and humans that the vast majority of *ex vivo*-derived DCs administered *s.c.* fail to migrate from vaccination sites to regional LNs (25, 26).

Here, we report that *s.c.*-growing B16 melanoma treated with SLC gene-modified DCs are readily infiltrated by host-derived T cells and that these T cells are primed locally within the tumor mass as measured by expression of activation markers and the cytokine IFN- γ . Our data further suggest that T-cell priming and the generation of functional antitumor effector cells can occur in the absence of a LN.

Received 7/27/01; accepted 10/19/01.

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¹ Supported by Grants 1 R01 CA71669, 1 R01 CA87019, 5 P01 CA59327, and M01-RR00042 from the National Cancer Institute/NIH, Grants DAMD17-96-1-6103 and DAAG55-97-1-0239 from the Department of Defense/United States Army, and gifts from C. J. and E. C. Aschauer, Abbott Laboratories, and the Dorothy and Herman Miller Immunology Research Endowment. C. J. K. was supported by Training Grant AI07413.

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⁴ The abbreviations used are: APC, antigen presenting cell; LN, lymph node; DC, dendritic cell; ELC, Epstein-Barr virus-induced molecule 1 ligand chemokine; SLC, secondary lymphoid tissue chemokine; TAA, tumor-associated antigen; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; CM, complete medium; GFP, green fluorescent protein; PE, phycoerythrin; PMA, phorbol myristate acetate; L α , lymphotoxin- α .

MATERIALS AND METHODS

Animals, Tumor Lines, and Adenoviruses. C57BL/6J mice (denoted B6) were purchased from Harlan Laboratories (Indianapolis, IN) and The Jackson Laboratory (Bar Harbor, ME). Lymphotoxin $\alpha^{-/-}$ (strain B6.129S2-Lt^{atm1Dch}) mice on the B6 background were purchased from The Jackson Laboratory. All animals were housed at the Animal Maintenance Facility at the University of Michigan Medical Center for at least 1 week prior to use and were 8–12 weeks of age. The B16-BL6 melanoma is of spontaneous origin in B6 mice and is poorly immunogenic (27). Recombinant adenoviruses encoding murine SLC or β -galactosidase have been described elsewhere (28, 29). pAdEasy1-GFP, containing the Ad-GFP genome, was a gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD; Ref. 30). All viruses were propagated as described (28).

Generation of Bone Marrow-derived DCs. Erythrocyte-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 1×10^6 cells/ml in CM (RPMI 1640 containing 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μ M sodium pyruvate, 2 mM fresh L-glutamine, 100 μ g/ml streptomycin, 100 units/ml penicillin, 50 μ g/ml gentamicin, 0.5 μ g/ml fungizone, and 5×10^{-5} M 2-mercaptoethanol). At day 3, fresh cytokines were added, and nonadherent cells were harvested on days 5–7 by gentle pipetting. DCs were enriched by density centrifugation over 14.5% (w/v) matrigel (Sigma Chemical Co., St. Louis, MO; Ref. 31). The low-density population was washed once in CM and once in RPMI 1640 containing 2% FCS prior to use. The resulting DC population was >85% positive for coexpression of MHC II, CD11c, CD40, CD80, and CD86 (data not shown).

Genetic Modification of DCs with Adenoviral Vectors. DCs were resuspended at a concentration of 1×10^7 cells/ml in RPMI 1640 + 2% FCS and placed in a 15-ml conical tube. Virus was added at a ratio of 15,000 vector particles/DC, the suspension was mixed well, and the tube was incubated at 37°C for 2 h. Nine volumes of complete medium with 10 ng/ml GM-CSF and 10 ng/ml IL-4 were then added, and the cells were transferred to tissue culture dishes. Cells were incubated for 18 h at 37°C, supernatants were recovered, and the cells were purified by incubation in PBS with 3 mM EDTA and gentle scraping. SLC production by gene-modified DCs was quantified using a microchemotaxis-based bioassay as described (28). Using an adenovirus encoding GFP, we determined a transfection efficiency of ~40% (data not shown). The cells were washed several times in HBSS, resuspended to 2×10^7 cells/ml, and irradiated with 2500 rads prior to injection into animals.

Treatment of Established B16-BL6 Tumors with Gene-modified DCs. B6 mice were injected s.c. with 1×10^5 B16 cells in the right flank. DCs (1×10^6) were injected into palpable tumors (≥ 9 mm²) on days 6 and 13. A cohort of mice were treated with daily intratumoral injections of recombinant SLC (3 μ g/dose in 100 μ l; kindly provided by Chiron Corp., Emeryville, CA) on days 6–10. Prior to harvest, tumor size was recorded by measuring the largest perpendicular diameters (in mm) with Vernier calipers.

Tumor and DLN Harvest. At the indicated time points, tumors were harvested, removed of extraneous tissue, and digested for 2 h at room temperature in 1 mg/ml type IV collagenase (Sigma Chemical Co.) with constant stirring. EDTA (10 mM) was added for the last 30 min to ensure release of DCs (32). Digested tumors were passed over a 70 μ m nylon mesh, washed once with HBSS, and depleted of erythrocytes. The samples were washed once in HBSS and resuspended in PBS + 1% BSA to $\sim 1 \times 10^6$ cells/ml. DLNs (inguinal) were mechanically disrupted, passed over 70 μ m nylon mesh and washed twice in HBSS prior to resuspension in PBS + 1% BSA to a final concentration of $\sim 1 \times 10^6$ cells/ml. Polystyrene beads (15 μ m diameter) were added to all samples to achieve a concentration of 5×10^5 beads/ml.

Fluorescence-activated Cell Sorter Analysis. All antibodies used were purchased from PharMingen (San Diego, CA). For analysis of T-cell subsets and DCs, samples were stained with PE-conjugated antibodies to CD4, CD8, and CD11c. To determine the level of activation in tumor-infiltrating lymphocytes, samples were stained with fluorescein (FITC)-conjugated antibodies to CD11b and B220, PE-conjugated antibodies to CD25 or CD62L, and Cy-Chrome-conjugated antibodies to CD4 or CD8 (FITC-conjugated antibodies were omitted from LN samples). Samples were analyzed by fluorescence-activated cell sorter with counting of 50,000 lymphocyte-sized events (based on splenocyte controls). The number of infiltrating DCs, CD4, or CD8 cells/tumor was determined by the following equation: (# of PE events/# beads

events) \times (5×10^5 beads/ml) \times cell sample volume (in ml). Because the tumors were of different sizes, the data were normalized to the tumor volume by dividing the total number of infiltrating cells by the tumor volume using the volume equation: V (in mm³) = $0.4(ab^2)$, where a is the long diameter and b is the short diameter. For analysis of T-cell subsets and activation in tumors, CD62L+ or CD25+ events that were also positive for CD4 or CD8 were counted in the CD11b-/B220- fraction of cells gated for lymphocyte size by forward and side scatter plot. We found that CD11b was expressed by most of the tumor cells (data not shown). We did not detect expression of either CD11b or B220 on T cells isolated from tumor or LN samples (data not shown).

Intracellular Cytokine Staining. Tumors were harvested from mice 7 and 9 days after treatment initiation and enzymatically digested as described above. Samples were resuspended in CM to $1\text{--}5 \times 10^6$ cells/ml and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma Chemical Co.) for 5 h at 37°C. Brefeldin A (10 μ g/ml; Sigma Chemical Co.) or monensin (3 μ M; Sigma Chemical Co.) was added for the last 3 h. To control for the possibility of any nonspecific mitogenic stimulus, LN cells from naïve mice were stimulated as above for comparison. Samples were washed once in HBSS and resuspended in PBS + 1% FBS to $\sim 1 \times 10^6$ cells/ml; polystyrene beads were added to a final concentration of 5×10^5 cells/ml. Samples were stained with FITC-conjugated antibodies to CD11b and B220 and Cy-Chrome-conjugated antibodies to CD4 or CD8. Samples were washed twice in PBS + 1% FBS and fixed in PBS containing 4% paraformaldehyde and 0.5% saponin (Sigma Chemical Co.) for 30 min at room temperature. Samples were washed twice in PBS + 1% FBS containing 0.5% saponin (permeabilization buffer) and stained with PE-conjugated antibodies to IFN- γ or IL-4 (or isotype control) for 30 min at 4°C. Cells were washed three times in permeabilization buffer and resuspended in ice-cold PBS prior to analysis by flow cytometry. Cells were analyzed for the presence of IFN- γ or IL-4 in the CD11b-/B220- fraction of lymphocyte gated events. Cell numbers were calculated as described above.

DC Migration Analysis. Cultured DCs were harvested 18 h after infection with adenovirus. The cells were washed once in CM and stained with PKH26 red dye (Sigma Chemical Co.) according to the manufacturer's instructions. Cells were washed twice in HBSS, resuspended to 2×10^7 cells/ml, and irradiated with 2,500 rads. Mice bearing 6-day s.c. tumors were injected with 1×10^6 labeled DCs; 24 h later, tumors and DLNs were harvested and processed as described above. Labeled DCs were detected by FL-2 channel fluorescence (567-nm dye emission) \pm costaining with fluorescein-conjugated antibodies to CD11c. The number of injected DCs in both the tumor and DLNs was calculated as described above, and data are presented as the percentage of injected DCs present in the tumor or LN.

Statistical Analysis. For comparisons of treatment groups, a one-way ANOVA (followed by a Newman-Keuls post hoc test) was performed using tumor size or cell number measurements taken at each time point. For comparisons of two treatment groups, Student's t test was performed. All statistical analysis was performed using GraphPad Prism software. Statistical significance was achieved when $P < 0.05$.

RESULTS

Retention of Intratumorally Administered DCs. To follow the migration of gene-modified DCs, we labeled DCs genetically modified to express SLC (denoted DC-SLC) or the control protein β -galactosidase (denoted control DC) with the dye PKH-26 (567-nm emission) prior to injection into s.c. B16 melanoma. Tumors and DLNs were harvested 24 h after injection and analyzed for the presence of labeled cells. In tumors injected with DC-SLC, 40% of injected cells remained in the tumor 1 day later, significantly more than labeled control DCs (Fig. 1). It is unlikely that the PKH-26+ cells represent uptake of dye or labeled DCs by tumor cells or resident macrophages, because the dye-containing cells expressed the DC marker CD11c (data not shown). Furthermore, these values may be underestimates because of the likelihood that the recovery process is not able to fully retrieve all viable DCs within the tumor digest. The increased retention of the SLC gene-modified DCs was likely the result of the expression of SLC by the DCs because SLC expressing

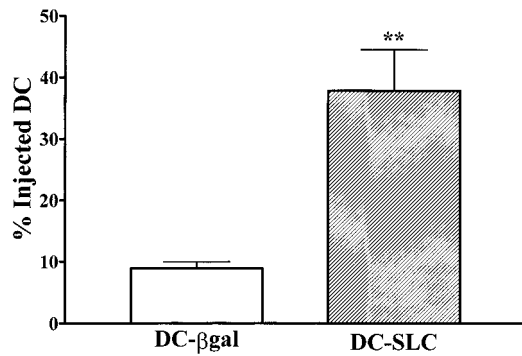


Fig. 1. Retention of gene-modified DCs in treated tumors. Mice bearing 6-day s.c. B16 tumors were injected with 1×10^6 genetically modified DCs that had been labeled with PKH26 dye prior to injection. Tumors were harvested 24 h after injection, stained with FITC-conjugated anti-CD11c, and analyzed for the presence of labeled cells by flow cytometry. Cell percentages were quantified as described in "Materials and Methods." Bars, SE.

DCs retain their ability to migrate along an SLC gradient *in vitro* (data not shown).

Despite the expression of CCR7, migration of bone marrow-derived DCs to DLNs after s.c. administration is minimal, on the order of <1% of injected cells (25, 26). To determine the trafficking of intratumorally administered DCs, we analyzed for the presence of labeled, gene-modified DCs in the tumor DLN 24 h after administration. We found no differences in percentages of DC-SLC and control DC in the DLN ($P = 0.841$; Table 1). In both cases, fewer than 1% of the injected DCs were present in the DLN. LNs were also analyzed at 72 h after injection, and again no differences in the accumulated labeled DCs were found (data not shown).

SLC Gene-modified DCs Promote T-Cell Tumor Infiltration. Because DC-SLC remained in the tumor site after intratumoral administration without subsequent migration to the DLN, we sought to investigate the antitumor effect elicited at the tumor site by these genetically modified APCs. We treated mice bearing s.c. B16 melanoma with intratumoral injections of DC-SLC or control DC on days 6 and 9. The SLC production by gene-modified DCs was $517.4 \pm 59.13 \text{ ng}/1 \times 10^6 \text{ cells}/18 \text{ h}$, as measured by a microchemotaxis-based bioassay (data not shown). Mice treated intratumorally with either control DC or rSLC ($3 \mu\text{g}$ daily from days 6 to 10) experienced a modest inhibition of tumor growth over the course of study (Fig. 2). In contrast, mice treated with DC-SLC demonstrated marked tumor growth inhibition, which was significantly greater than either control DC or rSLC (Fig. 2). Tumor growth inhibition by DC-SLC was evident for 28 days after tumor challenge (data not shown).

We analyzed single-cell suspensions from s.c.-growing B16 melanoma for the presence of infiltrating lymphocytes at 24 h and 4, 7, and 9 days after the initiation of DC-SLC treatment; for comparison, we also analyzed tumors for the presence of T cells after treatment with rSLC. rSLC induces a significant but transient influx of T cells into s.c. tumors (28). The presence of infiltrating T-cell subsets was determined by flow cytometry using antibodies specific for CD4 and CD8. The total number of infiltrating T cells was normalized to the tumor volume because tumor size varied between and within treatment groups (Fig. 2). Concurrently, we calculated the cellularity of the LN (inguinal) draining the s.c. tumor from the same mice.

Significant influx of both CD4 (Fig. 3A) and CD8 (Fig. 3B) T cells into tumors was seen 24 h after injections of DC-SLC but not control DC nor rSLC. T-cell influx into tumors increased in all treatment groups over the first 7 days after treatment initiation, whereas saline-injected tumors exhibited no change in the relative number of tumor-

infiltrating T cells (Fig. 3, A and B). By 7 days after the initiation of treatment, DC-SLC-treated tumors contained an average of 2.94×10^6 CD4+ and 1.45×10^6 CD8+ T cells compared with 0.42×10^6 CD4+ and 0.55×10^6 CD8+ T cells in saline-treated tumors. Although both rSLC and control DC could induce the influx of T cells 4 and 7 days after treatment initiation, DC-SLC resulted in 2–4-fold higher numbers of infiltrating T cells ($P < 0.05$ and $P < 0.001$ for 4 and 7 days, respectively). Equal numbers of the CD4+ and CD8+ cells were detected in the tumors after either rSLC or control DC treatment tumors, but a 2:1 CD4:CD8 ratio was present in those injected with DC-SLC. Preferential migration of CD4+ T cells in response to SLC has also been observed *in vitro* (28).

A strikingly different pattern of residing T-cell subsets was observed within the DLN. Twenty-four h after the first treatment, CD4+ and CD8+ T-cell numbers in the LNs, unlike those in treated tumor, were unchanged in groups receiving rSLC, control DC, or DC-SLC versus saline (Fig. 3, C and D). Treatment of tumors with DC-SLC resulted in a transient increase in LN cellularity at 4 days after treatment initiation. CD4+ (Fig. 3C) and CD8+ (Fig. 3D) cell numbers increased 3–4-fold during this course of time; however, T-cell numbers returned to the levels seen in saline-treated animals by 7 days after treatment. Interestingly, a delayed but sustained increase in LN cellularity was seen in mice receiving intratumoral injections of rSLC or control DC (Fig. 3, C and D). Collectively, our results suggest that direct administration of SLC-secreting DCs results in a rapid and sustained influx of T cells within the tumor while having only a transient effect on the cellularity of the DLN.

Table 1 *In vivo* migration of DCs from the intratumoral injection site to the DLN

PKH26-labeled DCs (1×10^6) were injected into 6-day-old s.c. tumors. DLNs were harvested 24 h later, stained with FITC-CD11c, and analyzed by flow cytometry. Labeled DCs present in LN samples were enumerated as described in "Materials and Methods" and presented as the percentage of injected cells. Each value represents the data from a single tumor-bearing animal.

DC-GFP	DC-SLC
0.23 ^a	2.51
0.17	0.17
0.29	0.15
0.01	0.16
0.01	0.09
0.14 ^b (0.06) ^c	0.62 (0.47)

^a Percentage of injected cells.

^b Mean.

^c SE.

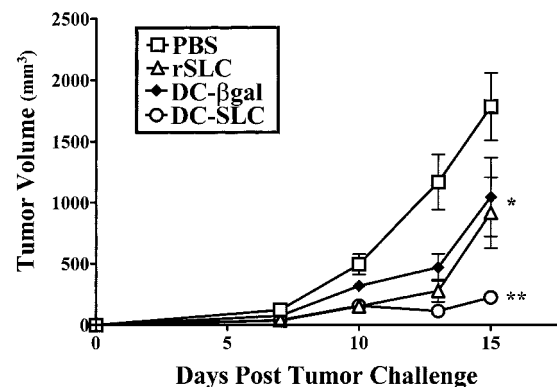


Fig. 2. Treatment of established B16 melanoma with rSLC and gene-modified DC. Tumors were established in B6 mice by s.c. injection with 1×10^5 viable cells. Six days after tumor challenge, mice were treated with daily injections of rSLC ($3 \mu\text{g}/\text{dose}$) from days 6–10 or 1×10^6 gene-modified DCs on days 6 and 9. Tumors were monitored for growth by perpendicular diameter measurement. Tumor volumes (means; bars, SE) are presented in mm^3 . *, $P < 0.05$ for DC-βgal and rSLC versus PBS; **, $P < 0.01$ for DC-SLC versus all other groups. $n = 6$ –10 mice/time point.

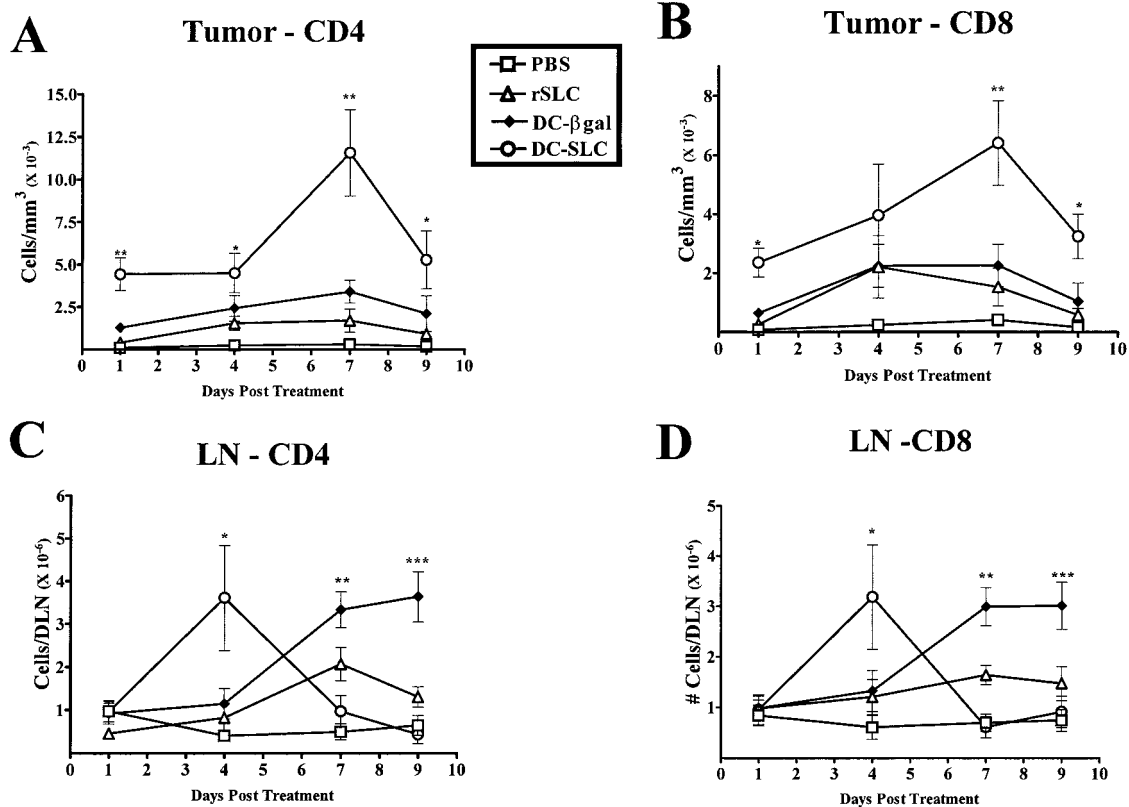


Fig. 3. Phenotypic analysis of T cells within tumor and DLN. Tumors (A and B) and DLNs (inguinal; C and D) were excised, and single-cell suspensions were prepared by enzymatic digestion (tumors) or mechanical disruption (LNs). Cells were stained for the presence of CD4+ (A and C) and CD8+ (B and D) T cells with PE-conjugated antibodies. Specific cell numbers were analyzed as described in "Materials and Methods" and are presented as cells/mm³ of tumor or per DLN. *, **, and ***, at least $P < 0.05$, $P < 0.01$, and $P < 0.001$ for DC-SLC versus all other groups, respectively (A and B). *, $P < 0.05$ for DC-SLC versus all other groups; ** and ***, $P < 0.01$ and $P < 0.001$ for DC-βgal versus DC-SLC and PBS, respectively (C and D). $n = 4-10$ mice/time point.

Tumor-infiltrating Lymphocytes Are Activated and Secrete IFN- γ . The question of the activation status and function of the tumor-infiltrating T cells in our experiments is raised by the chemotactic properties of SLC. SLC can induce the migration of naïve T cells as measured by their expression of CD62L (33). Indeed, after i.p. injection of rSLC, >80% of the T cells harvested from peritoneal exudate are CD62L+.⁵ Therefore, we determined whether the T-cell infiltration of tumors mediated by DC-SLC was comprised mainly of naïve CD4+ and CD8+ T cells by analyzing the excised tumors for the expression of CD62L. When tumors were analyzed 24 h after injection of DC-SLC, CD62L was expressed on 11.3% (± 2.5) and 48.3% (± 4.3) of CD4+ and CD8+ T cells, respectively (data not shown). Furthermore, there were no significant differences in the expression of CD62L on either T-cell subset in infiltrates isolated from control treated tumors or from tumors injected with control DCs when compared with DC-SLC treatment (data not shown). Therefore, although SLC is chemotactic for naïve T cells under certain circumstances, the majority of the enhanced tumor infiltration in response to DC-SLC is made up of T cells lacking expression of this naïve T-cell marker.

One explanation for the relatively low levels of CD62L expression on the tumor-infiltrating T cells isolated 24 h after injection of DC-SLC is that these lymphocytes have been activated prior to or upon their arrival at the tumor. We analyzed the infiltrating T cells for expression of CD25, which appears within 24 h of T-cell activation (34). DC-SLC-treated tumors contained significantly more "early" activated CD4+ and CD8+ T cells as determined by expression of

CD25 (Fig. 4). Expression of CD25 was found on ~30% of the CD4+ and CD8+ T cells in tumors treated with DC-SLC 24 h after the first injection of DCs and remained at this level until the last time point analyzed (Fig. 4). In the mice receiving control DC, rSLC, or saline, CD25 expression was also seen on ~30% of T cells 24 h after the first injection but had dropped to <15% of CD4+ and <10% of CD8+ T cells 3 days later. Furthermore, there was no significant increase in the number of activated CD4+ or CD8+ T cells in tumors of control DC- or rSLC-treated mice at the 24-h time point (Fig. 4). The decline in the relative expression of CD25 on T cells in the control DC-treated tumors is paralleled by an increase in the absolute number of activated T cells found in the DLN of these animals (data not shown).

We also analyzed the tumor-infiltrating lymphocytes for the production of IFN- γ . Our previous work had demonstrated a correlation between T-cell production of IFN- γ and antitumor therapeutic efficacy *in vivo* (35). To enumerate the number of effector T cells infiltrating the tumor, we assayed for the production of IFN- γ by flow cytometry (Fig. 5). Tumors isolated 7 and 9 days after the first injection of DCs were stained for the presence of intracellular IFN- γ in CD4+ and CD8+ T cells after stimulation with PMA and ionomycin for 5 h. In T cells isolated from DC-SLC-treated tumors, IFN- γ was expressed by ~25% of CD4+ T cells and ~30% of CD8+ cells (data not shown). Because we failed to detect IFN- γ -producing CD4+ (Fig. 5) and CD8+ (not shown) T cells in LNs of naïve animals upon stimulation with PMA and ionomycin, the cytokine-producing T cells within the tumor represent effector cells and not naïve cells. Using the same method for quantifying cell number as described above, we found that, in tumors treated with DC-SLC, the relative concentration

⁵ C. J. Kirk and J. J. Mulé, unpublished results.

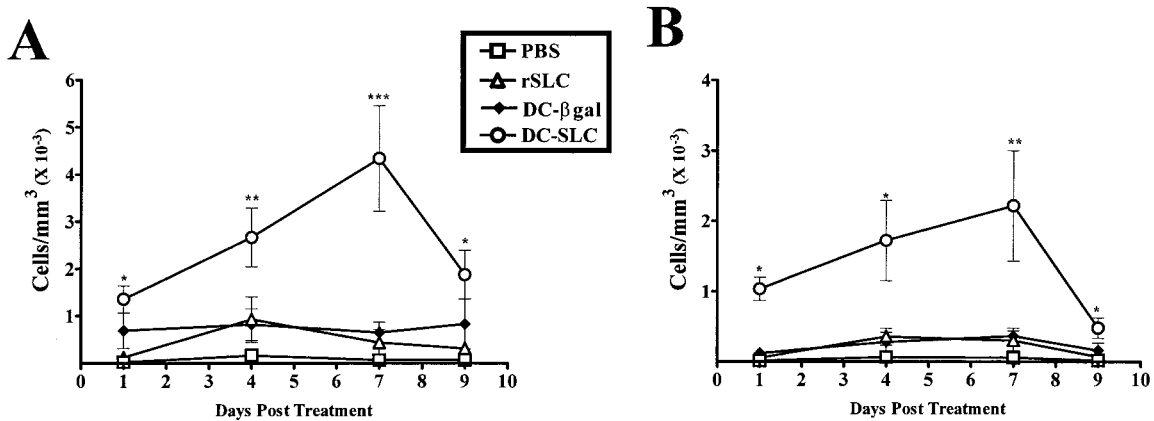


Fig. 4. Detection of recently activated T cells within treated tumors. Tumors were enzymatically digested and analyzed for the presence of CD25 in the CD4 (A) and CD8 (B) T-cell subsets with PE-conjugated anti-CD25- and Cy-Chrome-conjugated antibodies. Analysis was carried out in the CD11b⁺/B220⁺ population, and cell numbers were normalized to the tumor volume. *, **, and ***, at least $P < 0.05$, $P < 0.01$, and $P < 0.001$ for DC-SLC versus all other groups, respectively. $n = 4-6$ mice/time point; bars, SE.

of IFN- γ -producing cells was 3–5-fold higher than in control DC-treated tumors and up to 50-fold higher than in saline-treated tumors (Fig. 6). There was not a significant increase in the number of IFN- γ -secreting T-cells in mice treated with either control DC or rSLC. We did not detect an appreciable presence of IL-4-producing T cells in any tumors evaluated (data not shown). Taken together, these data suggest that treatment of tumors with DC-SLC results in the accumulation of activated, IFN- γ -producing effector cells at the tumor site.

T-Cell Priming Is Not Dependent on the Presence of a DLN.

One question that arises from our results is the specific location where the infiltrating T cells are primed. Because DC-SLC promotes T-cell influx within 24 h and a substantial portion of the cells display an activated phenotype, it is possible that these cells are primed within the tumor mass. Furthermore, because we detected only a transient increase in the cellularity of the DLN, it is also possible that the activated T-cells detected at 24 h differentiate within the tumor to become the IFN- γ -producing effectors at 7 days. To test these possibilities, we established s.c. B16 melanoma in both conventional B6

mice and those deficient in LT α (LT α ^{-/-}). LT α ^{-/-} mice lack all peripheral LNs and display an altered splenic architecture; however, these mice retain normal levels of circulating T lymphocytes (36). Indeed, we confirmed the lack of any LN structures upon necropsy. Tumor-bearing animals were treated with gene-modified DCs as described above on days 6 and 9, and tumors were harvested 24 h and 7 days after the initiation of treatment. As with B6 mice, treatment of tumors in LT α ^{-/-} mice with DC-SLC resulted in an influx of both CD4⁺ and CD8⁺ T cells within 24 h (data not shown). Furthermore, CD25 was expressed on similar levels of tumor-infiltrating CD4⁺ T cells in DC-SLC-treated tumors of LT α ^{-/-} and B6 mice ($17.8\% \pm 1.7$ and $16.6\% \pm 1.9$ for B6 mice and LT α ^{-/-} mice, respectively; data not shown).

We also analyzed tumors 7 days after the first treatment with gene-modified DCs to detect the presence of cytokine-secreting effector cells. Treatment of s.c. tumors in LT α ^{-/-} animals resulted in the expression of IFN- γ by both CD4⁺ and CD8⁺ T cells (Fig. 7). In B6 mice, $22.6\% \pm 0.7$ of CD4⁺ cells and $48.3\% \pm 11.1$ of CD8⁺ cells expressed IFN- γ . Of interest, similar percentages of T cells expressing

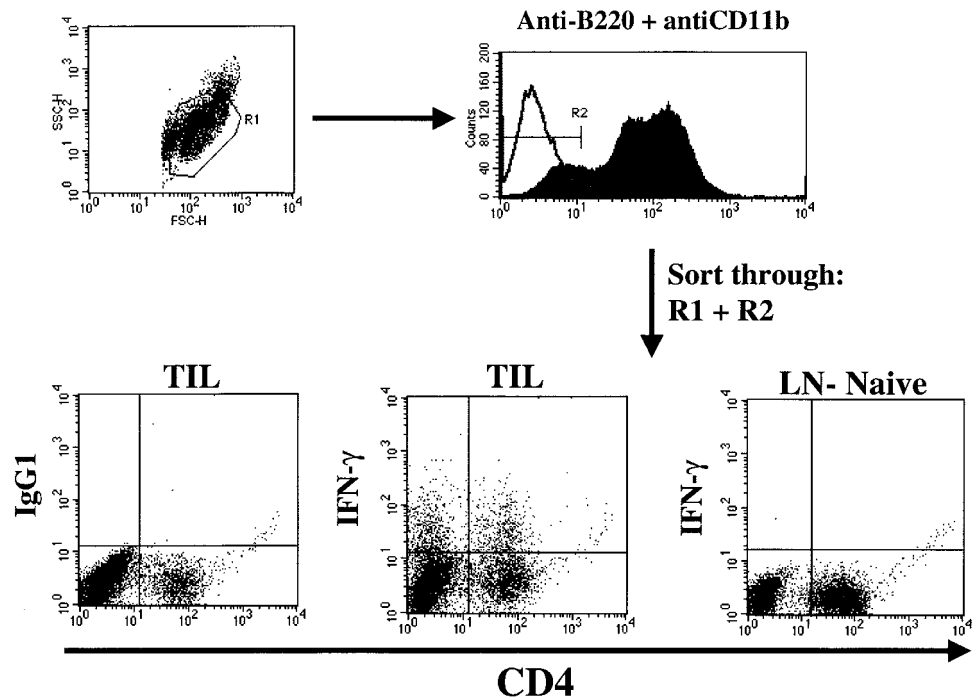


Fig. 5. Specificity of IFN- γ production by T cells derived from treated tumors. Tumors (TIL, tumor-infiltrating lymphocytes) or LNs from naïve mice (LN) were harvested 7 days after treatment with DC-SLC, and single-cell suspensions were generated as described in "Materials and Methods." Samples were stimulated for 5 h by PMA and ionomycin. Samples were stained with FITC-conjugated antibodies to CD11b and B220 (or isotype control, black line) and Cy-Chrome-conjugated CD4. Cells were fixed, permeabilized, and stained with PE-conjugated antibodies to IFN- γ (or isotype control, IgG1). Cells were analyzed through a lymphocyte-sized gate (R1) and for cells negative for CD11b/B220 staining (R2).

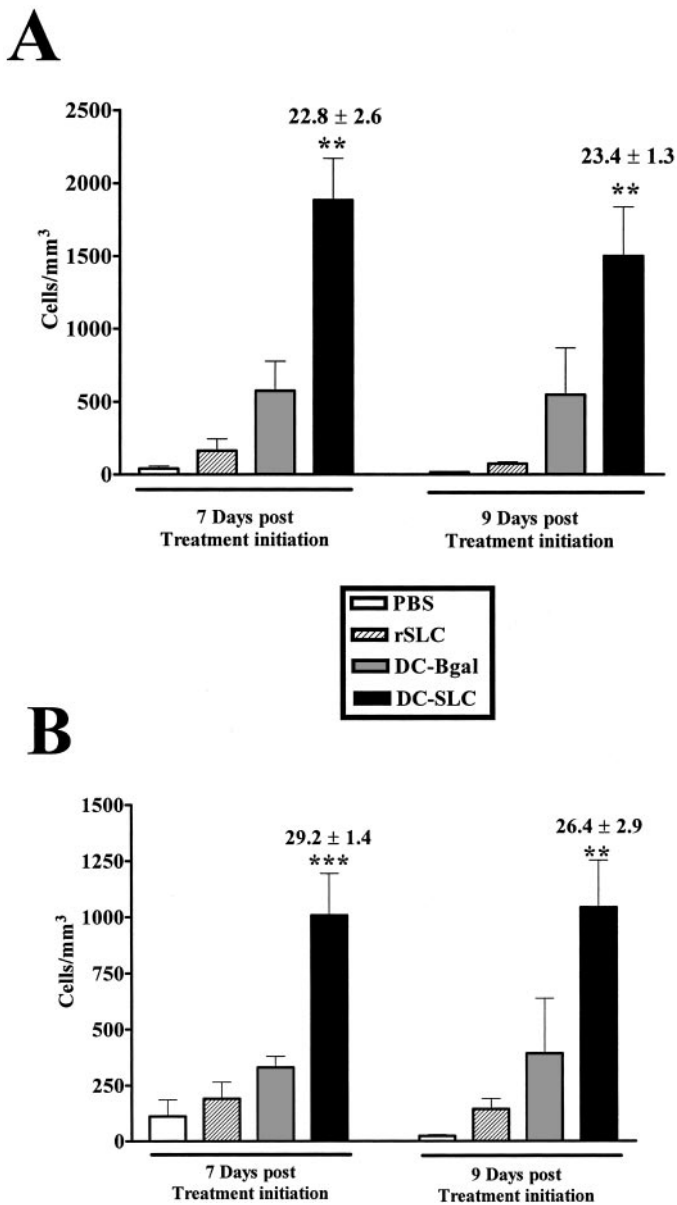


Fig. 6. Enumeration of the IFN- γ -producing T cells within treated tumors. Intracellular IFN- γ cytokine staining in the CD4 (A) and CD8 (B) subsets was performed on tumor samples as described in "Materials and Methods." Cell numbers were normalized to tumor volume and presented as cells/mm³. ** and ***, at least $P < 0.01$ and $P < 0.001$ for DC-SLC *versus* all other groups, respectively. $n = 4$ mice/time point. Numbers above columns for DC-SLC represent the percentage (bars, SE) of CD4+ (A) or CD8+ (B) cells producing IFN- γ .

this cytokine were also detected in DC-SLC-treated tumors of $Lt\alpha^{-/-}$ mice (18.0% \pm 1.5 and 40.3% \pm 3.3 of CD4+ and CD8+ T cells, respectively). Furthermore, DC-SLC-treated tumors in the $Lt\alpha$ gene knockout animals had a significant (*versus* saline) increase in the numbers of T cells present in the tumor at this time point (data not shown). Although, IFN- γ -producing cells were observed in tumors from saline-treated mice (Fig. 7), the small number of total T cells detected in these tumors likely renders the enumeration of cytokine production inaccurate.

Of particular note, DC-SLC treatment resulted in a significant inhibition of tumor growth in the $Lt\alpha^{-/-}$ mice that was comparable with conventional B6 mice. At 14 days after tumor challenge, tumors were 143.3 \pm 3.33 mm² and 151.7 \pm 7.27 mm² for saline-treated $Lt\alpha^{-/-}$ and B6 mice, respectively; treatment with DC-SLC resulted in

reduced tumor growth of 69.67 \pm 10.33 mm² in $Lt\alpha^{-/-}$ and 45.5 \pm 3.5 mm² in B6 animals ($P < 0.001$ for DC-SLC *versus* saline for both groups of mice). Taken together, our data demonstrate that treatment of s.c. B16 melanoma with DC-SLC results in the activation and differentiation of T cells into IFN- γ -secreting effectors in the absence of a DLN. Furthermore, inhibition of tumor growth by direct administration of DC-SLC does not require the presence of peripheral LNs.

The presence of DC-SLC, but not control DC, resulted in a significant influx of CD11c+ DCs within the tumor (Fig. 8). This influx of host-derived DCs was seen 24 h after injection of DC-SLC and was sustained throughout the course of the experiment (Fig. 7). The increase in the relative numbers of tumor-infiltrating DCs was attributable to the increased retention of DC-SLC (Fig. 1) and an influx of host-derived DCs because analysis of tumors treated with labeled DC-SLC contained unlabeled CD11c+ cells (data not shown). Therefore, the increase in lymph node cellularity mediated by treatment of tumors with control DC is not attributable to increased numbers of DCs migrating to the LN. Our data suggest that the DC-SLC induce an increase in T-cell infiltration to the tumor, in part through an increase in tumor retention of *ex vivo*-derived DCs and not through enhanced migration to the LN.

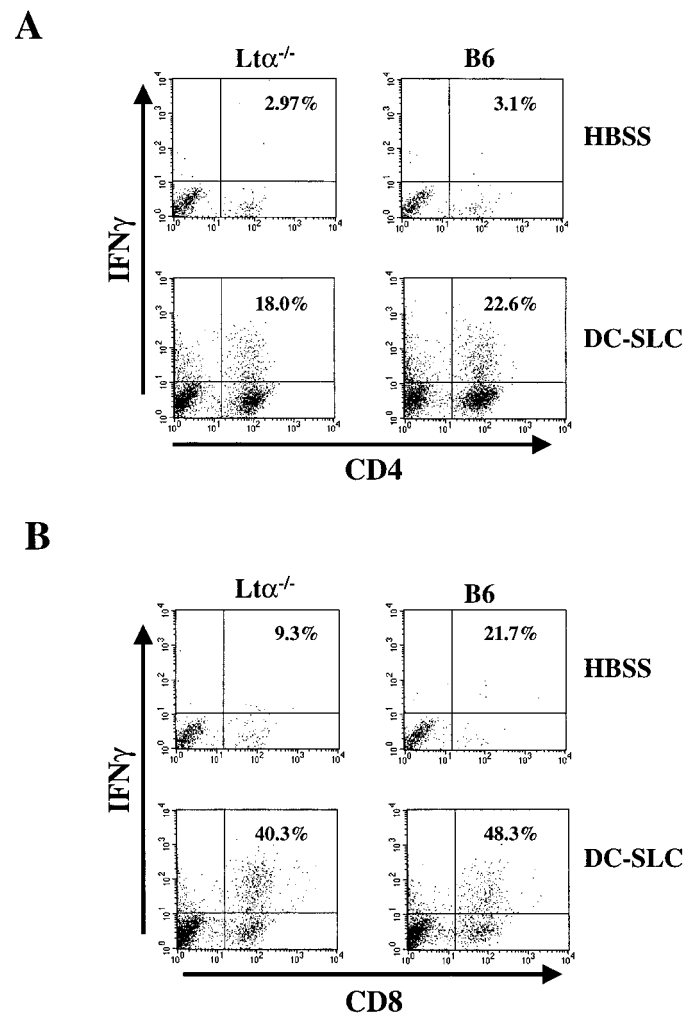


Fig. 7. Priming and differentiation of effector T cells within treated tumors in the absence of a LN. B16 tumors were established in either B6 or $Lt\alpha^{-/-}$ mice after injection of 1×10^5 tumor cells. Gene-modified DCs were given on days 6 and 9, and tumors were harvested on day 14 after tumor challenge. Intracellular staining for IFN- γ was performed as described for Fig. 4 for CD4+ (A) and CD8+ (B) T cells. The numbers in the upper right-hand quadrant of each dot plot represent the average percentage of T cells producing IFN- γ ($n = 3$ mice).

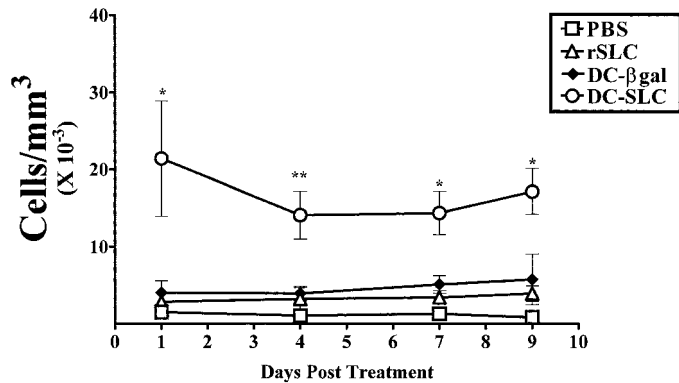


Fig. 8. Increased infiltration of DCs within treated tumors. Enzymatically digested tumors were analyzed for the presence of DCs using PE-conjugated anti-CD11c antibodies. Cell numbers were normalized to tumor volume and presented as cells/mm³. * and **, $P < 0.05$ and $P < 0.01$ for DC-SLC versus all other groups, respectively. $n = 4-6$ mice/group; bars, SE.

DISCUSSION

An implicit goal of treatment regimens designed to elicit an immune response against malignant cells is the migration of effector cells to the tumor mass. It is believed that the most effective immunotherapies against solid tumors will be those that result in a large, sustained tumor infiltration by tumor-specific effector cells. Indeed, in mouse models using T cells reactive to defined tumor antigens, tumor regression correlated with an early and sustained influx of CD8 T cells (37, 38). We report here the mechanisms underlying an effective antitumor treatment regimen based upon intratumoral delivery of DCs genetically modified to secrete the T-cell-attracting chemokine SLC. Intratumoral injections of SLC-secreting DCs promoted a rapid (*i.e.*, within 24 h), localized influx of T cells. Large numbers of these tumor-infiltrating T cells expressed an activated phenotype within 24 h and differentiated into IFN- γ -producing effector cells by 7 days. We also showed that increased T-cell numbers in the tumor was not associated with a sustained increase in the cellularity of the LN draining the tumor. In contrast, treatment of tumors with control DC, which elicited a modest antitumor effect, resulted in a substantial increase in the cellularity of the DLN but a far less pronounced T-cell infiltration into the tumor mass. These results suggest that increased T-cell numbers resulting from treatment of established tumor *per se* is not sufficient for maximum inhibition of tumor growth. In this treatment model, T cells must be localized within the tumor mass in sufficient numbers and be capable of effector function (*i.e.*, secretion of IFN- γ) to elicit the most potent antitumor response.

One question that arises from our results is the location of T-cell activation after treatment of tumors with DC-SLC. A tenet of immunology is that T-cell priming occurs in peripheral lymphoid organs such as LNs and the spleen. Naïve T cells interact with antigen-presenting DCs, via mutual attraction to CCR7 agonist chemokines, SLC and ELC, present in the LN (7). In our treatment model, we administered DCs genetically modified to secrete SLC directly into the tumor mass. When we analyzed the T cells for expression of the early activation marker CD25, we found that a significant proportion (~30%) of the infiltrating T cells expressed this activation marker (Fig. 4). Because the number and level of activation of T cells in the DLN did not change in the first 24 h of treatment with DC-SLC (Fig. 3, C and D), we conclude that the T cells are activated within the tumor mass and not in the DLN by migrating DCs. To test this model, DC-SLC was administered to tumors growing in $Lt\alpha^{-/-}$ mice that lack peripheral LNs (36). Even in the absence of a DLN, direct tumor administration with DC-SLC resulted in the presence of activated T

cells within 24 h and IFN- γ -secreting effector cells by 7 days. Of importance, these T-cell events corresponded with significant inhibition of tumor growth *in vivo*. Our data argue that the DC-SLC (and/or migrating endogenous DCs) are priming T cells directly within the tumor mass and are promoting their differentiation into cytokine-secreting effector cells. It remains plausible that DC-SLC are promoting the expansion of T cells activated during the first 6 days of tumor challenge. However, this possibility is unlikely because we did not detect cytokine-producing cells in the tumor or DLN of animals bearing 6-day tumors (data not shown), which argues that a detectable antitumor response was not mounted by this time point. It is also unlikely that the DC-SLC are selectively attracting and activating memory T cells, although a majority of the tumor-infiltrating lymphocytes have shed CD62L 24 h after DC administration. A more likely scenario, given the well-documented specificity of SLC for naïve T cells (3-5), is that the environment encountered by naïve T cells within the tumor results in signals leading to CD2L shedding (via direct antigen priming or bystander activation).

Because the DCs were not pulsed *ex vivo* with a source of TAA prior to administration, uptake and processing of these TAAs must have occurred within the tumor mass. Indeed, we have shown that dead or dying tumor cells within a growing mass can serve as a source of TAAs to DCs (23). It remains a possibility that a portion of the activated T cells represent adenoviral reactive cells primed by the gene-modified DCs. However, because the level of CD25 expression (~30% at 24 h) is equivalent between tumors treated with DC-SLC and rSLC, which contains no adenoviral components, we think that it is unlikely that antiviral T cells play a role in the antitumor response.

DC-SLC, but not control DC, mediated a significant inhibition in tumor growth in $Lt\alpha^{-/-}$ mice, suggesting that extranodal priming of T cells within the tumor is sufficient to mediate an effective antitumor response. To our knowledge, this is the first demonstration of T-cell priming outside a peripheral lymph node in the setting of a DC-based vaccine. Prior to this report, induction of a T-cell-mediated response of $Lt\alpha^{-/-}$ mice was demonstrable only after i.p. or i.v. administration of antigen, which likely involves splenic priming of T cells (39-41). Here, we demonstrate that T cells are capable of migrating to and being primed within s.c. tumor sites in response to SLC gene-modified DCs. Moreover, the choice of tumor model used in this study lends credence to the strength of the extranodal T-cell priming elicited by SLC-secreting DCs. B16 melanoma is a poorly immunogenic tumor, refractory to many treatment regimens that elicit potent antitumor responses against other tumor lines in B6 mice (42-44), which further underscores the significant T-cell antitumor response elicited by DC-SLC in the absence of regional LNs.

This model of extranodal T-cell activation is further supported by the fact that 40% of the injected DC-SLC remain in the tumor mass within the first 24 h after treatment, but only 0.6% migrate to the DLNs. Thus, the presence of large numbers of DCs (both host- and *ex vivo*-derived) within the tumor mass of DC-SLC-treated tumors should provide T cells with large numbers of functioning APCs capable of promoting T-cell expansion. Indeed, expression of CD25 by the accumulated T cells remains at 30% throughout the 9 days after treatment in DC-SLC-treated but not control DC- or rSLC-treated tumors, and significantly larger numbers of effector cells remained within the tumors almost 1 week after the last injection of DC-SLC. Extranodal priming may also play a role in the enhanced protection (*versus* control DC) from B16 tumor challenge in mice immunized with DC-SLC pulsed with the TAA TRP-2.⁶

The induction of large numbers of IFN- γ -producing T cells infil-

⁶ C. J. Kirk *et al.*, manuscript in preparation.

trating the regressing tumor mass in response to DC-SLC supports our previous findings that secretion of this cytokine is critical for eliciting an antitumor response *in vivo* (35). As with activation, we demonstrate that it is the presence of DC-SLC within the tumor and not in the DLN that is responsible for the induction of cytokine production by the tumor-infiltrating T cells. No significant differences in the number of IFN- γ -producing cells in tumor DLNs were seen between saline- and DC-SLC-treated tumors (data not shown), suggesting that the dramatic differences in cytokine-secreting T cells within the tumor is attributable to effector differentiation at that site. Because we did not detect IFN- γ -producing cells in the DLN prior to 7 days after treatment initiation, it is unlikely that the tumor-infiltrating T cells present at this time point represent recent migrants from the LN. It is not clear, however, what role, if any, host-derived DCs infiltrating in response to the SLC may play in intratumoral T-cell activation. Future studies with SLC gene-modified DCs derived from MHC class I or MHC class II knockout mice should allow for delineating the contributions played by injected DCs and endogenous, infiltrating DCs.

We believe that direct priming of tumor-reactive T cells by SLC-secreting DCs within the tumor mass has important clinical implications. As discussed above, the majority of DCs delivered intradermally or s.c. failed to migrate to regional LNs (25, 26). Indeed, the importance of LN migration by conventional DCs for the priming of naïve T cells has been underscored in an antigen-specific model (45). Therefore, much attention has been focused on manipulating DCs to enhance their migration to LNs to elicit optimal T-cell priming to specific antigen(s). For example, maturation of DCs with the tumor necrosis factor family members TRANCE and CD40L prior to s.c. administration was shown to improve LN migration (46, 47); nonetheless, <5% of these mature DCs was found in the regional LNs after vaccination. This study further underscores the difficulty in identifying strategies to manipulate *ex vivo*-generated DCs to effectively enhance their trafficking to LNs. Together with our previous report (28), our data demonstrate that expression of SLC by DCs can increase the effective number of DCs present in each immunization site as well as recruit lymphocytes from the periphery. By doing so, DCs can bypass the requirement for lymph node migration for effective priming of naïve T cells. It is also conceivable that direct intratumoral administration of DC-SLC might be used to more efficiently direct T cells administered systemically in adoptive transfer protocols, potentially improving their therapeutic efficacy. *In vivo* trafficking studies have shown that the majority of *ex vivo*-expanded, tumor-primed T cells fail to localize in tumor deposits upon systemic administration (48). Collectively, our data provide support for the first time that T cells can be primed and can differentiate into effector cells outside a defined lymphoid organ, and that the presence of an APC secreting a CCR7 agonist chemokine and a source of antigens (*i.e.*, the tumor mass) are sufficient for this extranodal priming.

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The Dynamics of the T-Cell Antitumor Response: Chemokine-secreting Dendritic Cells Can Prime Tumor-reactive T Cells Extranodally

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