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Lymphotactin Expression by Engineered Myeloma Cells Drives Tumor Regression: Mediation by CD4+ and CD8+ T Cells and Neutrophils Expressing XCR1 Receptor

Chantelle M. Cairns,* John R. Gordon,† Fang Li,† Maria E. Baca-Estrada,‡ Terence Moyana,§ and Jim Xiang²*

The C chemokine lymphotactin has been characterized as a T cell chemoattractant both in vitro and in vivo. To determine whether lymphotactin expression within tumors could influence tumor growth, we transfected an expression vector for lymphotactin into SP2/0 myeloma cells and tested their ability to form tumors in BALB/c and nude mice. Transfection did not alter cell growth in vitro. Whereas SP2/0 cells gave rise to a 100% tumor incidence, lymphotactin-expressing SP2/0-Lptn tumors invariably regressed in BALB/c mice and became infiltrated with CD4+ and CD8+ T cells and neutrophils. Regression of the SP2/0-Lptn tumors was associated with a type 1 cytokine response and dependent on both CD4+ and CD8+ T cells, but not NK cells. Both SP2/0 and SP2/0-Lptn tumors grew in nude mice, but growth of the latter tumors was retarded and associated with heavy neutrophil responses; this retardation of SP2/0-Lptn tumor growth was reversed by neutrophil depletion of the mice. Our data also indicate that mouse neutrophils express the lymphotactin receptor XCR1 and that lymphotactin specifically chemoattracts these cells in vitro. Thus, lymphotactin has natural adjuvant activities that may augment antitumor responses via effects on both T cells and neutrophils and thereby could be important in gene transfer immunotherapies for some cancers.


The trafficking of lymphocytes into tissues is a dynamic, multistep process. It involves both intra- and extravascular processes and ultimately, in the case of tissue reactions, migration of the lymphocytes along chemoattractant gradients (7, 8) established by chemokines produced at the site of inflammation (9). Chemokines are a superfamily of chemoattractant cytokines (10) produced by multiple cell types (e.g., leukocytes, endothelial cells, tumor cells) in response to an array of inflammatory stimuli (11). They are related genetically by a conserved four-cysteine motif, with the three major families of the superfamily defined by the spacing of the first two cysteines in this conserved motif. For the α family, the two cysteines are separated by any residue (CXC), whereas the analogous two cysteines of the β family are adjacent (CC), and the newly identified γ family (C) possesses only one cysteine at its N terminus (12). Generally, the CXC chemokines are potent activators and chemoattractants for neutrophils, whereas the CC and C chemokines have the potential to chemoattract monocytes and T lymphocytes (12). The T cell specificity of the CC and C chemokines suggests that they may play important roles in immunologic responses.

Various CXC and CC chemokine genes, such as IFN-γ-inducible protein-10 (IP-10),3 monocyte chemotactic protein (MCP)-1, MCP-3, T cell activation 3, RANTES, and macrophage-inhibitory protein (MIP) 1α have been transduced into a variety of experimental tumors (13–18). Transfer of genes encoding antiangiogenic CXC chemokines (e.g., IP-10) can block tumorigenicity in some cases (10, 11), whereas results obtained with analogous CC chemokine gene transfers were variable (15–18). Nevertheless, these findings indicate that the local secretion of some chemokines by engineered tumor cells has the potential to affect tumorigenicity (15, 17) and perhaps the induction of tumor-specific T cells (18). The C chemokine lymphotactin has the ability to induce T (19) or NK (20) cell migration in vitro and in vivo. Thus, experimental expression of the gene for this chemokine by tumor cells might be

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3 Abbreviations used in this paper: IP-10, IFN-γ-inducible protein-10; MIP, macrophage-inhibitory protein; MCP, monocyte chemotactic protein.
predicted to importantly affect the induction of tumor-specific im-

mune responses. In this study, we tested the effect on tumor growth of transferring a lymphotactin gene expression cassette into the highly tumorigenic mouse myeloma cell line SP2/0. Our results indicate that this procedure induces specific protective responses that are mediated by T cells, but also that lymphotactin receptor-expressing neutrophils display some antitumor activities that are readily discernable in T cell-deficient nude mice.

Materials and Methods

Abs, chemokines, tumor cell lines, and animals

Rat monoclonal anti-mouse CD3 (145-2C11), CD4 (GK1.5), CD8 (3.155), NK (PK136), and neutrophil (RB6-8C5) Abs were purified by affinity chromatography from the ascites of hybridoma cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA) or Dr. R. Coffman (DNAX, Palo Alto, CA). The following reagents were purchased commercially: rat anti-mouse B7-1 (1G10), B7-2 (GL1), and ICAM-1 (3E3), and mouse anti-H-2K (SF1-1.1) and IA (AMS-32.1) Abs, BD PharMin-
gen (San Diego, CA); FITC- and peroxidase-conjugated goat anti-rat and anti-mouse IgG Abs, Bio/Ca Scientific (Mississauga, Ontario, Canada); and recombinant mouse lymphotactin, R&D Systems (Minneapolis, MN).

Myeloma cell line SP2/0 and B cell lymphoma cell line A20 (BALB/c mouse origin) were obtained from the ATCC and maintained in complete medium (DMEM-10% FCS-gentamicin, 50 μg/ml). Female BALB/c and athymic nude mice (4–6 wk old) were obtained from our institutional Animal Resource Center and Charles River Laboratories (St. Constant, Quebec, Canada), respectively. All mice were maintained in the animal facility at the Saskatchewan Cancer Center (Saskatoon, Saskatchewan, Can-
da), and all experiments were conducted according to the guidelines of the Canadian Council for Animal Care.

Transfection of tumor cells with the expression vector pcILptn

A 10-kb cDNA fragment encoding the full open reading frame of the mouse lymphotactin gene was obtained from Dr. A. Zlotnik (DNAX) and ligated into the vector pcDNA3.1 (Invitrogen, Carlsbad, CA) to form pcDNA3.1-Lptn. For transfection, 2 × 10^5 SP2/0 cells were resuspended in 0.7 ml PBS, mixed with 0.3 ml PBS containing 10 μg pcDNA3.1-Lptn or pcDNA3.1 DNA, and then electroporated at 250 V/125 μF capacitance (Gene pulsor; Bio-Rad Laboratories, Richmond, CA). Two transfected cell lines, SP2/0-Lptn and SP2/0neo (control cell line), were obtained by select-
ing for growth in complete medium containing 2.0 mg/ml G418, respectively.

Analysis of mRNA expression

Northern analysis of lymphotactin expression.

Total cellular RNA was isolated from SP2/0-Lptn or control cells or tumors using a commercial kit (Qiagen, Mississauga, Ontario, Canada), denatured in formaldehyde, and electrophoresed on 1% agarose-formaldehyde gels. The integrity of the RNA samples in the gels was assessed under UV illumination; then the RNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). The EcoRI lymphotactin cDNA fragment was labeled with [32P]dCTP (Amersham, Arlington Heights, IL) by random priming and used to probe the blots. The membranes were hybridized overnight at 42°C in 6× SSC-5× Denhardt’s solution-0.5% SDS-20 μg/ml salmon sperm DNA. The filters were washed sequentially at 65°C in 3× SSC-0.1% SDS-1× SSC-0.1% SDS-0.1% SDS and then exposed to Kodak x-ray film until the desired exposures were obtained.

RNomeasure analysis of chemokine expression.

RNA was extracted from SP2/0 and SP2/0-Lptn tumors of nude mice as noted above and ana-
yzed using a commercial kit (RiboQuant MultiProbe RNaiAse kit; DB PharMingen). Briefly, in vitro transcription of the RNAs for an array of chemokines (mC5K MultiProbe Template Set; DB PharMingen) with [32P]UTP (Amersham) was conducted using with T7 RNA polymer-
ase followed by phenol-chloroform extraction and ethanol precipitation. The probe mixture was adjusted to 3 × 10^6 cpm/μl and hybridized to the tissue RNA samples (6 × 10^6 cpm antisense RNA, 5 μg sample RNA); then the hybridized samples were digested with RNase followed by pro-
teinase K treatment and phenol-chloroform extraction. After ethanol precip-
atration with 4 M ammonium acetate, the protected fragments were run on a 5.7% acrylamide-bisacrylamide urea gel, dried, and then visualized by autoradiography.

RT-PCR analysis of XCR1 expression.

Total RNA was obtained from nylon wool-purified T lymphocytes (>99% purity as determined using the anti-CD3 Ab by FACS, data not shown) and MACS-purified neutrophils. The neutrophils and RBC were first separated from the peripheral mono-
cytes with Ficoll-Paque gradient. The neutrophils were then purified by lysing the RBC with 0.84% ammonium chloride to yield a neutrophil purity of >95% and further purified by MACS using Dynabeads M-450 (Dynal, Lake Success, NY) coated with the antineutrophil Ab, to yield a final neutrophil purity of >99% as determined using the antineutrophil Ab by FACS (data not shown). The first-strand cDNA synthesis for the RT-PCR was performed with 5 μg RNA using a commercial kit (Stratagene, La Jolla, CA), following the PCR manufacturer’s instructions. Titers were specific for the lymphotactin receptor gene XCRI (sense primer, 5'-
tctc gctca ctgcc ttggt tg3'- antisense primer, 5'-tgact ggtgc tcggct ctg3'-21) and GAPDH (sense primer, 5'-caggt tgtct cctgc gactt-3'; antisense primer, 5'-ctgct gactc tgctg-3'). The PCR conditions comprised 1 cycle at 94°C (5 min), 54°C (1 min), and 72°C (1 min) and 40 cycles at 94°C (1 min), 54°C (1 min), and 72°C (1 min). All PCR products were resolved on 1% agarose gels with ethidium bromide staining.

Bioassays for lymphotactin

Cells. Splenic T lymphocytes were nylon wool purified from BALB/c mice as previously described (22) and resuspended in DMEM-0.1% BSA to 4 × 10^6 cells/ml. Mouse peripheral blood leukocytes were enriched by dextran sedimentation according to a standard protocol (23). Briefly, hepar-
in-anticoagulated mouse blood was mixed with equal volumes of 3% dex-
tran-HCl in PBS (Pharmacia Biotech, Uppsala, Sweden) in a 1:1 volumetric ratio. The RBC were allowed to sediment for 20 min at room temperature. The cells were then harvested from the leukocyte-rich plasma layer by centrifugation and washed and resuspended as above.

Chemotaxis assay.

Chemotactic responses of T lymphocytes and neutro-

phils to lymphotactin or the tumor extracts were examined using modified Boyden microchemotaxis chambers (Neuroprobe, Gaithersburg, MD) and polyvinylpyrrolidone-free 5-μm size polycarbonate filters, essentially as described (24). Protein extracts of the SP2/0-Lptn and control SP2/0 tumors were prepared in 10 mM Tris-0.2 mM CaCl2 (pH 7.2) as previously described (25). The culture supernatants of SP2/0-Lptn and SP2/0 cells, protein extract samples (15 mg/ml), standards (recombinant lymphotactin; 1–1000 ng/ml), or positive control bacterial tripeptide FMLP (1 nM–10 μM) were diluted in DMEM-0.1% BSA and placed in the lower chambers of the Boyden chambers, while the chemokines, tumor cell lines, or animals

and washed and resuspended at 37°C, the cells that had not migrated into the membranes were wiped from the upper surfaces; then the membranes were fixed and stained using a Diff-Quik kit (American Scientific Products, McGraw Hill, IL). For each sample, the numbers of cells associated with the membrane were enumerated by direct counting of at least nine ×40 objective fields; the results are expressed as the mean number of cells/×40 field (±SEM).

Essentially all (>99%) of the peripheral blood leukocytes that were ad-
herent to the lower surfaces of the membranes in the 20-min assays were neutrophils, as determined by morphologic examination.

Animal studies

Tumorigenicity studies.

In general, naive mice were inoculated s.c. on their right thighs with 1 × 10^6 SP2/0-Lptn, SP2/0neo, and SP2/0 tumor cells, respectively, and then monitored daily for tumor progression or re-

gression. Two weeks later, the animals were sacrificed, and the tumors were carefully removed, weighed, and examined histologically. Tumor vol-

umes (in cubic millimeters) were measured with a vernier caliper and cal-
bulated by the formula, tumor size = ab^2/2, where a is the larger and b is the smaller of the two dimensions (26). The basic experiments were per-
fomed in BALB/c mice (n = 10), whereas other experiments used BALB/c nude mice (n = 5), mice (n = 8). The T lymphocytes, NK cells, and neutrophils were depleted by injecting the mice i.p. on experi-

mental days 1, 2, and 5 with 1 mg of the appropriate specific rat Abs (GK1.5, 3.155, PK136, and RB6-8C5), respectively; control mice were similarly injected with isotype-matched rat Abs.

To examine the protective immunity conferred by tumor inoculation, mice were injected with 1 × 10^6 SP2/0 or A20 tumor cells were inoculated into the right thighs of BALB/c mice (n = 8) that had previously rejected SP2/0-Lptn tumors. In some experiments, these SP2/0-Lptn tumor-immune mice were depleted of CD4^+ and CD8^+ T cells before SP2/0 tumor cell challenge. The tumors were removed and examined as described above. Each animal experiment was performed twice, and in each case the results from the independent experiments were consistent. Student’s t test was used to examine the sta-
tistical significance of the animal experiment results (2).

Histopathology and immunohistochemistry.

To characterize the cellular infiltrates associated with SP2/0-Lptn tumor regression, 5 × 10^4 SP2/0-
Lptn cells were injected into the kidney capsules (adjacent to the adrenal gland) of the mice. At different times thereafter, the kidneys/adrenal glands were removed and processed for histology or immunohistochemistry as previously described (27). For histopathology, the tissues were fixed in 10% neutral-buffered formalin, routinely processed to paraffin sections, and stained with hematoxylin-eosin. For immunohistochemical analysis, frozen sections were fixed with cold acetone, endogenous peroxidase activity was blocked by treatment with methanol-3% H₂O₂ (10 min), and the non-specific staining was suppressed using 20% normal horse serum. Subsequently, the sections were incubated overnight at 4°C with either rat anti-mouse CD4 or CD8 Ab (10 μg/ml), then washed with PBS, and incubated with the biotinylated goat anti-rat IgG Ab for 30 min at room temperature. The sections were again washed and incubated in Vectastain avidin-biotin complex-peroxidase reagent (Vector Laboratories, Burlingame, CA) for 30 min; then the peroxidase activity was developed with freshly prepared 0.06% 3,3'-diaminobenzidine containing 0.1% hydrogen peroxide, and the sections counterstained with methyl green (0.1%). The sections were evaluated for the presence of brown diaminobenzidine precipitates indicative of CD4 and CD8 reactivity.

**Immunologic assays**

**Phenotypic analysis of SP2/0-Lptn cells.** We used FACS to quantitate the expression of MHC class I and II Ags, the costimulatory molecules B7-1 and B7-2, and ICAM-1 on SP2/0-Lptn tumor cells. To stain the cells, cells were first incubated for 1 h on ice with rat anti-B7-1, B7-2, or ICAM-1 or mouse anti-H-2Kd and Ia d Abs (each, 2 μg/ml), then washed three times with PBS, and incubated for 1 h more on ice with FITC-conjugated anti-rat or anti-mouse IgG Ab (5 μg/ml). After another three washes with PBS, the cells were analyzed using an Epics XL FACS (Coulter, Burlington, Ontario, Canada).

**Phenotyping the protective anti-SP2/0-Lptn response.** One week after inoculation of 1 × 10⁶ SP2/0-Lptn cells in the footpads of the mice, lymphocytes were harvested from their regional lymph nodes, washed, and resuspended in complete medium. The lymphocytes were cocultured (in quadruplicate) in 96-well plates with 6000-rad-irradiated SP2/0 tumor cells (5 × 10⁵ lymphocytes plus 2.5 × 10⁵ SP2/0 cells/well). The culture supernatants were harvested at 1 or 4 days and then pooled and assayed using commercial ELISA kits for IFN-γ and IL-4 (Endogen, Woburn, MA), respectively.

**Cytotoxic T cell assay.** Splenic lymphocytes were harvested from mice that had experienced SP2/0-Lptn tumor regression and from mice bearing SP2/0 tumors. The lymphocytes (5 × 10⁶) were cocultured for 5 days with 6000-rad-irradiated SP2/0 cells (1 × 10⁵) in 2 ml volumes of DMEM-10% FCS in 24-well plates (Costar, Cambridge, MA); then the T cells were harvested and used as effector cells in a chromium release assay. The target cells comprised 11Cr-labeled SP2/0 or A20 tumor cells, prepared by culturing the tumor cells for 6 h in the presence of 50 μCi sodium [11Cr]citrate (36 mCi/ml; Amersham) and then washing them twice with DMEM. For the assay, 1 × 10⁶ labeled target cells were incubated for 6 h with effector cells at various E:T ratios (triplicate cultures). The percentage specific lysis was calculated using the formula [(experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)] × 100. The counts released spontaneously (i.e., in the absence of effector cells) were <10% of specific lysis; the maximal release was determined by adding 1% Triton X-100 to a set of wells.

**Results**

**Lymphotactin-expressing SP2/0 tumor cells, but not parental strain SP2/0 tumors, are rejected by BALB/c mice**

Lymphotactin is a molecule known to chemoattract lymphocytes in vivo (19). To test whether expression of this chemokine within otherwise immunologically resistant tumors might render the tumors susceptible to immune attack, we transfected SP2/0 myeloma cells with a lymphotactin gene expression cassette and challenged BALB/c mice with these transfected or nontransfected SP2/0 cells. We had determined that the in vitro growth rate of the SP2/0-Lptn cells was similar to the control SP2/0neo and the parental SP2/0 cells (doubling times, 12.4, 12.0, and 11.8 h, respectively) and had characterized the expression of lymphotactin mRNA and product by the transfected cells in vitro. The SP2/0-Lptn, but not the SP2/0 cells expressed high levels of lymphotactin mRNA (Fig. 1A).

In vivo, s.c.-injected SP2/0 or SP2/0neo tumor cells grew aggressively in BALB/c mice, resulting in a tumor incidence of 100%, whereas no tumors were detected 2 wk after similar challenge of BALB/c mice with SP2/0-Lptn cells (Table 1). To follow the pathogenesis of the SP2/0-Lptn tumor response, we next injected these cells into the kidney capsules of BALB/c mice and biopsied the injection sites at various times thereafter. At 24 h postchallenge, no leukocyte responses were evident at the SP2/0-Lptn cell injection sites (Fig. 2A), but by 2 days postchallenge some lymphocytes and neutrophils started to infiltrate into the tumors (data not shown) and by 4 days postchallenge numerous lymphocytes as well as neutrophils had infiltrated into the tumors (Fig. 2C). Immunohistochemical analyses showed that these lymphocytes comprised both CD4⁺ and CD8⁺ cells (Fig. 3, A and B). However, no T cells were detected in a 4-day SP2/0 tumor growing in the kidney capsule of a control mouse either by using anti-CD3 Ab immunostaining (Fig. 3C) or by using anti-CD4 or CD8 Ab immunostaining (data not shown). Six days after tumor cell inoculation, multiple areas of necrosis were seen in the tumors (Fig. 2D), and by 9–10 day postchallenge the tumor cells were no longer detectable, although numerous lymphocytes, macrophages, and fibrin deposits were present (Fig. 2E). No cellular responses were observed in a 4-day SP2/0 tumor growing in the kidney capsule of a control mouse (Fig. 2A). Taken together, these data suggest that both CD4⁺ and CD8⁺ T cells as well as neutrophils were involved in the responses to the SP2/0-Lptn tumor cells in the BALB/c mice.

**Evidence that both T cells and neutrophils mediate anti-SP2/0-Lptn tumor responses**

To assess the possible cellular mechanisms responsible for SP2/0-Lptn tumor regression, we inoculated these cells into groups of

**Table 1. Tumor growth of SP2/0-Lptn cells in BALB/c and athymic mice**

<table>
<thead>
<tr>
<th>Tumor Cells</th>
<th>BALB/c mice</th>
<th>Athymic mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP2/0</td>
<td>10/10 (100)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>SP2/0-Lptn</td>
<td>0/10 (0)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>SP2/0-neo</td>
<td>10/10 (100)</td>
<td>8/8 (100)</td>
</tr>
</tbody>
</table>

*Naive BALB/c and athymic mice were inoculated s.c. with 1 × 10⁶ SP2/0, SP2/0neo, and SP2/0-Lptn tumor cells, respectively. Two weeks after tumor inoculation, the tumors were removed and confirmed as such by histologic analysis.
BALB/c mice that had been depleted of CD4+ or CD8+ lymphocytes, NK cells and neutrophils, respectively, and then tested their respective abilities to reject the tumors. We found that there was no SP2/0-Lptn tumor growth in the control Ab-treated, NK-depleted, and neutrophil-depleted mice, whereas the SP2/0-Lptn cells essentially grew as aggressively as SP2/0 cells in the CD4+ - and CD8+ - lymphocyte-depleted naive mice (Table II). This indicates that at least the initiation of immunologic rejection of SP2/0-Lptn tumor cells in naive mice in the initiation phase of immune responses is dependent on both CD4+ and CD8+ T cells. To confirm the roles of the T cells in the tumor rejection response, we repeated this SP2/0-Lptn challenge experiment in T cell-deficient nude mice. As...
expected, the incidence of Sp2/0-Lptn, SP2/0neo, and SP2/0 tumor growth in these nude mice was equivalent (Table I). However, we found that there was a significant retardation of SP2/0-Lptn tumor growth compared with the SP2/0 tumor growth in these nude mice (Fig. 4; p < 0.01). Histologic data revealed that there were dense infiltrations of neutrophils in an early stage (9 days) of SP2/0-Lptn tumors, and this was associated with extensive tumor necrosis in a later stage (12 days) (Fig. 2, F and G). To determine whether the neutrophil response was responsible for the retardation of SP2/0-Lptn tumor growth, we next challenged neutrophil-depleted nude mice with these cells and assessed the tumor growth. In these nude mice, there were no neutrophil responses to the tumor cells (Fig. 2F), and SP2/0-Lptn tumors grew as aggressively as SP2/0 tumors (Fig. 4). Taken together, these data confirm that T cells are important to SP2/0-Lptn tumor cell rejection but also clearly demonstrate that neutrophils play a role in this response.

Evidence that lymphotactin is a specific neutrophil chemoattractant

Lymphotactin has not been reported previously to affect neutrophils, although it is well known for its effects on lymphocytes (19). Our data confirmed that both recombinant lymphotactin and SP2/0-Lptn cell culture supernatants were able to chemoattract T cells in a dose-dependent manner. The amount of lymphotactin in SP2/0-Lptn cell culture supernatants was estimated to be ~500 pg/10^6 cells during 24 h in culture (Fig. 5A). To validate this apparent lymphotactin-mediated neutrophil response, we first confirmed using RT-PCR that highly purified mouse neutrophils (>99% purity) expressed substantial levels of the lymphotactin receptor XCR1 as did the mouse T lymphocytes (Fig. 1C). We then assessed the neutrophil chemotactic properties of recombinant lymphotactin, as well that of aqueous extracts of SP2/0-Lptn tumors resected from nude mice. Our data confirm that both recombinant lymphotactin and SP2/0-Lptn tumor extracts (but not SP2/0 tumor extracts) were able to chemoattract peripheral blood neutrophils from BALB/c mice in a dose-dependent fashion as did the chemotactic bacterial tripeptide FMLP (Fig. 5, B–D). To determine whether other high profile neutrophil chemokines were expressed within the SP2/0-Lptn tumors grown in nude mice, we probed the RNA extracted from the tumors with a panel of chemokine probes (MIP-1α, MIP-2, IP-10) using an RNase protection assay. There was no detectable expression within the SP2/0-Lptn tumors of MIP-1α, MIP-2, or IP-10 (i.e., neutrophil chemokines (13, 28, 29)), whereas there was significant expression of mRNA for lymphotactin.

![Figure 3](Image 84x376 to 244x735)

**FIGURE 3.** Immunohistochemical localization of CD4⁺ and CD8⁺ lymphocytes infiltrating SP2/0-Lptn tumors. A and B, Sections from a 4-day SP2/0-Lptn in the kidney capsule of a BALB/c mouse. A, Anti-CD4 Ab immunostaining. B, Anti-CD8 Ab staining. C, Section from a 4-day SP2/0 tumor in the kidney capsule of a BALB/c mouse with the anti-CD3 Ab immunostaining. The brown diaminobenzidine precipitates indicate the positive staining and provide evidence of both CD4⁺ and focal CD8⁺ T cell infiltration responses. Magnification, ×80 for all panels.

![Figure 4](Image 361x97 to 484x189)

**FIGURE 4.** Retardation SP2/0-Lptn tumor growth is mediated by neutrophils. We injected nude mice (n = 8) s.c. with 1 × 10^6 SP2/0-Lptn (▲) or SP2/0 (●) tumor cells and followed the growth of the tumors with time. Another group of eight neutrophil-depleted nude mice were similarly injected with SP2/0-Lptn tumor cells (○). Tumor size was measured as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Cell Population-Depleted</th>
<th>Tumor Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I: (naive mice)</td>
<td></td>
</tr>
<tr>
<td>CD4⁺ cells</td>
<td>7/8 (88)</td>
</tr>
<tr>
<td>CD8⁺ cells</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>NK cells</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Control Ab treatment</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Experiment II: (SP2/0-Lptn-immune mice)</td>
<td></td>
</tr>
<tr>
<td>CD4⁺ cells</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>CD8⁺ cells</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Control I (naive mice)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Control II (A20 cell challenge)</td>
<td>8/8 (100)</td>
</tr>
</tbody>
</table>

a In experiment I, aliquots of 1 × 10⁶ SP2/0-Lptn tumor cells were injected s.c. into neutrophil-, CD4⁺CD8⁺ T⁺, and NK cell-depleted naive mice, respectively. Control mice were treated with 1 mg isotype-matched rat Abs (n = 8). Tumor incidence was determined as in Table I. b In experiment II, aliquots of 1 × 10⁶ SP2/0 tumor cells were injected s.c. into CD4⁺ and CD8⁺ T cell-depleted mice that had previously immunologically rejected SP2/0-Lptn tumor-immune mice. Naive mice were used as control I group. A second control group comprised SP2/0 tumor-immune mice that were challenged with A20 tumor cells (1 × 10⁶ cells/mouse). n = 8.
**Vaccination with SP2/0-Lptn tumor cells induces protective immunity to challenge with highly tumorigenic SP2/0 tumor cells**

To gain insights into the immunologic basis of SP2/0-Lptn tumor regression, we assessed a number of parameters associated with this tumor regression. First, we used FACS to examine the expression by SP2/0 and SP2/0-Lptn cells of MHC class I and II Ags, the costimulatory molecules B7-1 and B7-2, and the adhesion molecule ICAM-1 (i.e., molecules important to immunogenic processes). Both SP2/0 and SP2/0-Lptn cells expressed MHC class I Ags, but MHC class II, B7-1, B7-2, or ICAM-1 molecules were not detectable in either population, and the SP2/0-Lptn cells expressed slightly but not remarkably enhanced MHC class I relative to the SP2/0 cells (data not shown). This indicates that the loss of tumorigenicity of SP2/0-Lptn tumor cells was not due to an alteration of immunogenicity. We next examined the phenotype of the SP2/0-Lptn cell-specific responses in SP2/0-Lptn-immune mice by assessing the IFN-γ/IL-4 cytokine profiles released by regional lymphocytes of tumor-exposed animals. The SP2/0-Lptn-responsive T cells in the animals undergoing SP2/0-Lptn tumor regression secreted high levels of IFN-γ, but little IL-4 (1120 ± 130 pg/ml).

**Table III. Cytokine secretion responses of regional lymphocytes from mice given SP2/0 or SP2/0-Lptn myeloid tumor cells**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>IL-4</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP2/0-Lptn</td>
<td>&lt;33</td>
<td>1220 ± 130</td>
</tr>
<tr>
<td>SP2/0</td>
<td>&lt;33</td>
<td>110 ± 45</td>
</tr>
</tbody>
</table>

* Mice were inoculated s.c. with 1 × 10⁶ SP2/0-Lptn or SP2/0 tumor cells. One week later, T cells were harvested from the draining regional lymph nodes and cocultured with irradiated SP2/0 tumor cells for 1 or 3 days; then the culture supernatants were assayed for IFN-γ and IL-4, respectively, by ELISA.

* Data represent the mean (±SD) picograms per 5 × 10⁶ T cells for IL-4 or IFN-γ from triplicate samples. The sensitivity of the IL-4 ELISA was 33 pg/ml.

* Significant (p < 0.01) vs SP2/0 group (Student’s t test).
IFN-γ (data not shown) or SP2/0 tumor-bearing mice secreted little anti-CD4-Ab-treated (incidence, 0 of 8) groups (Table II), but did not grow in the control Ab-treated (tumor incidence, 0 of 8) or immune mice of their respective lymphocyte subpopulations and CD4, anti-CD8, or irrelevant control Abs to deplete SP2/0-Lptn with the induction of such immune responses. First, we used anti-CD4-specific protection, we assessed a number of parameters associated tumor cells. Results clearly demonstrate a SP2/0 tumor-specific protective immunity in BALB/c mice previously vaccinated with SP2/0-Lptn cells, whereas the incidence of A20 growth was detectable (incidence, 0 of 8) in mice that had previously rejected the SP2/0-Lptn cells, whereas the incidence of A20 tumor growth in these mice was 100% (8 of 8) (Table II). These results clearly demonstrate a SP2/0 tumor-specific protective immunity in BALB/c mice previously vaccinated with SP2/0-Lptn tumor cells.

To gain insights into the immunologic basis of SP2/0-Lptn-inducible protection, we assessed a number of parameters associated with the induction of such immune responses. First, we used anti-CD4, anti-CD8, or irrelevant control Abs to deplete SP2/0-Lptn-immune mice of their respective lymphocyte subpopulations and then challenged the mice with SP2/0 cells. No SP2/0 tumor growth was detectable (incidence, 0 of 8) in mice that had previously rejected the SP2/0-Lptn cells, whereas the incidence of A20 tumor growth in these mice was 100% (8 of 8) (Table II). These results clearly demonstrate a SP2/0 tumor-specific protective immunity in BALB/c mice previously vaccinated with SP2/0-Lptn tumor cells.

FIGURE 6. SP2/0-Lptn tumor cell challenge up-regulates cytotoxic T lymphocyte activity in BALB/c mice. Splenic lymphocytes from mice either bearing SP2/0 tumors (▲) or just having rejected SP2/0-Lptn tumors (○) were cocultured with irradiated SP2/0 cells for 5 days and then tested for CTL activity against 51Cr-labeled SP2/0 target cells. The specificity of the CTL activity of the SP20-Lptn-immune lymphocytes was also confirmed in an assay using an alternate BALB/c tumor cell line, A20 (●), as target cells. Each point represents the mean of triplicate cultures. The SD of each point is <5% of the mean value.

pg/ml IFN-γ, <33 pg/ml IL-4), whereas analogous cells from naive (data not shown) or SP2/0 tumor-bearing mice secreted little IFN-γ (110 ± 45 pg/ml IFN-γ, <33 pg/ml IL-4) (Table III), suggesting that the anti-SP2/0-Lptn immune response was of a Th1 type.

We wished to determine whether the rejection of SP2/0-Lptn tumor cells that we observed in BALB/c mice would translate into immunologic protection of these animals from otherwise inevitable SP2/0 cell tumorigenesis. We challenged groups of BALB/c mice (n = 8), with SP2/0-Lptn tumor cells as above, allowed these SP2/0-Lptn tumors to undergo complete regression, and then challenged the mice with SP2/0 or A20 tumor cells. No SP2/0 tumor growth was detectable (incidence, 0 of 8) in mice that had previously rejected the SP2/0-Lptn cells, whereas the incidence of A20 tumor growth in these mice was 100% (8 of 8) (Table II). These results clearly demonstrate a SP2/0 tumor-specific protective immunity in BALB/c mice previously vaccinated with SP2/0-Lptn tumor cells.

To gain insights into the immunologic basis of SP2/0-Lptn-inducible protection, we assessed a number of parameters associated with the induction of such immune responses. First, we used anti-CD4, anti-CD8, or irrelevant control Abs to deplete SP2/0-Lptn-immune mice of their respective lymphocyte subpopulations and then challenged the mice with SP2/0 cells. The SP2/0-Lptn tumors did not grow in the control Ab-treated (tumor incidence, 0 of 8) or anti-CD4-Ab-treated (incidence, 0 of 8) groups (Table II), but significant tumor growth was clearly present in the CD8+ lymphocyte-depleted mice (tumor incidence, 8 of 8). Furthermore, following coculture for 5 days with irradiated SP2/0 cells, splenocytes from the SP2/0-Lptn-immune mice displayed highly significant cytotoxic activity against SP2/0 cells (78% specific killing; E:T ratio, 100) but not against irrelevant control A20 cells. Lymphocytes from mice bearing SP2/0 cell tumors showed only low SP2/0-specific killing (10%), and splenocytes from naive mice did not show any SP2/0-specific responses (data not shown). Thus, these data indicate that anti-SP2/0 tumor protection in the SP2/0-Lptn-vaccinated mice was largely mediated by SP2/0 tumor-specific CTLs.

Discussion

The C chemokine lymphotactin chemotactacts T cells both in vitro and in vivo (19, 20). Thus, it might be predicted that expression of lymphotactin within tumors might predispose the tumor cells to T cell-mediated rejection responses. However, at least in one case, experimental coinjection with tumor cells and fibroblasts engineered to express lymphotactin reportedly did not lead to inhibition of tumor growth, although inhibition could be observed with engineered fibroblasts coexpressing IL-2 with lymphotactin (31). In contrast, our data clearly indicate that mouse SP2/0 myeloma cells that had been engineered to secrete lymphotactin completely lost their ability to form solid tumor masses in vivo. Furthermore, this loss of tumorigenicity was dependent on both CD4+ and CD8+ T cells but not NK cells and was associated with a type 1 phenotype response as determined by IFN-γ secretion. Although lymphotactin is chemotactic for T cells, recently it was reported that lymphotactin can also differentially regulate cytokine secretion by CD4+ and CD8+ T cells (30). It dampens the IL-2 and IFN-γ responses of CD4+ T cells, although IFN-γ expression in the lymphotactin-stimulated CD4+ T cells still outstrips IL-4 release by 5-fold but also augments the IL-2 responses of CD8+ T cells (the IFN-γ responses of CD8+ T cells were not examined). It is feasible then that the IFN-γ expression observed in our study was attributable to both Th1 and Tc1 cells that were activated via the SP20-Lptn immunization. The CD4+ cells were critical for the induction of the tumor-specific immunity, because their depletion before initial exposure to the SP20-Lptn cells also depleted the ability to reject the tumor cells. The effector phase of the tumor rejection was mediated by CD8+ cells and not at all affected by CD4+ cell depletion. Previous studies on tumor resistance following chemokine gene transfer neither defined the role of T cells nor analyzed the type 1 or type 2 nature of these responses (31–34). Collectively, our results demonstrate that the C chemokine lymphotactin has natural adjuvant activities that result in augmented antitumor immune responses.

Neutrophil infiltration into tumors has been frequently reported in studies with tumor cells engineered to secrete cytokines and chemokines. Thus, the potential involvement of neutrophils in antitumor immune responses has begun to draw more attention. This neutrophil response has been documented in the context of tumor cells engineered to secrete IL-2, IL-4, IL-8, GM-CSF, MIP-1α, or MCP-3, wherein the engineered cells showed reduced tumorigenicity or underwent tumor regression in syngeneic mice (1, 17, 32–35). Nevertheless, neither the mechanism(s) of neutrophil recruitment nor the potential role(s) of these cells in antitumor responses were elucidated in these studies. Recently, Shinohara et al. demonstrated that expression of MIP-1α and MIP-2 was up-regulated in neutrophils that infiltrated GM-CSF-transfected tumors and that the observed neutrophil response preceded a subsequent macrophage effector response. Their data indicated that the neutrophil chemokines in fact mediated the chemotraction of the macrophages into the tumors (21), which illustrates one potential role that neutrophils can play in antitumor responses. More recently, Lee et al. (36) demonstrated that neutrophil infiltration occurred at injection sites of IL-8 transgene-expressing human ovarian cancer cells in nude mice and that this was associated with significantly reduced tumorigenicity in vivo. Our data clearly demonstrate that neutrophils express significant levels of the lymphotactin receptor XCR1, and this chemokine is chemotactic for not only T cells but also neutrophils, both in vitro and in vivo. Furthermore, we have documented that antineutrophil Abs can block the retardation of SP20-Lptn tumor growth observed in nude mice. Our study thus provides direct evidence that cooperates earlier suggestions that neutrophils may play some role in inhibition of tumor growth in mice. Although neutrophils recruited as a consequence of lymphotactin gene transfer exert some antitumor activity, they do not play a requisite role in tumor regression in fully
immunocompetent BALB/c mice, because in these animals CD4⁺CD8⁺ T cells are themselves able to accomplish this function. We did not address the mechanisms by which the neutrophils effect their antitumor activities in nude mice, but they may involve the release of granules, oxygen free radicals, or proteases, each of which could be pathogenic for tumor cells (37).

Recent clinical studies showed that CD8⁺ T cell infiltration of tumors contributed to better survival of patients (38). In systemic adoptive immunotherapy, it has generally been believed that the antitumor efficacy of the transferred T cells is, to a large extent, determined by their ability to extravasate into the tissues where tumors reside. Clinically, the therapeutic efficacy of this approach is still very limited (objective response rate of 30%; Ref. 39), most probably because the proportion of transferred T cells that accumulate within the tumors is rather small (40, 41). Because an efficient infiltration of tumors by systemically transferred tumor-reactive T lymphocytes is required for antitumor efficacy (42), the local secretion of lymphotactin at tumor sites (as could be effected, for example, by adenovirus-mediated lymphotactin gene transfer (43)) could perhaps increase the infiltration into tumors of adoptively transferred tumor-specific T cells and thus improve the efficiency of adoptive cellular immunotherapies. We have already shown, for example, that combined immunotherapies using both a cancer vaccine and gene transfer can be effective in enhancing responses against established tumors (44). In that study, we injected an adenovirus expressing TNF-α intratumorally and thereby enhanced T cell infiltration into tumor masses (44). It remains to be determined whether a similar approach using lymphotactin gene transfer would also induce effective responses against established tumors, but the roles in immunologic responses demonstrated herein and by others for this chemokine suggest that this may well be a fruitful avenue of investigation.

References


