

# Secondary Lymphoid Tissue Chemokine Mediates T Cell-Dependent Antitumor Responses In Vivo<sup>1</sup>

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Secondary lymphoid tissue chemokine (SLC, also referred to as Exodus 2 or 6Ckine) is a recently identified high endothelial-derived CC chemokine. The ability of SLC to chemoattract both Th1 lymphocytes and dendritic cells formed the rationale to evaluate this chemokine in cancer immunotherapy. Intratumoral injection of recombinant SLC evidenced potent antitumor responses and led to complete tumor eradication in 40% of treated mice. SLC-mediated antitumor responses were lymphocyte dependent as evidenced by the fact that this therapy did not alter tumor growth in SCID mice. Studies performed in CD4 and CD8 knockout mice also revealed a requirement for both CD4 and CD8 lymphocyte subsets for SLC-mediated tumor regression. In immunocompetent mice, intratumoral SLC injection led to a significant increase in CD4 and CD8 T lymphocytes and dendritic cells, infiltrating both the tumor and the draining lymph nodes. These cell infiltrates were accompanied by the enhanced elaboration of Th1 cytokines and chemokines monokine induced by IFN- $\gamma$  and IFN- $\gamma$ -inducible protein 10 but a concomitant decrease in immunosuppressive cytokines at the tumor site. In response to irradiated autologous tumor, splenic and lymph node-derived cells from SLC-treated tumor-bearing mice secreted significantly more IFN- $\gamma$ , GM-CSF, and IL-12 and reduced levels of IL-10 than did diluent-treated tumor-bearing mice. After stimulation with irradiated autologous tumor, lymph node-derived lymphocytes from SLC-treated tumor-bearing mice demonstrated enhanced cytolytic capacity, suggesting the generation of systemic immune responses. These findings provide a strong rationale for further evaluation of SLC in tumor immunity and its use in cancer immunotherapy. *The Journal of Immunology*, 2000, 164: 4558–4563.

Effective antitumor responses require both APC and lymphocyte effectors (1). Because tumor cells often have limited expression of MHC Ags and lack costimulatory molecules, they are ineffective APC (2). In addition, tumor cells produce immune inhibitory factors that promote escape from immune surveillance (3, 4). Consequently, it has been suggested that effective anticancer immunity may be achieved by recruiting professional host APC for tumor Ag presentation to promote specific T cell activation (5). Thus, chemokines that attract both DC and lymphocyte effectors to tumor sites could serve as potent agents in cancer immunotherapy.

Chemokines are a group of homologous yet functionally divergent proteins that directly mediate leukocyte migration and activation and play a role in regulating angiogenesis (6). Chemokines also function in maintaining immune homeostasis and secondary lymphoid organ architecture (7). Secondary lymphoid organ che-

mokine (SLC),<sup>3</sup> a CC chemokine expressed by high endothelial venules and in T cell zones of spleen and lymph nodes, strongly attracts naive T cells and mature dendritic cells (DC) (8–15). DC are uniquely potent APC involved in the initiation of immune responses (16). Serving as immune system sentinels, DC are responsible for Ag acquisition in the periphery and subsequent transport to T cell areas in lymphoid organs where they prime specific immune responses. SLC recruits both naive lymphocytes and Ag-stimulated DC into T cell zones of secondary lymphoid organs, colocalizing these early immune response constituents and culminating in cognate T cell activation (8). The importance of SLC in T cell homing was recently demonstrated by Gunn et al. (17). Mutant *plt/plt* mice that have a defective gene encoding SLC had impaired T cell homing to secondary lymphoid organs. SLC mediates its effects through two specific G protein-coupled seven-transmembrane domain chemokine receptors, CCR7 and CXCR3 (18, 19). Whereas CCR7 is expressed on naive T cells and mature DC, CXCR3 is expressed preferentially on Th1 cytokine-producing lymphocytes with memory phenotype (18, 19). Thus, we speculated that the capacity of SLC to facilitate the colocalization of both DC and T cells could reverse tumor-mediated immune suppression and orchestrate effective cell-mediated immune responses. A potentially effective pathway to restore Ag presentation is the establishment of a chemotactic gradient that favors localization of activated DC within the tumor site. We hypothesized that intratumoral injection of SLC would lead to chemoattraction of

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<sup>3</sup> Abbreviations used in this paper: SLC, secondary lymphoid organ chemokine; DC, dendritic cells; 3LL, Lewis lung carcinoma; VEGF, vascular endothelial growth factor; MIG, monokine induced by IFN- $\gamma$ ; IP-10, IFN- $\gamma$ -inducible protein 10; EIA, enzyme immunoassay.

mature DC and naive T cells to the tumor site and produce immune-dependent tumor reduction. In this study, utilizing two well-established lung cancer models, we show that intratumoral injection of SLC evidenced significant reductions in tumor volumes with complete tumor eradication in 40% of the mice.

## Materials and Methods

### Cell culture and tumorigenesis models

Two weakly immunogenic lung cancers, line 1 alveolar carcinoma (L1C2, H-2<sup>d</sup>) and Lewis lung carcinoma (3LL, H-2<sup>b</sup>), were utilized for assessment of antitumor responses *in vivo*. The cells were routinely cultured as monolayers in 25-cm<sup>2</sup> tissue culture flasks containing RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS (Gemini Bioproducts, Calabasas, CA), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2 mM glutamine (JRH Biosciences, Lenexa, KS) and maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. The cell lines were *Mycoplasma* free, and cells were utilized up to the tenth passage before thawing frozen stock cells from liquid N<sub>2</sub>. For tumorigenesis experiments, 10<sup>5</sup> 3LL or L1C2 tumor cells were inoculated by s.c. injection in the right suprascapular area of C57BL/6 or BALB/c mice, and tumor volume was monitored three times per week. Five-day-old established tumors were treated with intratumoral injection of 0.5 μg of murine recombinant SLC or PBS diluent (Pepro Tech, Rocky Hill, NJ) administered three times per week for 2 weeks. The endotoxin level reported by the manufacturer was <0.1 ng/μg (1 EU/μg) of SLC. The amount of SLC (0.5 μg) used for injection was determined by the *in vitro* biological activity data provided by the manufacturer. Maximal chemotactic activity of SLC for total murine T cells was 100 ng/ml. For *in vivo* evaluation of SLC-mediated antitumor properties, we utilized 5-fold more than this amount for each intratumoral injection. Tumorigenesis experiments were also performed in which equivalent amounts of murine serum albumin were utilized (Sigma, St. Louis, MO) as an irrelevant protein for control injections. Experiments were also performed in which the SLC was administered at the time of tumor inoculation. To determine the importance of the immune system in mediating antitumor responses after SLC administration, tumorigenesis experiments were conducted in SCID beige CB17 mice. SLC was administered s.c. at the time of tumor inoculation and then three times per week. CD4 and CD8 knockout mice were utilized to determine the contribution of CD4 and CD8 cells in tumor eradication. Two bisecting diameters of each tumor were measured with calipers. The volume was calculated using the formula (0.4 *ab*<sup>2</sup>), with *a* as the larger diameter and *b* as the smaller diameter.

### Cytokine determination from tumor nodules, lymph nodes, and spleens

The cytokine profiles in tumors, lymph nodes, and spleens were determined in both SLC and diluent-treated mice as previously described (4). Nonnecrotic tumors were harvested, cut into small pieces, and passed through a sieve (Bellco Glass, Vineland, NJ). Tumor-draining lymph nodes and spleens were harvested from SLC-treated tumor-bearing, control tumor-bearing, and normal control mice. Lymph nodes and spleens were teased apart, RBC depleted with double-distilled H<sub>2</sub>O, and brought to tonicity with 1× PBS. Tumor nodules were evaluated for the production of IL-10, IL-12, GM-CSF, IFN-γ, TGF-β, vascular endothelial growth factor (VEGF), monokine induced by IFN-γ (MIG), and IP-10 by ELISA and PGE<sub>2</sub> by enzyme immunoassay (EIA) in the supernatants after an overnight culture. Tumor-derived cytokine and PGE<sub>2</sub> concentrations were corrected for total protein by Bradford assay (Sigma, St. Louis, MO). For cytokine determinations after secondary stimulation with irradiated tumor cells (5 × 10<sup>6</sup> cells/ml), splenic or lymph node-derived lymphocytes were cocultured with irradiated 3LL (10<sup>5</sup> cells/ml) at a ratio of 50:1 in a total volume of 5 ml. After an overnight culture, supernatants were harvested and GM-CSF, IFN-γ, IL-12, and IL-10 determined by ELISA.

### Cytokine ELISA

Cytokine protein concentrations from tumor nodules, lymph nodes and spleens were determined by ELISA as previously described (20). Briefly, 96-well Costar (Cambridge, MA) plates were coated overnight with 4 μg/ml of the appropriate anti-mouse mAb to the cytokine being measured. The wells of the plate were blocked with 10% fetal bovine serum (Gemini Bioproducts) in PBS for 30 min. The plate was then incubated with the Ag for 1 h, and excess Ag was washed off with PBS-Tween. The plate was incubated with 2 μg/ml biotinylated mAb to the appropriate cytokine (PharMingen, San Diego, CA) for 30 min, and excess Ab was washed off with PBS-Tween. The plates were incubated with avidin peroxidase, and after incubation in OPD substrate to the desired extinction, the subsequent

change in color was read at 490 nm with a Microplate Reader (Molecular Dynamics, Sunnyvale, CA). The recombinant cytokines used as standards in the assay were obtained from PharMingen. IL-12 (Biosource) and VEGF (Oncogene Research Products, Cambridge, MA) were determined by kits according to the manufacturer's instructions. MIG and IP-10 were quantified by a modification of a double ligand method as previously described (21). The MIG and IP-10 Abs and protein were from R&D (Minneapolis, MN). The sensitivities of the IL-10, GM-CSF, IFN-γ, TGF-β, MIG, and IP-10 ELISA were 15 pg/ml. For IL-12 and VEGF, the sensitivities were 5 pg/ml.

### PGE<sub>2</sub> EIA

PGE<sub>2</sub> concentrations were determined using a kit from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's instructions as previously described (3). The EIA plates were read by a Molecular Dynamics Microplate Reader.

### Cytolytic experiments

Cytolytic activity was assessed as previously described (4). To quantify tumor cytotoxicity after a secondary stimulation with irradiated tumor cells, lymph node-derived lymphocytes (5 × 10<sup>6</sup> cells/ml) from SLC-treated and diluent tumor-bearing mice were cultured with irradiated 3LL (10<sup>5</sup> cells/ml) tumors at a ratio of 50:1 in a total volume of 5 ml. After a 5-day culture, the lytic capacity of lymph node-derived lymphocytes were determined against chromium-labeled (<sup>51</sup>Cr, Amersham Arlington, Heights, IL; sp. act. 250–500 mCi/mg) 3LL targets at varying E:T ratios for 4 h in 96-well plates. Spontaneous release and maximum release with 5% Triton X also were assessed. After the 4-h incubation, supernatants were removed and activity was determined with a gamma counter (Beckman, Fullerton, CA). The percent specific lysis was calculated by the formula: % lysis = 100 × (experimental cpm – spontaneous release)/(maximum release – spontaneous release).

### Flow cytometry

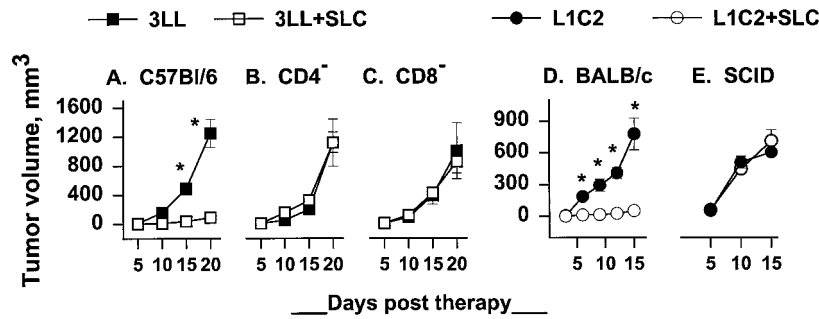
For flow cytometric experiments, two or three fluorochromes (PE, FITC, and Tri-color) (PharMingen) were used to gate on the CD3 T lymphocyte population of tumor nodule single-cell suspensions. DCs were defined as the CD11c and DEC 205 bright populations within tumor nodules and lymph nodes. Cells were identified as lymphocytes or DC by gating based on forward and side scatter profiles. Flow cytometric analyses were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) in the University of California, Los Angeles, Jonsson Cancer Center Flow Cytometry Core Facility. Between 5,000 and 15,000 gated events were collected and analyzed using Cell Quest software (Becton Dickinson).

### Intracellular cytokine analysis

T lymphocytes from single-cell suspensions of tumor nodules and lymph nodes of SLC-treated and diluent-treated 3LL tumor-bearing mice were depleted of RBC with distilled, deionized H<sub>2</sub>O and were evaluated for the presence of intracytoplasmic GM-CSF and IFN-γ. Cell suspensions were treated with the protein transport inhibitor kit GolgiPlug (PharMingen) according to the manufacturer's instructions. Cells were harvested and washed twice in 2% FBS-PBS. Cells (5 × 10<sup>5</sup>) cells were resuspended in 200 μl of 2% FBS-PBS with 0.5 μg FITC-conjugated mAb specific for cell surface Ags CD3, CD4, and CD8 for 30 min at 4°C. After two washes in 2% FBS-PBS, cells were fixed, permeabilized, and washed using the Cytofix/Cytoperm Kit (PharMingen) following the manufacturer's protocol. The cell pellet was resuspended in 100 μl Perm/Wash solution and stained with 0.25 μg PE-conjugated anti-GM-CSF and anti-IFN-γ mAb for intracellular staining. Cells were incubated at room temperature in the dark for 30 min, washed twice, resuspended in 300 μl PBS, 2% paraformaldehyde solution, and analyzed by flow cytometry.

## Results and Discussion

In this study, we tested the antitumor properties of SLC utilizing two murine lung cancer models. In both models, intratumoral SLC administration caused significant reduction in tumor volumes compared with diluent-treated tumor-bearing control mice (*p* < 0.01), and 40% of mice showed complete tumor eradication (Figs. 1, A and D). To determine whether the decrease in tumor volumes resulted from a direct effect of SLC on L1C2 and 3LL, the *in vitro* proliferation of the tumor cells was assessed in the presence of SLC. SLC (200 ng/ml) was added to 10<sup>5</sup> L1C2 and 3LL cells



**FIGURE 1.** SLC mediates antitumor responses in immune competent mice: requirement for CD4 and CD8 lymphocyte subsets. 3LL (H-2<sup>d</sup>) or L1C2 (H-2<sup>b</sup>) cells ( $10^5$ ) were inoculated s.c. into the right suprascapular area in C57BL/6 and BALB/c mice. Five days after tumor establishment, 0.5  $\mu$ g of murine recombinant SLC per injection or PBS diluent (1 $\times$ ) was administered three times per week intratumorally. Equivalent amounts of murine serum albumin was used as an irrelevant protein for control injections, and it did not alter the tumor volumes. Tumor volume was monitored three times per week ( $n = 10$ –12 mice/group). Intratumoral SLC administration led to significant reduction in tumor volumes compared with untreated tumor-bearing mice ( $p < 0.01$ ). In the SLC treatment group, 40% of mice showed complete tumor eradication (A and D). SLC-mediated antitumor responses are lymphocyte dependent as evidenced by the fact that this therapy did not alter tumor growth in SCID mice (Fig. 1E). Studies performed in CD4 and CD8 knockout mice also showed a requirement for both CD4 and CD8 effector subsets for SLC-mediated tumor regression (Fig. 1, B and C).

plated in 12-well Costar plates, and cell numbers were monitored daily for 3 days. SLC did not alter the in vitro proliferation rates of these tumor cells (data not shown).

To evaluate the role of host immunity in SLC-mediated antitumor responses, SLC was injected intratumorally in tumor-bearing SCID beige CB17 mice. SLC administration did not alter tumor volumes in SCID mice (Fig. 1E). Similarly, in CD4 and CD8 knockout mice, SLC failed to reduce tumor volumes, indicating that SLC-mediated antitumor responses were both CD4 and CD8 dependent (Fig. 1, B and C).

On the basis of previous reports indicating that tumor progression can be modified by host cytokine profiles (22, 23), we evaluated the cytokine production from tumor nodules after intratumoral SLC administration. The following cytokines were measured: VEGF, IL-10, PGE<sub>2</sub>, TGF- $\beta$ , IFN- $\gamma$ , GM-CSF, IL-12, MIG, and IP-10 (Table I). The production of these cytokines were evaluated for the following reasons. The tumor site has been documented to be an abundant source of PGE-2, VEGF, IL-10, and TGF- $\beta$ , and the presence of these molecules at the tumor site have been shown to suppress immune responses (3, 24, 25). VEGF, PGE<sub>2</sub>, and TGF- $\beta$  have also previously been documented to promote angiogenesis (26–28). Abs to VEGF, TGF- $\beta$ , PGE-2 and IL-10 have the capacity to suppress tumor growth in in vivo model systems. VEGF has also been shown to interfere with DC maturation (25). Both IL-10 and TGF $\beta$  are immune inhibitory cytokines that may potentially suppress Ag presentation and antagonize CTL generation and macrophage activities, thus enabling the tumor to escape immune detection (4, 24). Compared with tumor nodules from diluent-treated tumor-bearing controls, mice treated intratumorally with SLC had significant reductions of PGE<sub>2</sub> (3.5-fold), VEGF (4-fold), IL-10 (2-fold) and TGF- $\beta$  (2.3-fold) (Table I). An

overall decrease in IL-10 and TGF $\beta$  at the tumor site after SLC administration may have promoted Ag presentation and CTL generation. The decrease in VEGF and TGF- $\beta$  at the tumor site after SLC administration may have contributed to an inhibition of angiogenesis. In contrast, there was a significant increase in IFN- $\gamma$  (5-fold), GM-CSF (10-fold), IL-12 (2-fold), MIG (6.6-fold), and IP-10 (2-fold) after SLC administration (Table I).

Although IL-12 is a key inducer of type 1 cytokines, IFN- $\gamma$  is a type 1 cytokine that promotes cell-mediated immunity. Increases in IL-12 (2-fold) could explain the relative increase in IFN- $\gamma$  (5-fold) at the tumor site of SLC-treated mice (Table I). The tumor cells used for this study do not make detectable levels of IL-12 (data not shown). We therefore anticipate that macrophages and DC are the predominant sources of IL-12 at the tumor site.

MIG and IP-10 are potent angiostatic factors that are induced by IFN- $\gamma$  and may be responsible, in part, for IL-12-mediated tumor reduction (29–31). Hence, an increase in IFN- $\gamma$  at the tumor site of SLC-treated mice could explain the relative increase in MIG (6.6-fold) and IP-10 (2-fold) (Table I). Both MIG and IP-10 are chemotactic for stimulated CXCR3-expressing T lymphocytes, and this could also increase IFN- $\gamma$  at the tumor site (32). An increase in GM-CSF (10-fold) in the tumor nodules of SLC treated mice could enhance DC maturation and Ag presentation (16).

Based on the current results, it is not yet clear that the decrease in immunosuppressive cytokines and concomitant increase in type 1 cytokines is a direct effect of SLC on the cells resident within the tumor nodules. Alternatively, these changes could be a result of SLC-recruited T cells and DC. To begin to address this question, we evaluated the production of type 1 and immunosuppressive cytokines from tumor- and lymph node-derived cells in response to SLC in vitro. Tumor cells ( $1 \times 10^6$ ) or lymph node-derived cells

**Table I.** Intratumoral SLC administration promotes Th1 cytokine and antiangiogenic chemokine release and a decline in immunosuppressive mediators<sup>a</sup>

| Groups        | PGE <sub>2</sub> | VEGF          | IFN- $\gamma$ | GM-CSF      | IL-10         | IL-12       | TGF- $\beta$    | MIG              | IP-10               |
|---------------|------------------|---------------|---------------|-------------|---------------|-------------|-----------------|------------------|---------------------|
| 3LL + diluent | 28,510 $\pm$ 400 | 757 $\pm$ 26  | 104 $\pm$ 15  | <5          | 244 $\pm$ 40  | 24 $\pm$ 6  | 5,820 $\pm$ 578 | 1,000 $\pm$ 200  | 15,100 $\pm$ 1,100  |
| 3LL + SLC     | 8,737 $\pm$ 210* | 222 $\pm$ 34* | 549 $\pm$ 16* | 56 $\pm$ 11 | 112 $\pm$ 13* | 42 $\pm$ 1* | 2,473 $\pm$ 26* | 6,600 $\pm$ 100* | 39,400 $\pm$ 2,300* |

<sup>a</sup> Cytokine profiles in tumors were determined in mice treated intratumorally with SLC and compared with those in diluent-treated control mice bearing tumors. Nonnecrotic tumors were harvested, cut into small pieces, and passed through a sieve. Tumors were evaluated for the presence of IL-10, IL-12, GM-CSF, IFN- $\gamma$ , TGF- $\beta$ , VEGF, MIG, and IP-10 by ELISA and for PGE<sub>2</sub> by EIA in the supernatants after overnight culture. Cytokine, PGE<sub>2</sub>, and VEGF determinations from the tumors were corrected for total protein by Bradford assay. Results are expressed as picograms per milligram total protein/24 h. Compared with tumor nodules from diluent-treated tumor-bearing controls, mice treated intratumorally with SLC had significant reductions of PGE<sub>2</sub>, VEGF, IL-10, and TGF- $\beta$  but an increase in IFN- $\gamma$ , GM-CSF, IL-12, MIG, and IP-10. Experiments were repeated twice.

\*,  $p < 0.01$  compared with diluent-treated tumor-bearing mice,  $n = 6$  mice/group.

Table II. SLC increases the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte subsets secreting IFN- $\gamma$  and GM-CSF and CD11c + DEC205-expressing DC<sup>a</sup>

| Groups    | CD4 <sup>+</sup> |      |        |      | CD8 <sup>+</sup> |     |        |      | CD11c + DEC205 |      |
|-----------|------------------|------|--------|------|------------------|-----|--------|------|----------------|------|
|           | IFN- $\gamma$    |      | GM-CSF |      | IFN- $\gamma$    |     | GM-CSF |      | %              | MCF  |
|           | %                | MCF  | %      | MCF  | %                | MCF | %      | MCF  |                |      |
| Tumor     |                  |      |        |      |                  |     |        |      |                |      |
| 3LL       | 3.6              | 193  | 4.8    | 31   | 1.9              | 389 | 1.7    | 44   | 4              | 103  |
| 3LL + SLC | 7.4*             | 187  | 5.3    | 52*  | 3.2*             | 306 | 2.7*   | 47   | 13*            | 108  |
| LN        |                  |      |        |      |                  |     |        |      |                |      |
| 3LL       | 0.9              | 86   | 0.8    | 50   | 0.9              | 191 | 0.5    | 78   | 32             | 79   |
| 3LL + SLC | 1.7*             | 340* | 1.2*   | 181* | 1.7*             | 181 | 1.3*   | 107* | 40*            | 118* |

<sup>a</sup> Single-cell suspensions of tumor nodules and lymph nodes from SLC and diluent-treated tumor-bearing mice were prepared. Intracytoplasmic staining for GM-CSF and IFN- $\gamma$  and cell surface staining for CD4 and CD8 T lymphocytes were evaluated by flow cytometry. DC that stained positively for cell surface markers CD11c and DEC205 in lymph node and tumor nodule single-cell suspensions were also evaluated. Cells were identified as lymphocytes or DC by gating based on the forward and side scatter profiles; 15,000 gated events were collected and analyzed using Cell Quest software. Within the gated T lymphocyte population, intratumoral injection of SLC led to an increase in the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> cells secreting GM-CSF and IFN- $\gamma$  in the tumor nodules and lymph nodes compared with those of diluent-treated tumor-bearing control mice. Within the gated DC population, there was a significant increase in the frequency of DC in the SLC-treated tumor-bearing mice compared with the diluent-treated control tumor-bearing mice. For DC staining, MCF is for DEC205. MCF, mean channel fluorescence. Experiments were repeated twice.

\*,  $p < 0.01$  ( $n = 6$  mice/group).

( $5 \times 10^6$ ) were cocultured with SLC (200 ng/ml) for 24 h for cytokine determinations. SLC did not affect tumor cell production of VEGF, TGF- $\beta$ , IL-10, or PGE<sub>2</sub> (data not shown). Compared with the control untreated lymph node cells SLC significantly increased lymph node-derived IL-12 ( $288 \pm 15$  pg/ml vs  $400 \pm 7$  pg/ml) while decreasing IL-10 ( $110 \pm 5$  pg/ml vs  $67 \pm 1$  pg/ml), PGE<sub>2</sub> ( $210 \pm 4$  pg/ml vs  $70 \pm 2$  pg/ml), and TGF- $\beta$  ( $258 \pm 9$  pg/ml vs  $158 \pm 7$  pg/ml) production in an overnight in vitro culture. SLC did not alter lymph node-derived lymphocyte production of IFN- $\gamma$  and GM-CSF in vitro (data not shown). Because SLC is documented to have antiangiogenic effects (33, 34), the tumor reductions observed in these models may be due to T cell-dependent immunity as well as a participation by T cells in inhibiting angiogenesis (30). Further studies will be necessary to delineate the cell types and proteins critical for the decrease in immunosuppressive cytokines and the increase in type 1 cytokines after SLC administration.

To determine whether the increase in GM-CSF and IFN- $\gamma$  in the tumor nodules in response to SLC could be explained by an increase in the frequency of CD4 and CD8 T cell subsets secreting these cytokines, flow cytometric analyses were performed. CD3 T cells that stained positively for cell surface markers CD4 or CD8 were evaluated in single-cell suspensions from tumor nodules. In

the tumor nodules of SLC-treated mice, within the gated T lymphocyte population, there was a significant increase in the frequency of CD4 and CD8 T lymphocytes in comparison to diluent-treated mice (25 and 33% vs 15 and 11%, respectively;  $p < 0.01$ ). The GM-CSF and IFN- $\gamma$  profile of CD4 and CD8 T cells at the tumor sites and lymph nodes were determined by intracytoplasmic staining. SLC administration resulted in an increased frequency of CD4 and CD8 T lymphocytes from tumor nodules and lymph nodes secreting GM-CSF and IFN- $\gamma$  (Table II).

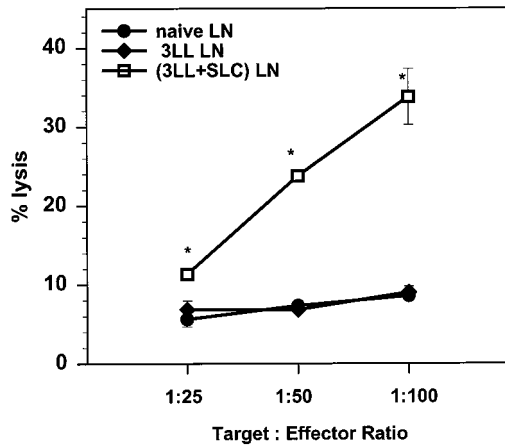
DC are uniquely potent APC involved in the initiation of immune responses, and it is well documented that SLC strongly attracts mature DC (10, 16). Because intratumoral SLC administration led to significant tumor reduction, we questioned whether intratumoral SLC administration led to enhanced DC infiltration of tumor nodules and lymph nodes. Single-cell suspensions of tumor nodules and lymph nodes from SLC and diluent-treated tumor-bearing mice were stained for the DC surface markers CD11c and DEC205. In the SLC-treated tumor-bearing mice, there was an increase in both the frequency and mean channel fluorescence intensities of DC for cell surface staining of CD11c and DEC205 in the tumor nodules and lymph nodes in comparison with diluent-treated 3LL tumor-bearing mice (Table II). These findings indicate

Table III. Specific systemic induction of type 1 cytokines and down-regulation of IL-10 after SLC treatment<sup>a</sup>

| Group                            | IFN- $\gamma$   | GM-CSF      | IL-10         | IL-12        |
|----------------------------------|-----------------|-------------|---------------|--------------|
| Splenocytes                      |                 |             |               |              |
| Mice without tumor, constitutive | 220 $\pm$ 30    | 0           | 0             | 78 $\pm$ 1   |
| Stimulated with 3LL              | 85 $\pm$ 19     | 37 $\pm$ 2  | 0             | 16 $\pm$ 3   |
| Diluent-treated, constitutive    | 117 $\pm$ 41    | 0           | 18 $\pm$ 1    | 60 $\pm$ 12  |
| Stimulated with 3LL              | 97 $\pm$ 13     | 23 $\pm$ 9* | 1566 $\pm$ 93 | 51 $\pm$ 1   |
| SLC-treated, constitutive        | 113 $\pm$ 21    | 0           | 16 $\pm$ 1    | 111 $\pm$ 1  |
| Stimulated with 3LL              | 2731 $\pm$ 99*  | 71 $\pm$ 2* | 181 $\pm$ 14* | 206 $\pm$ 7* |
| Lymph node                       |                 |             |               |              |
| Mice without tumor, constitutive | 248 $\pm$ 1     | 16 $\pm$ 1  | 0             | 60 $\pm$ 1   |
| Stimulated with 3LL              | 87 $\pm$ 12     | 34 $\pm$ 7  | 0             | 48 $\pm$ 1   |
| Diluent-treated, constitutive    | 166 $\pm$ 16    | 16 $\pm$ 1  | 297 $\pm$ 10  | 25 $\pm$ 1   |
| Stimulated with 3LL              | 96 $\pm$ 17     | 52 $\pm$ 8  | 816 $\pm$ 25  | 84 $\pm$ 6   |
| SLC-treated, constitutive        | 256 $\pm$ 10    | 19 $\pm$ 1  | 0             | 25 $\pm$ 1   |
| Stimulated with 3LL              | 1242 $\pm$ 270* | 49 $\pm$ 6  | 133 $\pm$ 17* | 108 $\pm$ 3* |

<sup>a</sup> Splenic or lymph node-derived lymphocytes ( $5 \times 10^6$  cells/ml) were cultured with irradiated 3LL ( $10^5$  cells/ml) tumors at a ratio of 50:1 in a total volume of 5 ml. After overnight culture, supernatants were harvested, and GM-CSF, IFN- $\gamma$ , IL-12, and IL-10 were determined by ELISA. After stimulation with irradiated tumor cells, splenocytes and lymph node-derived cells from SLC-treated mice secreted significantly enhanced levels of IFN- $\gamma$ , GM-CSF, and IL-12 but reduced levels of IL-10 compared with diluent-treated tumor-bearing mice. Results are expressed as picograms per milliliter. Experiments were repeated twice.

\*,  $p < 0.01$  compared with diluent-treated mice and SLC constitutive levels;  $n = 6$  mice/group.



**FIGURE 2.** Intratumoral SLC administration augments the cytolytic capacity of lymph node (LN)-derived lymphocytes. The cytolytic capacity of lymph node-derived lymphocytes from SLC-treated and diluent control tumor-bearing mice was determined after 1 week of stimulation with irradiated 3LL tumors. Lymph node-derived lymphocytes ( $5 \times 10^6$  cells/ml) were cultured with irradiated 3LL ( $10^5$  cells/ml) tumors at a ratio of 50:1 in a total volume of 5 ml. After a 5-day culture, the lymph node-derived lymphocytes cytolytic capacity was assessed against  $^{51}\text{Cr}$ -labeled 3LL tumor targets. After intratumoral SLC administration, the cytolytic capacity of LN-DC was significantly enhanced above that of lymphocytes from diluent-treated tumor-bearing mice. \*,  $p < 0.01$ .

that intratumoral SLC administration effectively recruited DC to the tumor site

We next asked whether intratumoral SLC administration could induce significant systemic immune responses. To address this question, lymph node and splenocytes from SLC and diluent-treated tumor-bearing mice were cocultured with irradiated tumor cells for 24 h, and GM-CSF, IFN- $\gamma$ , IL-10, and IL-12 levels were determined by ELISA. After secondary stimulation with irradiated tumor cells, splenocytes and lymph node-derived cells from SLC-treated tumor-bearing mice secreted significantly increased levels of IFN- $\gamma$  (13- to 28-fold), GM-CSF (3-fold spleen only) and IL-12 (1.3- to 4-fold). In contrast, IL-10 secretion was reduced (6- to 9-fold) in SLC-treated mice (Table III). Moreover, intratumoral SLC administration led to enhanced lymph node-derived lymphocyte cytolytic activity against the parental tumor cells (Fig. 2). We speculate that the phenotype of the effector cell population in the cytolytic experiments is CD8<sup>+</sup> T lymphocytes because SLC did not affect tumor growth in SCID mice. However, tumorigenesis experiments utilizing CD4 and CD8 knockout mice demonstrate the importance of both CD4 and CD8 T lymphocytes subsets for effective tumor reduction. Because CD4 T lymphocytes can also act as cytolytic effectors (35, 36), further studies will be required to delineate the role of CD4 T lymphocytes in SLC-mediated tumor reduction.

The results of this study indicate that intratumoral SLC administration leads to colocalization of both DC and T lymphocytes within tumor nodules and T cell dependent tumor rejection. These findings provide a strong rationale for further evaluation of SLC in tumor immunity and its use in cancer immunotherapy.

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