EBV-Induced Molecule 1 Ligand Chemokine (ELC/CCL19) Promotes IFN-γ-Dependent Antitumor Responses in a Lung Cancer Model

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The antitumor efficacy of EBV-induced molecule 1 ligand CC chemokine (ELC/CCL19) was evaluated in a murine lung cancer model. The ability of ELC/CCL19 to chemoattract both dendritic cells and T lymphocytes formed the rationale for this study. Compared with diluent-treated tumor-bearing mice, intratumoral injection of recombinant ELC/CCL19 led to significant systemic reduction in tumor volumes ($p < 0.01$). ELC/CCL19-treated mice exhibited an increased influx of CD4 and CD8 T cell subsets as well as dendritic cells at the tumor sites. These cell infiltrates were accompanied by increases in IFN-γ, MIG/CXCL9, IP-10/CXCL10, GM-CSF, and IL-12 but a concomitant decrease in the immunosuppressive molecules PGE_2 and TGFβ. Transfer of T lymphocytes from ELC/CCL19 treated tumor-bearing mice conferred the antitumor therapeutic efficacy of ELC/CCL19 to naive mice. ELC/CCL19 treated tumor-bearing mice showed enhanced frequency of tumor specific T lymphocytes secreting IFN-γ. In vivo depletion of IFN-γ, MIG/CXCL9, or IP-10/CXCL10 significantly reduced the antitumor efficacy of ELC/CCL19. These findings provide a strong rationale for further evaluation of ELC/CCL19 in tumor immunity and its use in cancer immunotherapy. *The Journal of Immunology,* **2003, 171: 6457–6465.**

Effective antitumor responses require both APCs and lymphocyte effectors (1). Although lung cancers express tumor Ags (2), they are ineffective as APCs (3) because tumor cells often have limited MHC Ag expression, defective TAP, and lack costimulatory molecules (4). In addition, tumor cells produce immune inhibitory factors that promote escape from immune surveillance (5, 6). Recruiting professional host APCs for tumor Ag presentation to promote specific T cell activation may also achieve anti-cancer immunity (7). A potentially effective pathway to restore Ag presentation is the establishment of a chemotactic gradient that favors localization of dendritic cells (DCs)[3] within the tumor site (8). Our efforts to produce an effective cancer immune therapy are therefore focused on methods to restore tumor Ag presentation by using chemokines that attract DCs and lymphocyte effectors to the tumor site.

EBV-induced molecule 1 ligand chemokine (ELC/CCL19), a CC chemokine expressed in T cell zones of spleen and lymph nodes, strongly attracts naive T cells and mature DCs (9–12). ELC/CCL19 mediates its effects through the specific G protein-coupled seven-transmembrane domain chemokine receptor, CCR7 that is expressed by naive T cells, DC, NK, and B cells (13). Based on the capacity of ELC/CCL19 to facilitate the colocalization of both DC and T cells, we speculated that intratumoral ELC/CCL19 administration might reverse tumor-mediated immune suppression and orchestrate effective cell-mediated immune responses. We hypothesized that intratumoral injections of ELC/CCL19 would lead to chemoattraction of DC and T cells to the source of the tumor Ags and induce immune-dependent tumor reduction. In this study, we show that intratumoral injection of ELC/CCL19 shows an IFN-γ-dependent reduction in tumor burden.

Materials and Methods

Animals

BALB/c female mice, 6-8 wk old, were obtained from Charles River Breeding Laboratories (Wilmington, MA) and maintained in the West Los Angeles Veteran's Administration Animal Research Facility, Los Angeles, CA (Association for Assessment and Accredidation of Laboratory Animal Care accredited). The institutional animal studies committee approved all experiments.

Reagents

Recombinant ELC/CCL19 was purchased from R&D Systems (Minneapolis, MN). The endotoxin level reported by the manufacturer was <0.1 ng/μg of ELC/CCL19. Murine serum albumin (Sigma-Aldrich, St. Louis, MO) was used as control injections. Murine Ab pairs and recombinant cytokines for GM-CSF, IFN-γ, TGFβ and IL-10 were obtained from BD PharMingen (San Diego, CA). IL-12 determination was performed with a kit from BioSource International (Camarillo, CA) according to the manufacturer's instructions. Ab pairs and recombinant MIG/CXCL9 (monokine induced by IFN-γ) and IP-10/CXCL10 (IFN-γ inducible protein 10) were obtained from R&D Systems (Minneapolis, MN). PGE_2 kit was obtained from Cayman Chemicals (Ann Arbor, MI). Quantitative ELISPOT for

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[3] Abbreviations used in this paper: DC, dendritic cell; ELC/CCL19, EBV-induced molecule 1 ligand CC chemokine; L1C2, murine line 1 alveolar lung tumor; EIA, enzyme immunoassay.

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IFN-γ was performed using a kit from BD PharMingen. For flow cytometry analyses, PE-conjugated Abs (CD3, CD8, CD25) or FITC-conjugated Abs (CD4 and CD11c) were obtained from BD PharMingen. CXCR3 was detected by rabbit-anti-CXCR3 (Zymed Laboratories, San Francisco, CA) and FITC-conjugated polyclonal anti-rabbit IgG (BD PharMingen). Polyclonal goat anti-murine MIG/CXCL9 and anti-murine IP-10/CXCL10 specific anti-serum were produced and characterized as previously described (14). Anti-mouse IFN-γ monoclonal (R4-462, American Type Culture Collection, Manassas, VA) neutralizing Ab was purified by affinity chromatography from *scid* mice ascites, which was generated 3–4 wk after i.p. injection of 10^6 R4-462 hybridoma cells per mouse (15).

Cell culture

Murine Line 1 alveolar lung tumor (L1C2) and WEHI cell lines were obtained from American Type Culture Collection. The cells were routinely cultured as monolayers in 75 cm^3 tissue culture flasks containing RPMI 1640 (Irvine) supplemented with 10% FBS (Gemini Bio-products, 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_2 in air. The cell lines were *Mycoplasma* free, and cells were used up to the tenth passage before thawing frozen stock cells from liquid N_2.

Tumorigenesis experiments

A total of 1.5×10^5 L1C2 tumor cells were injected s.c. in the right suprascapular area of BALB/c mice and tumor volume was monitored three times per week. Two bisecting diameters of each tumor were measured with calipers. The volume was calculated using the formula $V = 0.4ab$ (2), where a is the larger diameter and b is the smaller diameter. Five-day-old established tumors were treated with intratumoral injection of 0.5 μg of murine recombinant ELC/CCL19 or diluent administered three times a week for 2 wk. For the evaluation of ELC/CCL19 mediated systemic antitumor responses, L1C2 cells were inoculated subcutaneously in the right flank (1.5×10^5 cells) and left flank (2×10^4 cells). All treatments were administered intratumorally (0.5 μg) into the right flank three times a week.

In vivo cytokine neutralization

For in vivo neutralization experiments, 1.5×10^5 L1C2 tumor cells were inoculated by s.c. injection in the right suprascapular area of BALB/c mice. Five day established tumors were treated with an intratumoral injection of 0.5 μg of recombinant murine ELC/CCL19 or murine serum albumin (used as an irrelevant protein control for injections) and were administered three times per week for 2 wk. Twenty four hours before ELC/CCL19 treatment, and then three times a week, mice were injected i.p. with 1 ml/dose of anti-IP-10/CXCL10, or anti-MIG/CXCL9, or 100 μg/dose of purified monoclonal anti-IFN-γ or appropriate control Abs (goat IgG and rat IgG) at equivalent doses for the duration of the experiment. In response to these Abs, there was a significant reduction of the respective cytokines in vivo (see Fig. 6*B*). Tumor volumes were assessed three times per week. Cytokine profiles and splenocyte IFN-γ ELISPOT were performed 14 days after treatment.

Lymphocyte transfer

A total of 1.5×10^5 L1C2 tumor cells were inoculated by s.c. injection in the right suprascapular area of BALB/c mice. Five day established tumors were treated four times with intratumoral injection of 0.5 μg of recombinant murine ELC/CCL19 or murine serum albumin. For adoptive transfer experiments, T lymphocytes were isolated from spleens of ELC/CCL19 or diluent-treated tumor-bearing mice by Miltenyi Biotec beads (Auburn, CA) using the manufacturer's protocol and transferred (3.5×10^7) to naive mice 24 h before L1C2 tumor inoculation (1.5×10^5) and again 1 wk following tumor inoculation. Tumor volumes were assessed three times per week.

Flow cytometry

On day 12 post tumor inoculation, flow cytometric analyses of tumor nodules for T cell and DC markers were performed with a FACScan cytometer (BD Biosciences, San Jose, CA) in the University of California, Los Angeles, Jonsson Cancer Center Flow Cytometry Core Facility (Los Angeles, CA). Non-necrotic tumors were harvested, cut into small pieces in RPMI 1640, and passed through a sieve (Bellco Glass, Vineland, NJ). Tumor leukocytes were isolated by digesting tumor tissue in collagenase IV (Sigma-Aldrich) in RPMI 1640 for 30 min with stirring at 37ºC. A 10-ml syringe with a blunt-ended 16-gauge needle was used to break down the tissue further. The cell suspension was strained through a disposable plastic strainer (Fisher, Pittsburg, PA) to separate free lymphocytes from tissue matrix. The cells were pelleted at 2,000 rpm for 10 min and cell pellets washed twice to remove collagenase. Leukocytes were further purified using a discontinuous Percoll (Sigma-Aldrich) gradient, collecting at the 35–60% interface following centrifugation at 1,500 rpm for 20 min at 4ºC without brake. The collected cells were washed twice in PBS and stained for flow cytometric evaluation. Because we have Percoll-purified, the percentage of leukocytes in the cell population is approximately >95%. Cells were identified as lymphocytes or DCs by gating based on forward and side scatter profiles. $CD11c^+$ DCs were defined as the bright populations within tumor nodules. A total of 10,000-gated events were collected and analyzed using CellQuest software BD Biosciences). For staining, two or three fluorochromes (PE, FITC, PerCP) were used to gate on the CD3, CD4, and CD8 T lymphocyte population or $CD11c^+$ DC in single cell suspensions from tumor nodule. For CXCR3 expression, T cells were doubly stained for CD3 and CXCR3 cell surface markers.

Cytokine determination from tumor nodules

The cytokine profiles in tumors were determined in both ELC/CCL19 and diluent-treated mice as previously described (6). On day 12 post-tumor inoculation non-necrotic tumors were harvested, cut into small pieces, homogenized, and passed through a sieve (Bellco Glass). Tumor nodule homogenates were evaluated for the production of IL-10, IL-12, GM-CSF, IFN-γ, TGFβ, monokine induced by IFN-γ (MIG/CXCL9), and IP-10/CXCL10 by ELISA and PGE_2 by enzyme immunoassay (EIA) and results are expressed as picograms per milligram of total protein. Tumor-derived cytokine and PGE_2 concentrations were corrected for total protein by Bradford assay (Sigma-Aldrich).

Cytokine ELISA

Cytokine protein concentrations from tumor nodules were determined by ELISA as previously described (16). The plates were read at 490 nm with a Micro Plate (Amersham Biosciences, Sunnyvale, CA). MIG/CXCL9 and IP-10/CXCL10 were quantified by a modification of a double ligand method as previously described (17). The TGF-β ELISA measured TGF-β1. The sensitivities of the IL-10, GM-CSF, IFN-γ, TGFβ, MIG/CXCL9, and IP-10/CXCL10 ELISAs were 15 pg/ml. The sensitivity for IL-12 was 5 pg/ml.

PGE_2 EIA

PGE_2 concentrations were determined using a kit from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer's instructions as previously described (5). An Amersham Biosciences Micro Plate Reader read the EIA plates.

ELISPOT

To evaluate the specificity of the ELC/CCL19 treatment, IFN-γ ELISPOT assay was performed to determine the frequency of splenocyte producing IFN-γ in response to irradiated specific tumors. On day 14 post- tumor inoculation, spleens from ELC/CCL19 or diluent control treated mice were harvested, crushed, RBC depleted, filtered through a 10 μm filter (Millipore, Minneapolis, MN), and coincubated with either irradiated specific L1C2 cell line or the non-specific syngeneic WEHI cell line at a ratio of 10 lymphocyte effectors:1 stimulator for 24 h. A single cell suspension of L1C2 or WEHI tumor cells (10^6 cells/ml) was irradiated with 80 Gy of gamma irradiation in a ^{137}Cs gamma irradiator. Spots were quantified with an Immunospot Image Analyzer (Cellular Technologies, Cleveland, OH) at the University of California, Los Angeles Immunology Core Facility (Los Angeles, CA).

Histology

On day 12 post-tumor inoculation, non-necrotic tumors were isolated, placed in 10% formalin, and embedded in paraffin. Sections (5 μm) were prepared for H&E staining and histopathological examination.

Statistical analyses

Groups of 6–8 mice were used in each experiment. Statistical analyses of the data were performed using the unpaired two-tailed Student's *t* test for all significant data presented in the manuscript.

Results

ELC/CCL19 mediates potent systemic antitumor responses in vivo

The antitumor efficacy of ELC/CCL19 was evaluated in BALB/c mice with established L1C2 tumors. Intratumoral administration of

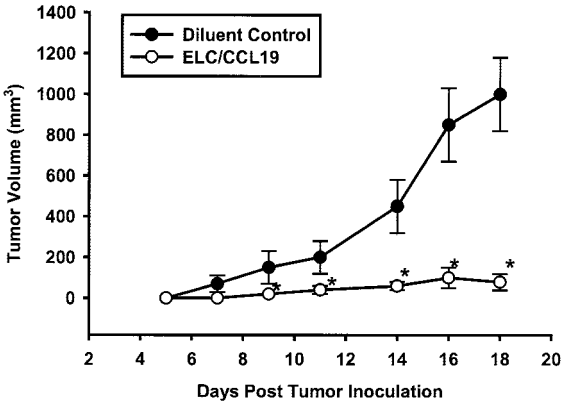

FIGURE 1. ELC/CCL19 has potent antitumor responses in vivo. A total of 1.5×10^5 L1C2 tumor cells were inoculated subcutaneously into the right suprascapular area in BALB/c mice. Five days after the tumor was established, mice were treated intratumorally with recombinant ELC/CCL19 (0.5 μg/dose/mouse, three times per week). Compared with diluent-treated controls, ELC/CCL19 therapy led to a significant reduction in tumor volumes (*, $p < 0.01$, $n = 8$ mice/group).

ELC/CCL19 (0.5 μg) three times per week caused a significant reduction in tumor volumes compared with diluent-treated tumor-bearing control mice (Fig. 1, $p < 0.01$). A comparison of the tumor growth curve slopes in the diluent vs ELC/CCL19 treated tumor bearing mice shows a 6-fold decrease in the tumor growth rate in the first week of ELC/CCL19 treatment. During the second week of ELC/CCL19 administration, there was a 19-fold reduction in tumor growth rate compared with the diluent control. To determine the extent of systemic antitumor responses generated as a result of intratumoral ELC/CCL19 injections, BALB/c mice were simultaneously inoculated with 10^5 L1C2 cells in the right flank and with 2×10^4 cells in the left flank. Therapeutic injections were administered in the right flank tumors only. Compared with diluent controls, ELC/CCL19 led to slowing in the growth rates of the bilateral tumors (Fig. 2, *A* and *B*, $p < 0.01$). In the bilateral tumor model, comparison of the tumor growth curve slopes in the diluent vs ELC/CCL19 treated tumor-bearing mice showed an approximate 3-fold overall decrease in the tumor growth rate on both the treatment as well as the contralateral side during ELC/CCL19 treatment.

ELC/CCL19 enhances frequency of T cell subsets and DC at the tumor site

H&E staining of tumors showed an enhanced mononuclear infiltration following intratumoral ELC/CCL19 therapy compared with control (Fig. 3*A*). Because ELC/CCL19 is chemotactic for both T cells and DCs, we hypothesized that intratumoral injections of ELC/CCL19 would elicit migration of these cell types to the tumor site. Flow cytometric evaluation of single cell suspensions of tumor nodules showed that compared to diluent treated controls ELC/CCL19 administration led to an increase in the frequency of $CD4^+$ (66%), $CD8^+$ (57%), and $CD3^+CXCR3^+$ (50%) T cells as well as the DCs expressing $CD11c^+$ (40%) infiltrating the tumors (Fig. 3*B*).

ELC/CCL19 therapy promotes type-1 cytokine and chemokine release as well as a decline in the immunosuppressive molecules TGFβ and PGE_2

On the basis of previous reports indicating that tumor progression can be modified by host cytokine profiles (18, 19), we evaluated the cytokine production from tumor sites. Cytokine profiles at the tumor sites of BALB/c mice treated with ELC/CCL19 were quantified. Compared with diluent-treated controls, the tumor sites of ELC/CCL19 treated mice had enhanced elaboration of IFN-γ (5-fold), MIG/CXCL9 (5-fold), IP-10/CXCL10 (5-fold), GM-CSF(4-fold), and IL-12 (5-fold), but a decrease in the immunosuppressive molecules TGFβ (2-fold) and PGE_2 (2-fold) (Fig. 4, *A* and *B*, $p < 0.05$).

ELC/CCL19 therapy induces specific T cell responses

To evaluate the specificity of the ELC/CCL19 treatment, IFN-γ ELISPOT assay was performed on splenocytes from ELC/CCL19

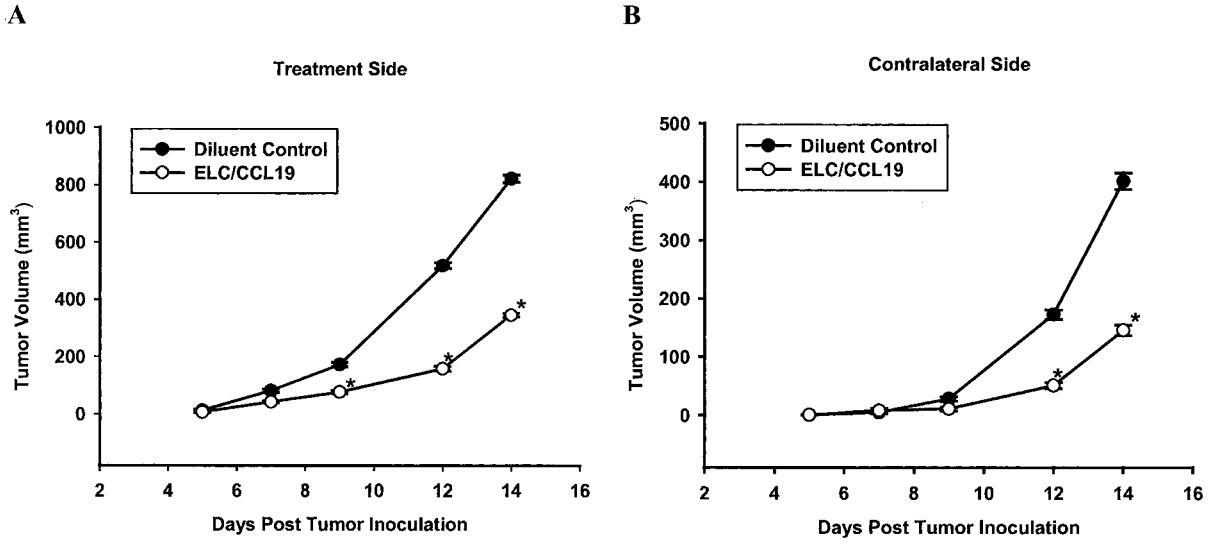

FIGURE 2. *A* and *B*, Intratumoral ELC/CCL19 leads to reduction in growth rates of bilateral tumors. A total of 1.5×10^5 L1C2 tumor cells were inoculated on the right suprascapular area and 2×10^4 L1C2 tumor cells on the left in BALB/c mice. Five days after tumors were established, mice were treated intratumorally with recombinant ELC/CCL19 (0.5 μg/dose/mouse, three times per week) on the right side only. Compared with diluent controls, ELC/CCL19 therapy led to systemic resolution of the bilateral tumors (*, $p < 0.01$, $n = 6$ mice/group).

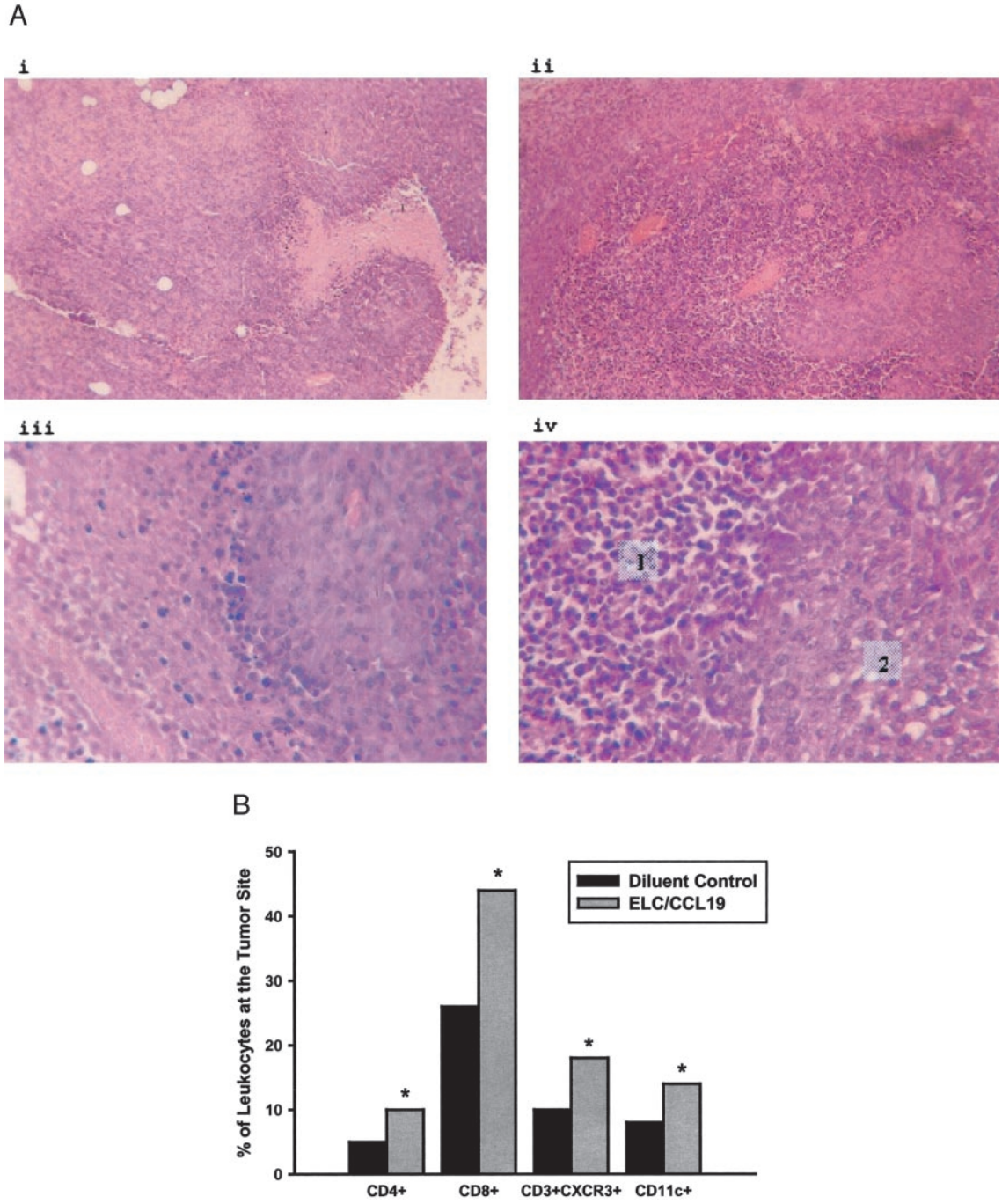

FIGURE 3. A. Intratumoral ELC/CCL19 therapy is accompanied by increased mononuclear infiltration. H&E staining of paraffin-embedded tumor sections (*Ai* and *Aii* ×100, *Aiii* and *Aiv* ×400 magnification). *Ai* and *Aiii*, diluent-treated control; *Aii* and *Aiv*, ELC-treated. Extensive mononuclear infiltration is evident in regressing tumors from ELC-treated mice. Minimal infiltration is evident within the tumor of the diluent-treated control mice (1. mononuclear infiltration, 2. tumor). *B*, ELC/CCL19 enhances influx of T cell subsets and DCs at the tumor site. Five-day-old established tumors were treated with intratumoral injection of 0.5 μg of murine recombinant ELC/CCL19 every other day for four days before tumors were harvested for flow cytometric analysis. Single cell suspensions of non-necrotic tumor nodules were prepared from ELC/CCL19 and diluent-treated mice. Cell surface staining or T cell markers CD4, CD8, and the chemokine receptor CXCR3 as well as the DC marker CD11c were evaluated by flow cytometry. Cells were identified as lymphocytes or DC by gating on the forward and side scatter profiles; 10,000 gated events were collected and analyzed using CellQuest software. Within the gated T lymphocyte population, intratumoral injection of ELC/CCL19 led to an increase in the frequency of $CD4^+$, $CD8^+$, and $CXCR3^+$ events compared with the diluent control (*, $p < 0.001$). Within the gated DC population, intratumoral injection of ELC/CCL19 led to an increase in the frequency of $CD11c^+$ events compared with the diluent control (*, $p < 0.001$) ($n = 8$ mice/group).

and diluent treated tumor-bearing controls. On day 14 post-tumor inoculation, splenocytes were restimulated overnight with irradiated autologous L1C2 or irrelevant syngeneic WEHI control tumors at a ratio of 10:1. Splenocytes from tumor bearing mice treated with ELC/CCL19 had significantly increased frequency of tumor specific IFN-γ producing cells ($p < 0.01$). There were minimal responses to the control syngeneic tumor WEHI (Fig. 5).

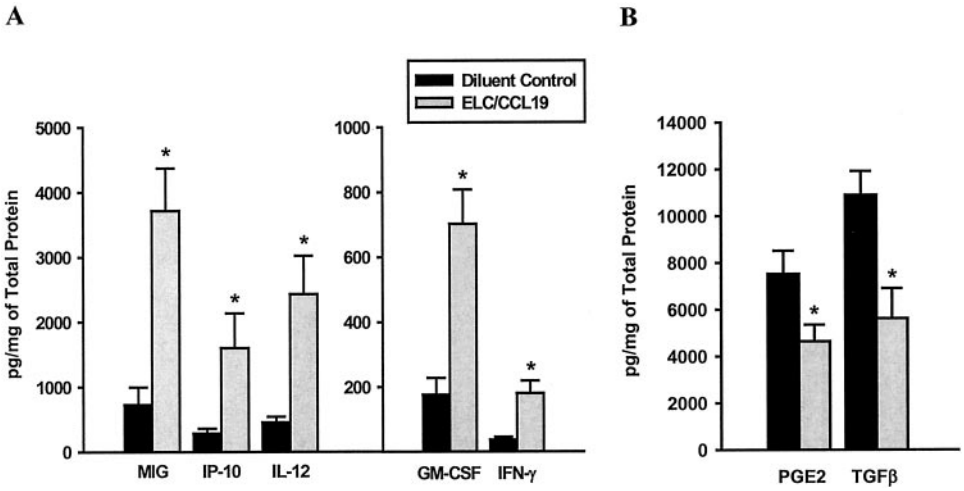

FIGURE 4. *A* and *B*, ELC/CCL19 therapy leads to an induction of Th1 cytokines and a decrease in immunosuppressive molecules. Five day-old established tumors were treated with intratumoral injection of 0.5 μg of murine recombinant ELC/CCL19 every other day for 4 days before tumors were harvested for cytokine analysis. Non-necrotic tumors were harvested, cut into small pieces, homogenized and passed through a sieve. Tumors homogenates were evaluated for the presence of GM-CSF, IFN-γ, MIG/CXCL9, IP-10/CXCL10, IL-12, and TGF-β by ELISA and PGE_2 by EIA. The cytokine and PGE_2 measurements were normalized to total protein determined in the homogenates by the Bradford kit from Sigma-Aldrich. Results are expressed as picogram per milligram of total protein. Compared with tumor nodules from the control group, mice treated intratumorally with ELC/CCL19 had significant increase in GM-CSF, IFN-γ, MIG/CXCL9, IP-10/CXCL10, and IL-12, but a decrease in the immunosuppressive molecules PGE_2 and TGFβ ($*$, $p < 0.01$ compared with control tumor group, $n = 6$ mice/group).

ELC/CCL19 mediated antitumor responses require IFN-γ, MIG/CCL9 and IP-10/CCL10

To determine the importance of MIG/CXCL9, IP-10/CXCL10, and IFN-γ in the ELC/CCL19 mediated antitumor response, these cytokines were depleted in ELC/CCL19 treated mice. Anti-IP-10/CXCL10 and anti-MIG/CXCL9 each partially yet significantly inhibited whereas anti-IFN-γ completely inhibited the antitumor efficacy of ELC/CCL19 (Fig. 6*A*, $*$, $p < 0.01$ compared with the control Ab group). Neutralization of IFN-γ caused a significant decrease in both MIG/CXCL9 and IP-10/CXCL10 consistent with previous studies indicating that these chemokines are largely IFN-γ dependent (20). Thus, an increase in IFN-γ at the tumor site of ELC/CCL19 treated mice could explain the relative increases in IP-10/CXCL10 and MIG/CXCL9. The converse was also observed; IFN-γ production at the tumor site was found to be MIG/CXCL9- and IP-10/CXCL10-dependent as indicated by the fact that neutralization of these cytokines caused a significant decrease in IFN-γ (Fig. 6*B*). Neutralization of any one of these cytokines caused a concomitant decrease in all three cytokines, thus indicating that IFN-γ, MIG/CXCL9, and IP-10/CXCL10 are interdependent in the ELC/CCL19 mediated antitumor responses. In addition, neutralization of any one of these cytokines in vivo led to a decrease in the frequency of tumor specific T cells producing IFN-γ (Fig. 6*C*).

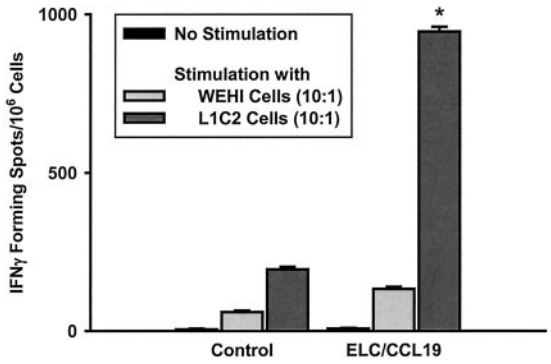

FIGURE 5. ELC/CCL19 therapy induces specific T cell response. Ag specific T cell response was determined by ELISPOT in tumor-bearing mice treated with ELC/CCL19. On day 14, splenocytes were re-stimulated overnight with irradiated L1C2 cells at a ratio of 10:1. Mouse IFN-γ specific ELISPOT was performed and spots quantified with an Immunospot Image Analyzer. Splenocytes from ELC/CCL19 treated mice had a significantly greater frequency of specific T cells releasing IFN-γ when re-stimulated with irradiated L1C2 cells ($*$, $p < 0.01$). There were minimal responses to the syngeneic control tumor WEHI.

T lymphocytes from ELC/CCL19 treated mice transfer the antitumor therapeutic efficacy to naive mice

To determine whether the therapeutic efficacy of ELC/CCL19 could be transferred, 3.5×10^7 T cells from ELC/CCL19 treated tumor bearing mice were administered to naive mice one day before and 1 wk following tumor challenge. Transfer of T lymphocytes from ELC/CCL19 treated tumor-bearing mice conferred the therapeutic efficacy of ELC/CCL19 to naive mice challenged with tumors and caused a significant reduction in tumor growth rate compared with control transfer (Fig. 7).

Discussion

Host APCs are critical for the cross-presentation of tumor Ags (1). However, tumors have the capacity to limit APC maturation, function, and infiltration of the tumor site (3, 21–23). Thus molecules that attract host APCs and T cells could serve as potent agents for cancer immunotherapy. We have previously shown that intratumoral injection of recombinant SLC/CCL21 mediated a T cell dependent antitumor response in vivo (8). Because both SLC/CCL21 and ELC/CCL19 mediate their effects through the specific G protein-coupled seven-transmembrane domain chemokine receptor CCR 7 (13) and are important for colocalization of DCs and T cells for the T cell immune response (24), we determined the antitumor efficacy of recombinant ELC/CCL19 and the effector molecules important for mediating these responses.

ELC/CCL19 produced by a subset of DCs, and possibly by other non-lymphoid cells, in T cell areas of lymphoid tissue (25) strongly attracts naive T cells and DCs (9–12). Because DCs are

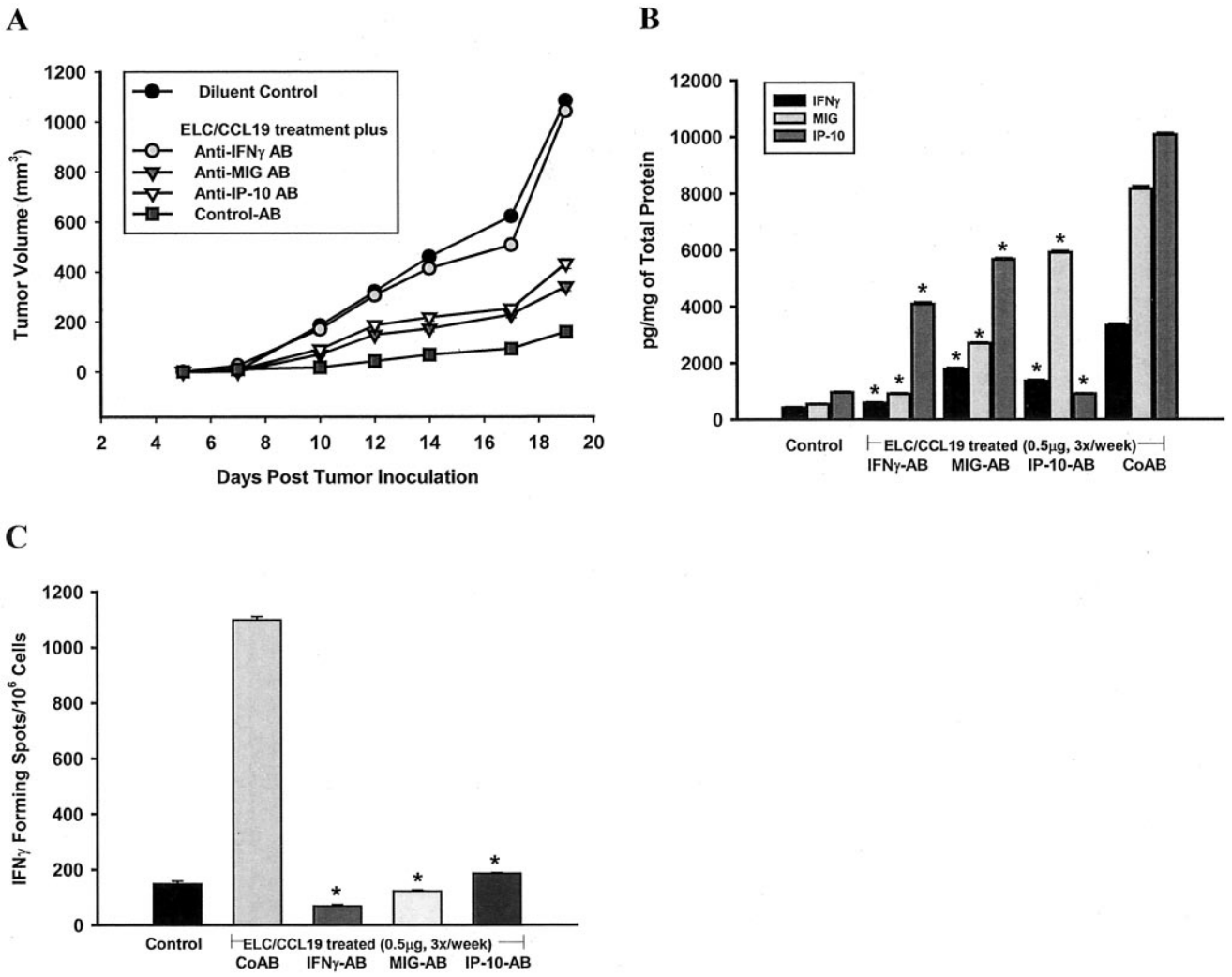

FIGURE 6. *A–C,* ELC/CCL19-mediated antitumor responses require IFN-γ, MIG/CXCL9 and IP-10/CXCL10. *A,* A total of 1.5×10^5 L1C2 tumors were implanted in BALB/c mice. Five days following tumor implantation, mice were treated intratumorally with recombinant murine ELC/CCL19 (0.5 μg) three times per week. One day before ELC/CCL19 administration, mice were given the respective cytokine Ab by i.p. injection. The Abs were administered three times per week. Abs to: IP-10/CXCL10, MIG/CXCL9 partially whereas IFN-γ completely inhibited the antitumor efficacy of ELC/CCL19 ($p < 0.01$ for anti-IL-10/CXCL10 and anti-MIG/CXCL9, $p < 0.001$ for anti-IFN-γ compared with the control Ab treated group) ($n = 8$ mice per group). *B,* ELC/CCL19 treated mice had a significant induction in IFN-γ, MIG/CXCL9 and IP-10/CXCL10 at the tumor site compared with diluent-treated control tumor bearing mice ($p < 0.001$). Assessment of cytokine production at the tumor site of ELC/CCL19 treated mice receiving anti-IFN-γ, anti-MIG/CXCL9 and anti-IP-10/CXCL10 showed an interdependence of IFN-γ, MIG/CXCL9 and IP-10/CXCL10: neutralization of any one of these cytokines in vivo caused a concomitant decrease in all three cytokines (*, $p < 0.01$ compared with the control Ab treated group). Results are expressed as picograms per milligram of total protein. Total protein was determined by the Bradford assay ($n = 8$ mice per group). *C,* ELC/CCL19 treated mice had a significant induction in IFN-γ producing tumor specific T cells compared with diluent-treated control tumor bearing mice ($p < 0.001$). Assessment of T lymphocytes producing IFN-γ from ELC/CCL19 treated mice receiving anti-IFN-γ, anti- MIG/CXCL9, and anti-IP-10/CXCL10 showed an interdependence of IFN-γ, MIG/CXCL9, and IP-10/CXCL10:neutralization of any one of these cytokines in vivo caused a decrease in tumor specific T cells producing IFN-γ (*, $p < 0.01$ compared with the control Ab treated group). There were minimal responses to non-specific tumors (data not shown) ($n = 8$ mice per group).

potent APCs that function as principal activators of T cells (24), the capacity of ELC/CCL19 to facilitate the colocalization of both DC and T cells may reverse tumor-mediated immune suppression and orchestrate effective cell-mediated immune responses. The expression of ELC/CCL19 in a breast cancer cell line mediated rejection of the transduced tumor through a mechanism involving NK and $CD4^+$ cells (26). Recent studies have shown that ELC/CCL19 released from polymer rods entrapped migratory Langerhans cells. The Langerhans cells, when loaded with tumor-associated Ags in situ, led to potent CTL activities and protective antitumor immunity (27). Nomura et al. (28) showed an enhancement of anti-tumor immunity by tumor cells transfected with ELC/CCL19 and stromal cell-derived factor-1α chemokine genes. Based on these properties, we speculated that ELC/CCL19 would be an important protein for evaluation in cancer immunotherapy.

The antitumor activity of ELC/CCL19 was determined in a transplantable model for lung cancer by injecting recombinant ELC/CCL19 intratumorally. The efficacy of injecting immune stimulators intratumorally for the treatment of cancer has been demonstrated in recent studies; intratumoral injection of recombinant SLC/CCL21 evidenced potent antitumor responses in murine lung cancer models (8, 29). In experimental models, DC injection into tumor masses has antitumor activity against micro metastases. However, if ex vivo generated DCs are genetically modified to express IL-12 (30, 31), IL-7 (7), CD40L (32), or IL-2 (33), they are highly efficacious against malignant tumors and elicit specific

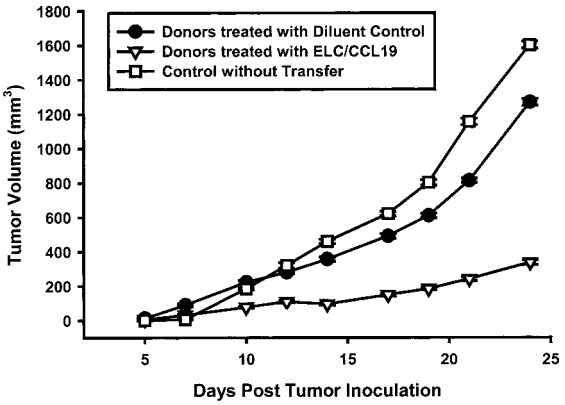

FIGURE 7. T lymphocytes from ELC/CCL19-treated mice transfer the antitumor therapeutic efficacy to naive mice. Intravenous transfer of splenic T lymphocytes from ELC/CCL19-treated mice to naive mice limited tumor growth. Splenic T lymphocytes from ELC/CCL19 or diluent-treated mice (3.5×10^7 cells) tumor-bearing mice were transferred to naive mice 24 h before and 1 wk following tumor challenge. Transfer of T lymphocytes from ELC/CCL19-treated tumor-bearing mice transferred the antitumor therapeutic efficacy of ELC/CCL19 to naive mice and caused a significant reduction in tumor growth rate compared with control transfer ($p < 0.01$ between the diluent and ELC/CCL19 transfer, $n = 8$ mice/group).

CTLs. Our rationale for injecting ELC/CCL19 intratumorally was to colocalize DCs and T cells at the source of tumor Ags where they can prime specific antitumor immune responses. This approach harnesses DCs to initiate antitumor immunity by using chemokine to attract endogenous DCs to tumors. Thus this strategy circumvents the ex vivo manipulation of DCs and tumor cells or tumor Ags. In many clinical situations access to intratumoral injection is achievable. The induction of an Ag-specific response requires the activation of naïve T lymphocytes by APCs bearing cognate Ag. T cell priming is thought to occur only in specialized compartments (i.e., secondary lymphoid organs such as the spleen and LNs). However, recent work by Kirk et al. (34) has shown that intratumoral administration of SLC gene-modified DCs are readily infiltrated by host-derived T cells and these T cells were primed within the tumor mass as measured by expression of activation markers and the cytokine IFN-γ. In addition, the data suggested that T cell priming and the generation of functional antitumor effector cells could occur in the absence of functional lymph nodes. Our results in this study show that this approach is effective in generating systemic antitumor responses. ELC/CCL19 injected intratumorally evidenced potent systemic antitumor responses. The reduced tumor burden in ELC/CCL19 treated mice was accompanied by extensive lymphocyte as well as DC infiltrates of the tumor sites.

The cytokine production at the tumor site was altered as a result of ELC/CCL19 therapy. The following cytokines were measured: PGE_2, TGFβ, IFN-γ, GM-CSF, IL-12, MIG/CXCL9, and IP-10/CXCL10. These cytokines were evaluated for the following reasons: the tumor site has been documented to be an abundant source of PGE_2 and TGFβ that have been shown to suppress immune responses (5, 35) and to promote angiogenesis (36, 37). Abs to TGFβ and PGE_2 suppress tumor growth in in vivo model systems (38, 39). TGFβ is known to suppress Ag presentation, antagonize CTL generation, and macrophage activation (35). ELC/CCL19-treated tumor-bearing mice showed significant reductions in PGE_2 and TGFβ at the tumor sites. Thus possible benefits of an ELC/CCL19-mediated decrease in these molecules include promotion of Ag presentation and CTL generation (35), as well as a limitation of angiogenesis (36, 37).

Apart from a decrease in TGFβ and PGE_2, the tumor sites of ELC/CCL19 treated mice revealed significant increases in IFN-γ, IL-12, IP-10/CXCL10, MIG/CXCL9, and GM-CSF. It is well documented that successful immunotherapy shifts tumor-specific T cell responses to a type-1 cytokine profile (40). Both IL-12 and IFN-γ mediate a range of biological effects that facilitate anticancer immunity. IL-12, a cytokine produced by macrophages (41) and DC (42), mediates potent antitumor effects that are the result of several actions involving the induction of CTL (43), Th1-mediated immune responses, and NK activation (41), as well as the impairment of tumor vascularization (44). An increase in GM-CSF in ELC/CCL19-treated mice could enhance DC maturation and Ag presentation (24). IP-10/CXCL10 and MIG/CXCL9 are CXC chemokines that chemoattract activated T cells expressing the CXCR3 chemokine receptor (45) and are known to have potent antitumor and antiangiogenic properties (46–49). MIG/CXCL9 and IP-10/CXCL10 are potent angiostatic factors that are induced by IFN-γ (48, 50, 51). The tumor reductions observed in this model might be due to T cell-dependent immunity, as well as participation by T cells secreting IFN-γ in inhibiting angiogenesis (51) by inducing MIG/CCL9 and IP-10/CCL10. Hence, an increase in IFN-γ at the tumor site of ELC/CCL19 treated mice could explain the relative increases in IP-10/CXCL10 and MIG/CCL9. Both MIG/CCL9 and IP-10/CCL10 are chemotactic for stimulated CXCR3-expressing T lymphocytes that could further amplify IFN-γ at the tumor site (52). Flow cytometric determinations revealed that both CD4 and CD8 cells as well as $CD3^+$ T cells expressing CXCR3 were increased at the in ELC/CCL19 treated mice. Braun et al. (26) had shown that ELC/CCL19 in a breast cancer cell line mediated rejection of the transduced tumor through a mechanism involving NK and $CD4^+$ cells. Future experiments will address the contribution of NK cells in intratumoral ELC mediated anti-tumor responses.

To determine the importance of MIG/CXL9, IP-10/CXCL10, and IFN-γ in the ELC/CCL19 mediated antitumor response, these cytokines were depleted in ELC/CCL19 treated mice. Anti-MIG/CXL9 or IP-10/CXL10 each partially yet significantly inhibited the antitumor response. Because MIG/CCL9 and IP-10/CCL10 share the same receptor (CXCR3), one possible explanation of a partial inhibition in tumor growth is that neutralization of one ligand may overexpose the receptor to the other ligand. An alternative explanation is that residual cytokines such as IFN-γ inducible T cell α chemoattractant (ITAC/CXCL11) present after in vivo neutralization of MIG/CXL9 and IP-10/CCL10 play interrelated roles in the recruitment of CXCR3 activated T cells in ELC/CCL19 mediated antitumor responses. In vivo depletion of IFN-γ completely inhibited the antitumor efficacy of ELC/CCL19. The fact that neutralization of IFN-γ was the most efficient at inhibiting ELC/CCL19 mediated antitumor response may be due to a decrease in the IFN-γ dependent CXCR3 ligands MIG/CXL9 and IP-10/CXCL10 indicating that these chemokines are largely IFN-γ dependent. One interesting question is whether IFN-γ, and therefore, presumably MIG and IP-10, are necessary in the afferent, efferent, or both phases of the immune response. This question could be addressed in future adoptive transfer experiments in which these cytokines are neutralized after transfer of lymphocytes from CCL19-treated tumor-bearing mice, i.e., is anti-IFN-γ treatment as effective at reversing the antitumor efficacy of CCL19 when administered after the establishment of a specific T cell response (is it necessary for the cytotoxic activity of elicited T cells, or in their recruitment to the tumor site?). CXCL9 (MIG) and CXCL10 (IP-10), although ligands of the CXCR3 receptor, do not appear to play redundant roles in the anti-tumor effect of CCL19

because inhibition of a single ligand significantly, though less effectively than anti-IFN-γ treatment, reduced tumor growth. The increase in type-1 cytokines may in part be due to an increase in specificity against the autologous tumor; splenocytes from ELC/CCL19 treated mice had a significantly increased frequency of tumor specific T cells producing IFN-γ. In addition, transfer of T lymphocytes from ELC/CCL19 treated tumor bearing mice transferred the antitumor therapeutic efficacy to naive mice.

The current study indicates that ELC/CCL19 administered intratumorally leads to the generation of tumor specific antitumor responses that are dependent on IFN-γ. The potent antitumor properties demonstrated in this model provide a strong rationale for further evaluation of ELC/CCL19 regulation of tumor immunity and its use in immunotherapy for lung cancer.

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