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# Purification of L-Selectin<sup>low</sup> Cells Promotes the Generation of Highly Potent CD4 Antitumor Effector T Lymphocytes<sup>1</sup>

Hiroshi Kagamu and Suyu Shu<sup>2</sup>

Successful adoptive immunotherapy of cancer requires the identification, isolation, and expansion of tumor-specific immune effector cells. A reliable source of tumor-immune lymphocytes is lymph nodes draining a growing tumor. After in vitro stimulation with anti-CD3 and expansion in IL-2, these cells are capable of mediating the regression of established tumors. In the absence of further Ag stimulation, we recently found that the down-regulation of the homing molecule L-selectin could serve as a surrogate marker for isolation of specific tumor-sensitized T cells. The L-selectin<sup>low</sup> (L-selectin<sup>-</sup>) T cells proliferated more vigorously than unfractionated or L-selectin<sup>high</sup> cells. In adoptive immunotherapy of established intracranial MCA 205 tumors, L-selectin<sup>-</sup> cells displayed at least 30-fold greater therapeutic efficacy than unfractionated cells. L-selectin<sup>high</sup> cells did not demonstrate any antitumor effects. Activated L-selectin<sup>-</sup> cells secreted a number of cytokines, including IFN- $\gamma$ , IL-2, IL-4, and IL-10, specifically when stimulated with cognate tumor cells. Further analysis revealed that CD4 T cells alone mediated tumor regression and secreted cytokines. Our results thus demonstrate that the purification of L-selectin<sup>-</sup> cells led to the generation of CD4 immune effector cells with unusually high therapeutic efficacy against chemically induced tumors. The lack of cytotoxicity and the ability to secrete cytokines suggest that these effector CD4 cells mediate antitumor effects through an indirect mechanism similar to the delayed hypersensitivity reaction. *The Journal of Immunology*, 1998, 160: 3444–3452.

In animal studies, T cell immunity to syngeneic tumors can influence the outcome of the disease. Attempts to treat autologous malignancy by augmenting T cell immune responses have focused on either active immunization or adoptive transfer of tumor-specific T cells grown to large numbers in vitro. Although adoptive immunotherapy has theoretical and experimental support to be the most effective immunologic approach, the difficulties associated with the identification and successful expansion of tumor Ag-specific T lymphocytes limit its clinical applications. Insufficient knowledge concerning the molecular and chemical structures of most Ags expressed by tumor cells that are recognized by host T cells has also impeded progress on the generation of highly purified T cells with defined specificity.

Despite its limitations, successful treatment of established tumors by the adoptive transfer of tumor-specific T cells has been demonstrated in a large number of animal models (1–3). For the development of clinically applicable approaches, an essential requirement has been the identification and isolation of tumor-reactive T cells during progressive tumor growth. A potential source of tumor-reactive effector cells has been T lymphocytes infiltrating a tumor mass. These tumor-infiltrating lymphocytes frequently exhibit unique cytolytic specificity for the autochthonous tumor cells, and the adoptive transfer of these cells into tumor-bearing animals mediates potent therapeutic effects (4, 5). The principles and methodology established in animal studies have been extrapolated for clinical treatment of metastatic solid tumors with encouraging re-

sults (6). However, the cell populations used for these studies probably contain significant numbers of T cells without relevant specificity.

In the past few years, we have been interested in utilizing the regional draining lymph nodes (LNs)<sup>3</sup> as an alternative source for generating tumor-sensitized T cells. Murine models have established that LNs draining a progressively growing tumor contain specifically sensitized cells (1, 7). When sequentially activated in vitro with anti-CD3 and IL-2, these cells undergo a functional change such that they mediate the regression of established tumors when adoptively transferred into mice bearing pulmonary metastases. Recently, we have demonstrated that the activated LN cells were capable of mediating the regression of tumors established in the brain as well as in the skin (8, 9). Although adoptive immunotherapy mediated by the LN cells is immunologically specific for the tumor that stimulates the draining LNs, the polyclonal nature of the anti-CD3-T-cell interaction must have also predisposed the culture system to the proliferation of T cells without specific antitumor reactivity.

In the absence of purified tumor antigenic proteins or peptides on many tumors, the expression of the homing molecule L-selectin (CD62L) on T cells may serve as a surrogate marker for identifying tumor-specific immune cells. In a previous paper (10), we have isolated potent tumor-reactive T cells from tumor-draining LNs based on the expression of L-selectin. Upon activation in vitro with anti-CD3 and IL-2, T cells with down-regulated L-selectin (L-selectin<sup>-</sup>) developed into potent immune effector cells capable of mediating the regression of established intracranial MCA 205 tumors. The L-selectin<sup>+</sup> LN T cells, on the other hand, did not demonstrate detectable antitumor effects. In the present study, we analyzed the specificity of the in vivo antitumor reaction mediated by the purified L-selectin<sup>-</sup> T cells and correlated their in vivo activity with specific in vitro secretion of a variety of cytokines.

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<sup>3</sup> Abbreviations used in this paper: LN, lymph node; L-selectin<sup>+</sup>, L-selectin<sup>high</sup>; L-selectin<sup>-</sup>, L-selectin<sup>low</sup>; CM, complete medium; LAK, lymphokine-activated killer.

The presence of MHC class I molecules on most tumor cells and the readily demonstrable cytolytic activity of class I-restricted CD8 T cells have prompted extensive investigation for the use of CTLs as probes to clone tumor-associated target peptides and the utilization of the peptides for active immunotherapy to elicit CD8 cytolytic immune responses (11, 12). Although the generation of CD8 cytolytic responses often requires the helper function of CD4 cells, examples of tumor rejection by noncytolytic CD4 T cells in the absence of a contribution by cytolytic CD8 T cells have been scarce and mostly restricted to virally induced tumors (13). The potential of CD4 cells as sole effector cells in mediating tumor eradication has largely been unexplored. In the current study, phenotype analysis of L-selectin<sup>-</sup> cells revealed that CD4 T cells alone possessed all the antitumor reactivities and their therapeutic efficacy was at least 30-fold greater than cells generated from the unfractionated LN cell population. These results document the sensitization of CD4 T cells for recognizing distinct, nonshared tumor Ags of a chemically induced syngeneic murine fibrosarcoma, and the potential utility of these cells for adoptive immunotherapy.

## Materials and Methods

### Mice

Female C57BL/6N (B6) mice were purchased from the Biologic Testing Branch, Frederick Cancer Research and Developmental Center, National Cancer Institute (Frederick, MD). They were maintained in a specific pathogen-free environment and were used at the age of 8 to 10 wk.

### Tumors

The MCA 205 fibrosarcoma, syngeneic to B6 mice, was induced with 3-methylcholanthrene (14). The tumor has been maintained *in vivo* by serial s.c. transplantation in syngeneic mice and was used within the 10th transplantation generation. Single cell suspensions were prepared from solid tumors by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 U/ml hyaluronidase (Sigma, St. Louis, MO) for 3 h at room temperature as previously described (7). Another syngeneic fibrosarcoma, MCA 207, was also maintained *in vivo* and used for specificity control.

### mAb and flow cytometry

Hybridomas producing mAb against the murine CD3 $\epsilon$ -chain (145-2C11), CD4 (GK1.5), CD8 (2.43), and the murine L-selectin (MEL-14) were obtained from the American Type Culture Collection (Rockville, MD). Phycoerythrin-conjugated anti-Thy 1.2 (30-H12), FITC-conjugated anti-CD4 (GK1.5), Cy-Chrome-conjugated anti-CD8 $\alpha$ -chain (53-6.7) and phycoerythrin-conjugated anti-L-selectin (MEL-14) mAb were purchased from PharMingen (San Diego, CA). Analyses of cell surface phenotypes were conducted by direct immunofluorescence staining of 0.5 to 1  $\times$  10<sup>6</sup> cells with conjugated mAb. In each sample, 10,000 cells were analyzed by a FACScan flow microfluorometer (Becton Dickinson, Sunnyvale, CA).

### Tumor-draining LN cells and anti-CD3/IL-2 activation

B6 mice were inoculated s.c. with 1.5  $\times$  10<sup>6</sup> MCA 205 tumor cells in both flanks. Twelve days later, tumor-draining inguinal LNs were harvested and single cell suspensions were prepared mechanically by teasing with needles and pressing tissue fragments with the blunt end of a 10-ml plastic syringe. LN cells were activated on 24-well plates precoated with the anti-CD3 mAb. Each well contained 3 to 4  $\times$  10<sup>6</sup> cells in 2 ml of complete medium (CM). CM consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1  $\mu$ M sodium pyruvate, 2 mM fresh L-glutamine, 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, 50  $\mu$ g/ml gentamicin, 0.5  $\mu$ g/ml fungizone (all from Life Technologies, Grand Island, NY), and 5  $\times$  10<sup>-5</sup> M 2-ME (Sigma). After 2 days of incubation at 37°C in 5% CO<sub>2</sub>, activated cells were suspended in 4 U/ml of human rIL-2 (Chiron, Emeryville, CA) at 1 to 2  $\times$  10<sup>5</sup>/ml and cultured in 24-well plates or gas-permeable culture bags (Baxter Healthcare, Deerfield, IL) for 3 days. Cells were harvested, washed, and resuspended in HBSS for adoptive immunotherapy or in CM for ELISA.

### Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Robbins Scientific, Sunnyvale, CA). After a 45-min incubation at 37°C, the nonadherent elution contained 90 to 95% Thy1.2<sup>+</sup>

T cells. Purified T cells were further fractionated into two subpopulations based on the expression of L-selectin. Cells were first incubated for 20 min at 4°C with the L-selectin hybridoma ascites fluid at 1:1000 dilution. The cells were washed free of unbound Ab. In all, 3 to 4  $\times$  10<sup>7</sup> cells in 4 ml of CM were plated on a T-25 flask, which was precoated with goat anti-rat Ig Ab (American Qualex, San Clemente, CA). After 1 h of incubation at 4°C, nonadherent (L-selectin<sup>-</sup>) cells were collected by gentle rocking. These cells were incubated on a new goat anti-rat Ig Ab-coated flask to yield highly purified (>90%) L-selectin<sup>-</sup> cells. Adherent cells were collected from the first incubation flask with a cell scraper after rinsing twice with PBS. Greater than 95% of the recovered adherent cells were L-selectin<sup>+</sup>. In some experiments, activated L-selectin<sup>-</sup> cells (40  $\times$  10<sup>6</sup>/ml) were further separated into CD4 and CD8 cells by incubating at 4°C with either anti-CD4 hybridoma ascites fluid at 1:100 dilution or anti-CD8 ascites fluid at 1:1000 dilution for 20 min. Ab-coated cells were then depleted magnetically utilizing sheep anti-rat Ig Ab-coated DynaBeads M-450 (DynaL, Lake Success, NY) at a 4:1 beads to cell ratio, according to the manufacturer's instructions.

### Adoptive immunotherapy

B6 mice were inoculated intracranially in the right hemisphere with 0.8 to 1  $\times$  10<sup>5</sup> MCA 205 tumor cells in 10  $\mu$ l of HBSS to establish brain metastases (15). Three to four days after tumor inoculation, mice were sublethally irradiated (500 R) followed by infusion of effector T cells suspended in 1.0 ml of HBSS through the tail vein. Mice were followed for evidence of intracerebral tumor growth and survival time was recorded.

Therapeutic efficacy of effector cells was also assessed in the treatment of metastases in the lung. In this model, mice were inoculated i.v. with 3  $\times$  10<sup>5</sup> MCA 205 tumor cells suspended in 1.0 ml of HBSS to establish pulmonary metastases. Three days later, cells were given i.v. in 1.0 ml of HBSS through the tail vein. On day 21 after tumor inoculation, all mice were sacrificed for enumeration of tumor nodules on the surface of the lung, as described (7).

### Cytokine assays

A total of 2  $\times$  10<sup>6</sup> of anti-CD3/IL-2-activated L-selectin<sup>-</sup> or L-selectin<sup>+</sup> cells derived from MCA 205 tumor-draining LNs were stimulated by either 5  $\times$  10<sup>5</sup> of 5000 R irradiated MCA 205, MCA 207 tumor cells, or immobilized anti-CD3 mAb for 24 h in 2 ml of CM in 24-well plates at 37°C. Supernatants were harvested and the concentrations of IFN- $\gamma$ , IL-2, IL-4, and IL-10 were measured by ELISA using paired mAbs and standards purchased from PharMingen.

### In vitro cytotoxicity assay

The 4-h <sup>51</sup>Cr release assay was used to determine the cytotoxic reactivity of the anti-CD3/IL-2-activated unfractionated, L-selectin<sup>-</sup>, and L-selectin<sup>+</sup> T cells. The MCA 205 tumor cells (10<sup>7</sup>) were labeled with <sup>51</sup>Cr (Na<sup>51</sup>CrO<sub>4</sub>; 100  $\mu$ Ci; Dupont, Wilmington, DE) at 37°C for 1 h and washed three times in CM. Target cells (10<sup>4</sup>) were incubated with various numbers of effector cells at 37°C in a volume of 0.2 ml of CM for 4 h. The supernatant was collected (Titertek Collecting System, Flow Laboratories, McLean, VA) and the samples were counted in a gamma counter. The percent lysis was calculated as follows: % lysis = (experimental cpm - spontaneous cpm)/maximal cpm - spontaneous cpm  $\times$  100.

Nonspecific lymphokine-activated killer (LAK) cells were generated from B6 normal spleen cells by incubating 2  $\times$  10<sup>6</sup> cells/ml in CM containing 1000 U/ml IL-2 for 3 days and used as cytotoxic effector cell control.

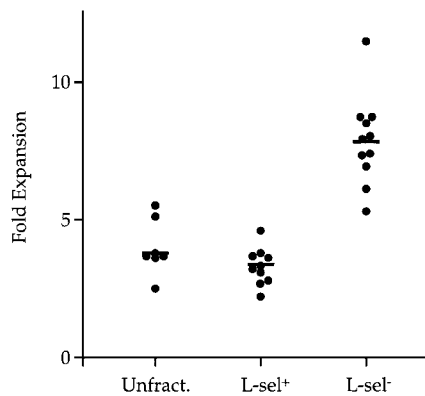
### Statistical analysis

The significance of differences in survival times between different groups was analyzed by the Cox-Mantel test. A two-sided *p* value of <0.05 was considered significant.

## Results

### Antitumor efficacy and specificity of adoptive immunotherapy mediated by activated L-selectin<sup>-</sup> T cells derived from tumor-draining LNs

Inguinal LNs draining a progressive MCA 205 tumor for 12 days contained approximately 40% T lymphocytes. On average, 20.3  $\pm$  3.5% of the T cells were identified to be L-selectin<sup>-</sup> cells. Upon purification by negative selection with Ab-coated flasks, the cells were cultured by the anti-CD3/IL-2 method for 5 days. The first evidence suggesting that L-selectin<sup>-</sup> cells represented cells at an



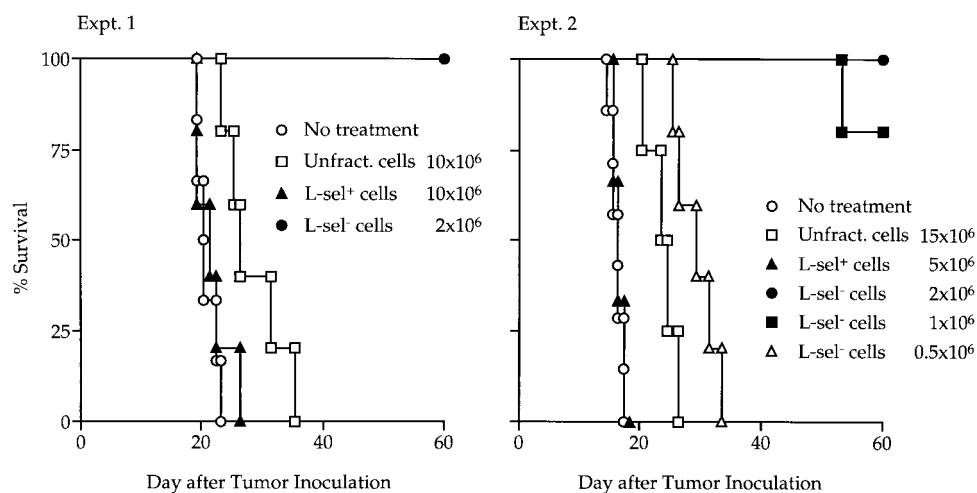
**FIGURE 1.** Proliferation of unfractionated, purified L-selectin<sup>+</sup>, and L-selectin<sup>-</sup> tumor-draining LN cells in response to anti-CD3 activation and IL-2 expansion.

activated stage came from their high proliferation rate in response to anti-CD3 and IL-2 stimulation. Figure 1 depicts the growth of unfractionated, L-selectin<sup>+</sup>, and L-selectin<sup>-</sup> cells in 11 independent experiments. L-selectin<sup>-</sup> cells increased an average of  $7.7 \pm 1$ -fold in 5 days, compared with  $3.7 \pm 1$ -fold and  $3.4 \pm 0.8$ -fold observed in L-selectin<sup>+</sup> and unfractionated LN cells, respectively. The therapeutic efficacy of these cells was analyzed quantitatively in adoptive immunotherapy of 3-day established intracranial MCA 205 tumors. As shown in Figure 2, the transfer of  $2 \times 10^6$  L-selectin<sup>-</sup> cells resulted in eradication of the tumor and long-term survival, whereas only minimal survival benefit resulted from the transfer of  $10 \times 10^6$  unfractionated cells (Expt. 1). In Experiment 2, a titration of L-selectin<sup>-</sup> cells in adoptive immunotherapy reveals that  $0.5 \times 10^6$  L-selectin<sup>-</sup> cells were therapeutically more effective than  $15 \times 10^6$  unfractionated LN cells. These data indicate that, on a per cell basis, L-selectin<sup>-</sup> cells are greater than 30 times more effective than unfractionated cells. L-selectin<sup>+</sup> cells, in

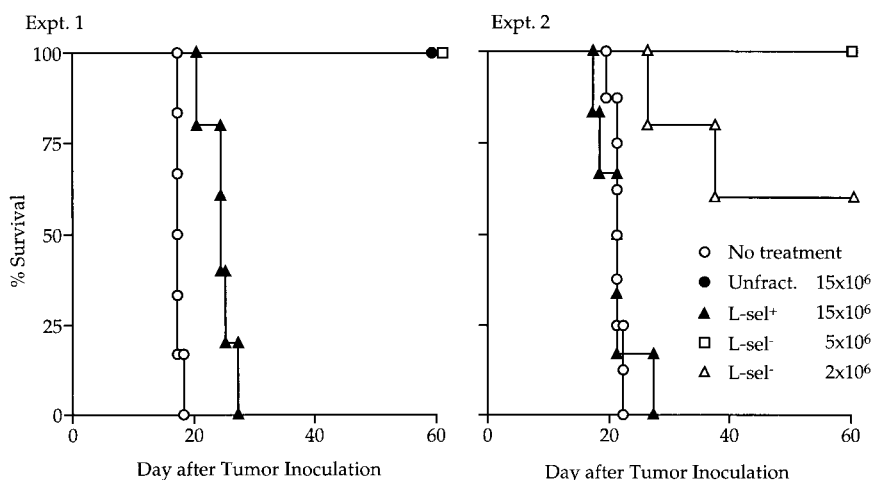
the range of 5 to  $10 \times 10^6$  cells, demonstrated no therapeutic effects when transferred to tumor-bearing mice.

Although tumor-draining LN T cells contained only ~20% L-selectin<sup>-</sup> cells, there was approximately 40% L-selectin<sup>-</sup> cells after in vitro activation. In addition, a significant proportion (~30%) of activated, purified L-selectin<sup>-</sup> cells regained the expression of L-selectin during the 5-day culture. We therefore analyzed the relative contribution of L-selectin<sup>+</sup> and L-selectin<sup>-</sup> cells separated after in vitro activation for their ability to mediate tumor regression. As demonstrated in two experiments in Figure 3, at the effector cell level, only L-selectin<sup>-</sup> cells were capable of mediating antitumor effects. Specifically, as few as  $2 \times 10^6$  isolated L-selectin<sup>-</sup> cells significantly prolonged the survival time and cured 8 of 10 tumor-bearing animals, while the transfer of as many as  $15 \times 10^6$  L-selectin<sup>+</sup> cells showed only minimum (Expt. 1) or no antitumor effects (Expt. 2). The antitumor reactivity mediated by the activated L-selectin<sup>-</sup> tumor-draining LN T cells is immunologically specific, as demonstrated in a criss-cross adoptive immunotherapy experiment (Fig. 4). L-selectin<sup>-</sup> cells derived from MCA 205 tumor-draining LNs mediated antitumor effects against the MCA 205 but not the MCA 207 tumor, whereas cells derived from MCA 207-draining LNs had potent antitumor reactivity against the MCA 207 tumor but only minimum reactivity against the MCA 205 tumor. Taken together, the above results clearly demonstrated that during the immune response to the growing tumor, a small subset of T cells down-regulated the LN homing receptor, L-selectin, as they differentiated to acquire specificity for tumor Ags. The immune cells proliferated vigorously in response to anti-CD3/IL-2 stimulation in vitro while maintaining their L-selectin<sup>-</sup> phenotype as effector cells for adoptive immunotherapy.

L-selectin<sup>-</sup> tumor-draining LN cells mediate potent antitumor effects against the experimental intracranial metastasis. Because the brain represents an immunologically privileged site and the entry of immune effector cells might be restricted by the presence of the blood-brain barrier, the L-selectin<sup>-</sup> cells might represent a subset of immune cells that were equipped to traffic to the central



**FIGURE 2.** Adoptive immunotherapy of 3-day established intracranial MCA 205 metastases with purified tumor-draining LN cells. Purified cells were activated by the anti-CD3/IL-2 method. All mice were treated with sublethal whole-body irradiation (500 R), followed by i.v. infusion of effector cells. In experiment 1, all groups consisted of five mice, except the no-treatment group, which contained six mice. The group that received  $2 \times 10^6$  L-selectin<sup>-</sup> (L-sel<sup>-</sup>) cells is significantly different ( $p \leq 0.01$ ) in survival time from all other groups. The group that received  $10 \times 10^6$  unfractionated (unfract.) cells is significantly different ( $p \leq 0.05$ ) from the no-treatment group. Mice that received  $10 \times 10^6$  L-selectin<sup>+</sup> (L-sel<sup>+</sup>) cells are not significantly different from the no-treatment group. In experiment 2, the groups ( $n = 5$ ) that received 1 or  $2 \times 10^6$  L-selectin<sup>-</sup> cells are significantly different ( $p \leq 0.01$ ) from all other groups. The group ( $n = 5$ ) that received  $0.5 \times 10^6$  L-selectin<sup>-</sup> cells is significantly different ( $p \leq 0.05$ ) from the no-treatment group ( $n = 7$ ). The group of mice ( $n = 4$ ) that received  $15 \times 10^6$  unfractionated cells is significantly different ( $p \leq 0.05$ ) from the no-treatment group. There is no significant difference between mice that received  $5 \times 10^6$  L-selectin<sup>+</sup> cells and the no-treatment group.



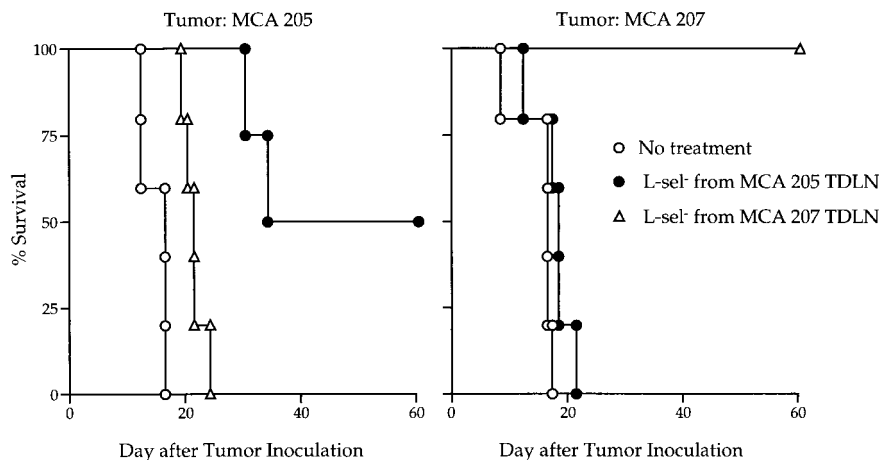
**FIGURE 3.** Antitumor effects of L-selectin<sup>+</sup> and L-selectin<sup>-</sup> cells separated after anti-CD3/IL-2 activation of whole tumor-draining LN cells. Experiments were designed similarly as those described in Figure 2. In experiment 1, all groups consisted of five mice except the no-treatment group, which contained six animals. The groups that received  $15 \times 10^6$  unfractionated or  $5 \times 10^6$  L-selectin<sup>-</sup> cells are significantly different ( $p \leq 0.01$ ) in survival time from mice that received no treatment or received  $15 \times 10^6$  L-selectin<sup>+</sup> cells. In experiment 2, the no-treatment control group consisted of eight mice, the group that received  $15 \times 10^6$  L-selectin<sup>+</sup> cells had six mice, and other groups consisted of five mice. The groups that received either  $5$  or  $2 \times 10^6$  L-selectin<sup>-</sup> cells are significantly different ( $p \leq 0.01$ ) from the no-treatment group or the group that received  $15 \times 10^6$  L-selectin<sup>+</sup> cells.

nervous system. To confirm that down-regulation of L-selectin is a general phenomenon of T cell immune response, we tested L-selectin<sup>-</sup> cells for their antitumor reactivity in a second model system, in which mice bearing established pulmonary metastases were treated with these cells (Table I). Mice were injected with MCA 205 tumor cells i.v. to establish pulmonary metastases. Three days later, they were treated by i.v. infusion of effector lymphocytes. The therapeutic efficacy of adoptive immunotherapy is proportional to the numbers of transferred cells. With unfractionated LN cells, the transfer of  $10 \times 10^6$  cells was capable of eradicating all metastatic nodules on the lung, whereas the transfer of  $3 \times 10^6$  unfractionated cells had no effects. The transfer of  $3 \times 10^6$  purified L-selectin<sup>-</sup> cells had potent antitumor reactivity against pulmonary metastases, whereas  $10 \times 10^6$  L-selectin<sup>+</sup> cells had no demonstrable antitumor effects. These results suggest that although the expression of L-selectin is best known to be associated with recirculation of lymphocytes through LNs, the down-regulation of

L-selectin on T cells in the LN after antigenic stimulation also represents an early physiologic signal of T cell priming.

#### *In vitro immunologic reactivities of L-selectin<sup>-</sup> LN T cells*

In previous studies (7), the anti-CD3/IL-2-activated tumor-draining LN cells lacked in vitro cytotoxicity despite their potent in vivo antitumor effects. Similarly, the isolated L-selectin<sup>-</sup> LN cells did not demonstrate cytotoxic effects against tumor target cells in the 4-h <sup>51</sup>Cr release assay (Fig. 5). The tumor target MCA 205 cells were readily lysed by LAK effector cells. In the absence of cytotoxicity, immune effector functions may be measured by the tumor-specific secretion of cytokines (16). We, therefore, examined the ability of L-selectin<sup>-</sup> cells to secrete cytokines upon stimulation with specific tumor cells as a potential mechanism in tumor regression. Purified L-selectin<sup>+</sup> and L-selectin<sup>-</sup> cells from LNs draining the MCA 205 tumor for 12 days were activated by the anti-CD3/IL-2 culture. The resulting cells were stimulated with



**FIGURE 4.** Specificity of adoptive immunotherapy mediated by the transfer of L-selectin<sup>-</sup> T cells. The experimental design is similar to that of the experiments described in Figure 2. Each mouse received  $2 \times 10^6$  activated, L-selectin<sup>-</sup> cells from either MCA 205 or MCA 207 tumor-draining LN. Each group consisted of five mice except the group of MCA 205 tumor-bearing mice treated with MCA 205 tumor-draining LN cells, which consisted of four mice. The groups of MCA 205 and MCA 207 intracranial tumor-bearing mice treated with cells derived from MCA 205 and MCA 207 tumor-draining LN, respectively, are significantly different ( $p \leq 0.01$ ) from all other groups. This experiment has been repeated with similar results.

Table I. Adoptive immunotherapy of 3-day established pulmonary MCA 205 metastases with tumor-draining LN cells separated based on L-selectin expression

Cells <sup>a</sup>	No. Transferred	No. Metastases <sup>b</sup>
	0	≥250
Unfractionated	10 × 10 <sup>6</sup>	0
Unfractionated	3 × 10 <sup>6</sup>	≥250
L-selectin <sup>+</sup>	10 × 10 <sup>6</sup>	≥250
L-selectin <sup>-</sup>	3 × 10 <sup>6</sup>	0

<sup>a</sup> Cells from inguinal LN draining a progressive MCA 205 tumor were separated and activated by the anti-CD3/IL-2 method and given i.v. to mice bearing 3-day pulmonary MCA 205 metastases.

<sup>b</sup> Lungs were harvested and metastases counted on day 21.

irradiated MCA 205 tumor cells for 24 h and the culture supernatants were assayed quantitatively for IFN- $\gamma$ , IL-2, IL-4, and IL-10 by ELISA. To assess the intrinsic ability to secrete cytokines by the LN cells, we also stimulated the purified cells with immobilized anti-CD3 mAb coated on the culture plates. L-selectin<sup>-</sup> cells secreted considerable amounts of all four cytokines when stimulated by anti-CD3. By contrast, L-selectin<sup>+</sup> cells were capable of secreting IFN- $\gamma$  and IL-2, but the amount of IL-4 and IL-10 released was minimal (Fig. 6). Upon stimulation with MCA 205 tumor cells, L-selectin<sup>-</sup> cells released substantially more cytokines than L-selectin<sup>+</sup> cells. In fact, L-selectin<sup>+</sup> cells did not secrete detectable IL-2, IL-4, or IL-10. The small amount of IL-10 released reflected secretion by MCA 205 tumor cells. The MCA 205 tumor did not secrete IFN- $\gamma$ , IL-2, or IL-4. The secretion of these cytokines by L-selectin<sup>-</sup> cells was tumor specific, as irradiated MCA 207 cells did not stimulate these lymphocytes to secrete a comparable amount of cytokines (Table II).

#### T cell phenotype of L-selectin<sup>-</sup> tumor-draining LN cells mediating antitumor effects

Freshly isolated tumor-draining LN cells contained 39 ± 7.8% T lymphocytes. Of the T cells, 51.5 ± 5.8% were CD4 and 41.3 ± 3.8% CD8 cells. Upon purification, L-selectin<sup>-</sup> T cells consisted of 62 ± 3.9% CD4 and 25.8 ± 0.8% CD8 cells. After activation, L-selectin<sup>-</sup> cells were composed of 25.5 ± 4.5% CD4 and 54.7 ±

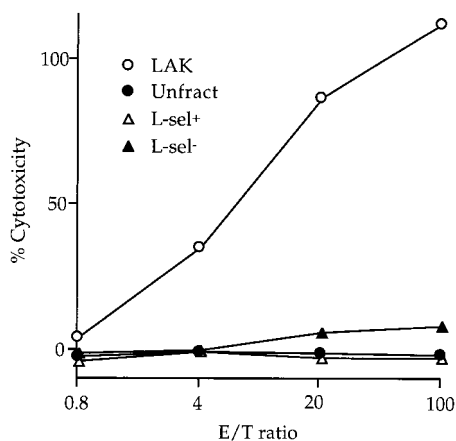


FIGURE 5. Lack of cytotoxicity of the activated tumor-draining LN cells. Cells from LN draining the MCA 205 tumor for 12 days were separated into L-selectin<sup>+</sup> and L-selectin<sup>-</sup> cells before activation by the anti-CD3/IL-2 method. LAK cells were prepared by incubating normal B6 spleen cells in 1000 U/ml of IL-2 for 3 days. The effector cells were tested against <sup>51</sup>Cr-labeled MCA 205 tumor cells in the cytotoxicity assay. This is an example of three independent experiments.

Table II. Specificity of cytokine secretion by activated L-selectin<sup>-</sup> tumor-draining LN T cells

Stimulation	Cytokine (pg/ml) <sup>a</sup>			
	IFN- $\gamma$	IL-2	IL-4	IL-10
None	<10	<10	<10	<10
MCA 205	971	52	<10	205
MCA207	248	<10	<10	44

<sup>a</sup> Cytokine release was measured by ELISA, and values reflect the secreted cytokines after subtraction of low background amounts measured from irradiated tumor cells alone.

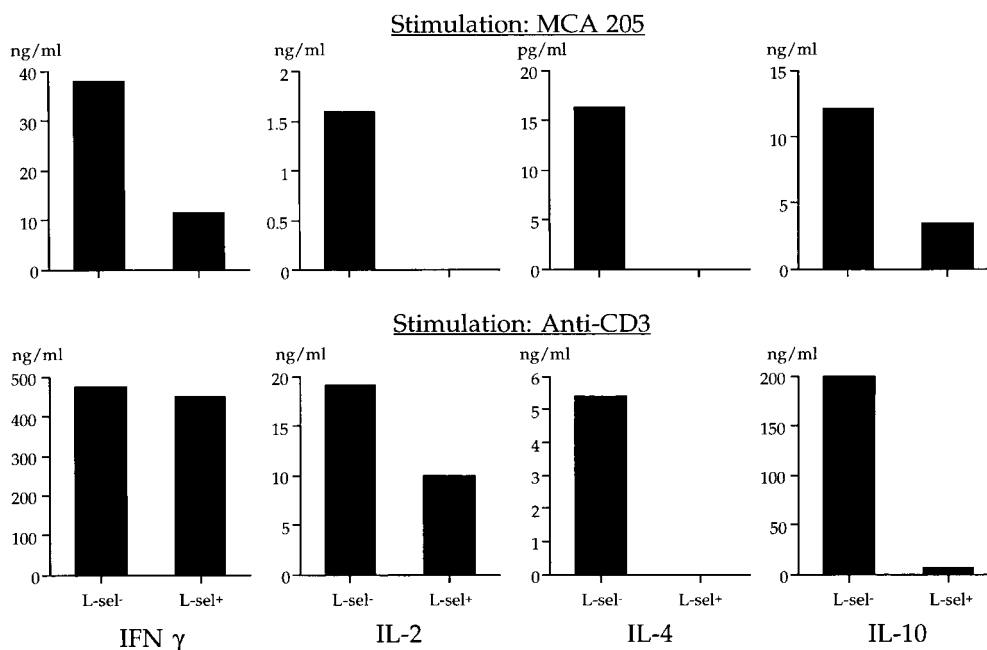
7.4% CD8 cells. Interestingly, a small subset (13.1 ± 6.7%) of activated L-selectin<sup>-</sup> cells expressed neither CD8 nor CD4 but apparently TCR $\alpha\beta$ <sup>+</sup>. To determine the subset of T cells responsible for mediating antitumor effects, we tested the therapeutic efficacy of L-selectin<sup>-</sup> cells after depleting either CD4 or CD8 cells. The results of phenotype analysis are depicted in Figure 7. In adoptive immunotherapy, CD8-depleted L-selectin<sup>-</sup> LN cells mediated potent antitumor effects, as all treated mice were cured of their tumors. Depletion of CD4 T cells, on the other hand, abrogated the effector function of the activated L-selectin<sup>-</sup> cells (Fig. 8A). These results suggest that CD4 T cells possessed antitumor reactivity. The small population of double negative T cells (see Fig. 7.) did not seem to play a significant role because the CD4-depleted population contained these cells but did not mediate tumor regression. To confirm this, we further separated CD8-depleted L-selectin<sup>-</sup> cells into CD4 and double negative cells using magnetic beads. To test their therapeutic efficacy, mice with 3-day established intracranial MCA 205 tumors were treated with these separated cells. While mice that received CD4 cells showed prolongation of survival, and three of four animals were apparently cured of the disease, mice that received double negative cells succumbed to the growing tumor with a survival time similar to that of nontreated mice (Fig. 8B). Thus, CD4 T cells constituted all the antitumor reactivity in purified L-selectin<sup>-</sup> tumor-draining LN cells.

#### Tumor-specific cytokine secretion by CD4 L-selectin<sup>-</sup> cells

In an attempt to correlate in vitro reactivity of CD4 L-selectin<sup>-</sup> LN cells with their in vivo therapeutic efficacy, we assessed the secretion of T cell cytokines by these cells upon stimulation with specific tumor cells (Fig. 9). Both purified CD4 (CD8-depleted) and CD8 (CD4-depleted) cells secreted large amounts of IFN- $\gamma$ , IL-2, IL-4, and IL-10 in response to anti-CD3 stimulation, indicating their intrinsic ability to synthesize cytokines. However, when stimulated with MCA 205 tumor cells, CD4 cells secreted large amounts of IFN- $\gamma$ , IL-2, and IL-10 and small amounts of IL-4. By contrast, CD8 cells released only negligible amounts of IL-2, IL-4, or IL-10. Although a significant quantity (4.5 ng/ml) of IFN- $\gamma$  was secreted by CD8 cells, this amount was less than that secreted by CD4 cells (8.1 ng/ml). The secretion of cytokines by CD4 cells appeared to be immunologically specific. Stimulation with the immunologically distinct tumor MCA 207 resulted in far less cytokine release (Table III). These results thus suggest that a correlation may exist between the in vitro tumor-specific stimulation of cytokine release and the in vivo therapeutic efficacy of these CD4 cells.

## Discussion

Previous studies have shown that activation of L-selectin<sup>+</sup> T cells in vitro by alloantigen, or polyclonally with Con A or anti-CD3, results in the down-regulation of L-selectin on the majority of T

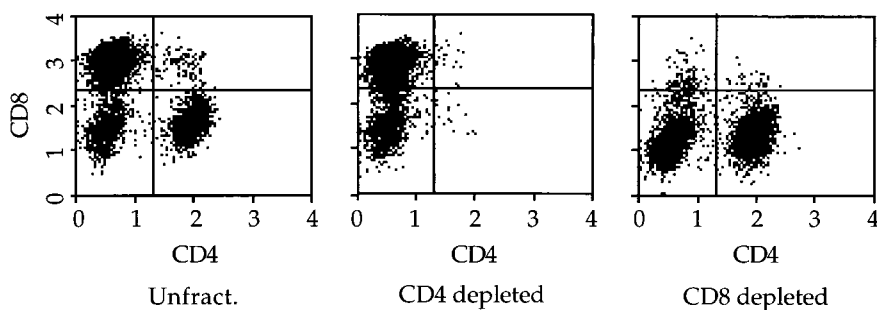


**FIGURE 6.** ELISA measurement of cytokine secretion in the medium of activated L-selectin<sup>+</sup> or L-selectin<sup>-</sup> tumor-draining LN cells stimulated with MCA 205 tumor cells or immobilized anti-CD3 mAb.

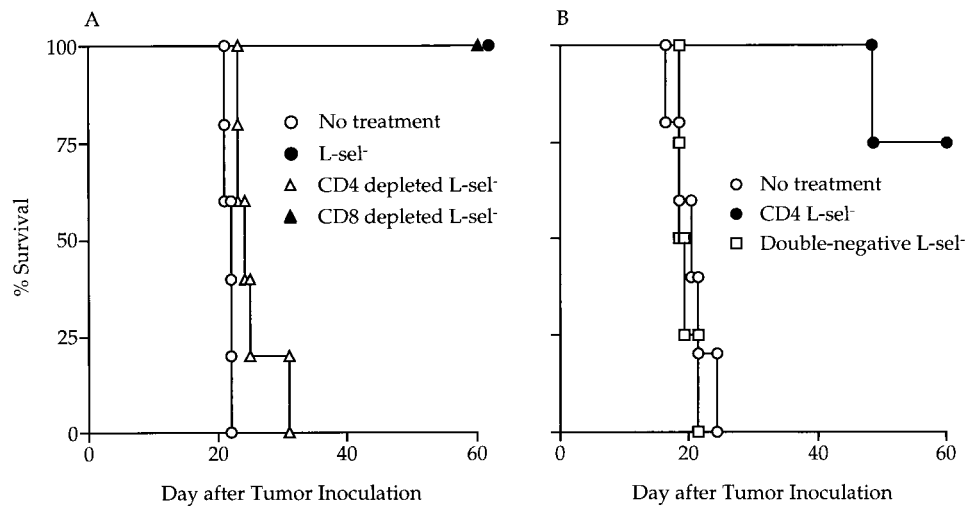
lymphocytes (17–19). Some evidence also suggests that down-regulation of L-selectin on T cells occurs *in vivo* during a normal immune response to soluble Ags and to allogeneic cells (20, 21). Our recent results described in a brief report (10) indicated that a small population of T cells with low expression of L-selectin in the tumor-draining LN were sensitized to the growing tumor. Although the homing receptor L-selectin on lymphocytes serves a primary function of regulating cell circulation to LNs through the binding to specialized high endothelial venules, the down-regulation of L-selectin on T cells also appears to reflect an early physiologic event of a T cell responding to antigenic stimulation (22). Experiments described in this report further examined the immunologic characteristics of L-selectin<sup>-</sup> T cells isolated from tumor-draining LNs. The results indicate that within LNs draining a progressively growing tumor, a population of CD4 cells differentiates into antigenically committed immune cells with a concomitant loss of surface L-selectin expression. Although these L-selectin<sup>-</sup> immune cells conceivably could result from the expansion of L-selectin<sup>-</sup> precursor cells, the presence of L-selectin on the vast majority of resting CD4 cells argues against this. These CD4 cells further differentiate and expand during *in vitro* stimulation with anti-CD3 and IL-2 into effector cells capable of mediating the re-

gression of established tumors when systemically transferred into animals. The exact stage of differentiation of these freshly harvested L-selectin<sup>-</sup> CD4 cells is not known. Direct testing of their antitumor efficacy in adoptive immunotherapy indicated that they possessed effector function to mediate tumor regression. However, on a per cell basis, freshly isolated L-selectin<sup>-</sup> cells were at least fourfold less effective than the same cells after anti-CD3/IL-2 stimulation (data not shown). These findings suggest that although non-specific, the *in vitro* culture may promote further maturation or differentiation of these CD4 cells.

Theoretically, both CD4 and CD8 T cell populations can mediate tumor-specific therapy. However, the requirements for generating and validating the specificity of CD4 and CD8 antitumor effector T cells are distinctly different. CD4 cells require MHC class II APCs to process and present tumor Ags whereas CD8 T cells require a viable stimulator cell such as tumor for presentation of the nominal target Ag but, in addition, require other cells for providing soluble accessory molecules such as IL-1. In recent years, the demonstration of a predominant role of cellular immune reactions played in the destruction of malignant tumors has concentrated efforts on the identification of tumor Ags recognized by T lymphocytes. Because CTLs are readily cloned from peripheral



**FIGURE 7.** FACS analysis of activated L-selectin<sup>-</sup> cells. CD4 cells represent 26, 0.2, and 65% of unfractionated, CD4-depleted, or CD8-depleted cell populations, respectively.

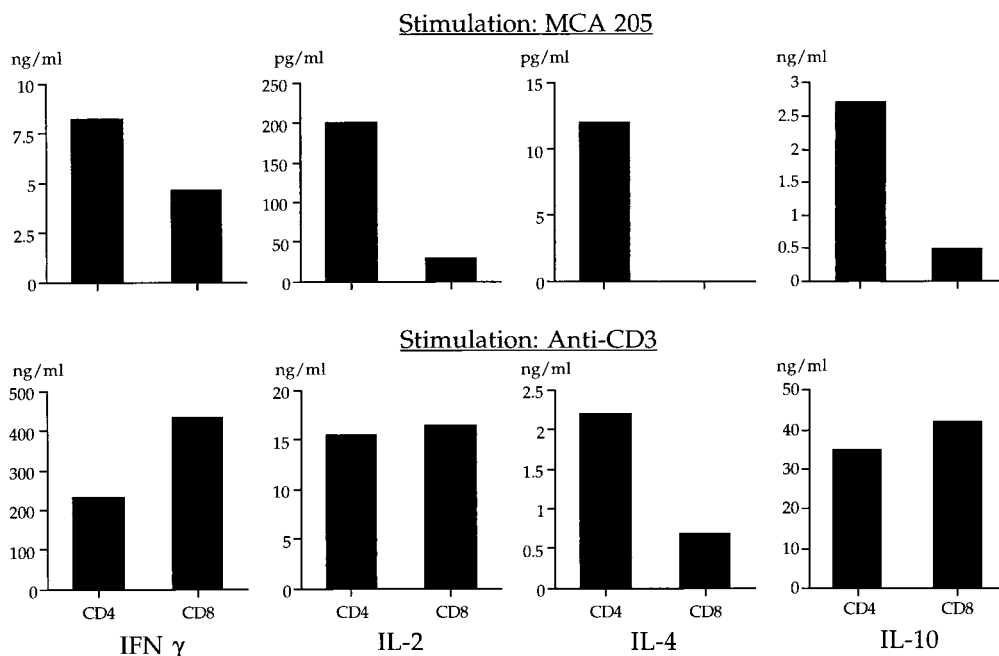


**FIGURE 8.** Phenotype of activated L-selectin<sup>-</sup> tumor-draining LN cells that mediates the regression of intracranial MCA 205 metastases. The experimental design is similar to that of the experiments described in Figure 2. All groups consisted of five mice unless otherwise indicated. *A*, Each mouse received  $2 \times 10^6$  of the indicated cells. The groups that received L-selectin<sup>-</sup> or CD8-depleted L-selectin<sup>-</sup> cells are significantly different ( $p \leq 0.01$ ) from the no-treatment group, and the group that received CD4-depleted L-selectin<sup>-</sup> cells is not significantly different from the no-treatment group. *B*, Each mouse was given  $0.5 \times 10^6$  of the indicated cells i.v. Mice that received CD4 L-selectin<sup>-</sup> cells ( $n = 4$ ) are significantly different ( $p \leq 0.01$ ) from mice that received no treatment or were treated with double negative cells ( $n = 4$ ). Results are representative of three independent experiments.

blood or tumor-infiltrating lymphocytes of melanoma patients, the majority of tumor antigenic peptides identified are associated with MHC class I molecules, which are recognized by CD8 T lymphocytes (11, 12, 23). While CD8 cells are demonstrably capable of mediating antitumor effects via perhaps direct cytotoxicity, examples of CD4-mediated tumor regression are rare. CD4 cells potentially offer several distinct advantages and can mediate tumor regression by a variety of mechanisms. For example, by secreting IL-2 and other cytokines, CD4 cells can activate and amplify existing host-specific CD8 CTLs as well as nonspecific NK or LAK cells. Secretion of other lymphokines such as TNF- $\alpha$ , IFN- $\gamma$ , and granulocyte-macrophage CSF by CD4 cells may activate macrophages that will directly or indirectly lyse tumor cells. CD4 T cells secrete

endogenous T cell growth factors such as IL-2, IL-4, and IL-7 and thus are capable of proliferating in response to antigenic stimulation without a requirement for exogenously provided growth factors. Our findings of the extraordinary efficacy of CD4 cells in mediating the regression of established tumors support the hypothesis that CD4 cells alone when sensitized appropriately can mediate potent antitumor effects.

The MCA tumors used for our experiments express abundant MHC class I Ags but they do not express MHC class II Ags on their surface. Since CD4 cells cannot directly interact with tumor cells either in the afferent or efferent phase of the immune response, host APCs must have ingested and processed the tumor-specific Ags and presented them in the context of the MHC class



**FIGURE 9.** ELISA measurement of cytokine secretion by activated L-selectin<sup>-</sup>, CD4, or CD8 T cells.



Table III. Specificity of cytokine secretion by CD4 cells from activated L-selectin<sup>-</sup> tumor-draining LN cells

Stimulation	Cytokine (pg/ml) <sup>a</sup>			
	IFN- $\gamma$	IL-2	IL-4	IL-10
None	<10	<10	<10	<10
MCA 205	8163	200	12	1192
MCA207	1194	<10	<10	71

<sup>a</sup> See Table II footnote.

II molecule to the immune system. Similarly, the antitumor effects mediated by the transferred CD4 cells must have been initiated through the presentation of tumor Ags by host APCs. The tumor-specific secretion of cytokines such IL-2 and IFN- $\gamma$  in vitro is consistent with the hypothesis that CD4 cell-mediated tumor destruction is the indirect result of cytokine production at the tumor site similar to that described for eliciting a delayed-type hypersensitivity reaction.

Alternative mechanisms of CD4 cell-mediated tumor regression include induction of CD8 CTLs with antitumor specificity. In previous studies, we demonstrated that tumor-draining LN T cells could differentiate into cytotoxic effector cells after stimulation with irradiated tumor cells in the presence of low concentrations of IL-2 (24, 25). The resulting cells were of CD8 phenotype exclusively and, upon adoptive transfer, they mediated specific antitumor effects. More recently, the method of anti-CD3/IL-2 stimulation has been investigated and used as a convenient means to generate therapeutic T cells from tumor-draining LNs (7). However, phenotype analysis indicated that in this situation, both CD4 and CD8 subsets of cells were required for antitumor efficacy (8, 26). The role of CD4 cells was to provide a helper function to CD8 cells, since CD8 cells alone could mediate tumor regression provided that exogenous IL-2 was administered as a conjunctive treatment (26). The current study clearly demonstrated that by separating L-selectin<sup>-</sup> T cells from tumor-draining LNs, CD4 cells developed independently into potent immune effector cells, which did not occur when unseparated LN cells containing L-selectin<sup>+</sup> cells were activated in vitro. Apparently, both the culture system and cell composition determine the type of effector cells generated. In the absence of L-selectin<sup>+</sup> cells, CD8 cells (L-selectin<sup>-</sup>) did not acquire effector cell function. This is in contrast to the activation of unfractionated LN cells, which contained both L-selectin<sup>+</sup> and L-selectin<sup>-</sup> T cells. Instead of developing into effector cells, CD4 cells probably served a major function to promote the generation of CD8 effector cells from L-selectin<sup>+</sup> precursor cells. If this interpretation is correct, it would be possible to generate CD8 effector cells from naive, resting L-selectin<sup>+</sup> CD8 lymphocytes when cocultured with L-selectin<sup>-</sup> CD4 T cells from tumor-draining LNs. This possibility is being investigated in our laboratory.

Perhaps the most significant finding in this study is the documentation, for the first time, of the potential of CD4 cells to mediate therapeutic effects against chemically induced tumors. The CD4 Th population contains two functionally distinct subpopulations that may be differentiated on the basis of the sets of lymphokines each produces. These Th subpopulations may be mutually inhibitory for each other during the evolution of an immune response (27). The activated L-selectin<sup>-</sup> CD4 cells secreted both Th1- and Th2- associated cytokines upon stimulation with specific tumor cells, suggesting that they consisted of a heterogeneous population of Th cells. To estimate the percentage of CD4 cells for cytoplasmic expression of IFN- $\gamma$ , IL-2, and IL-4, we found that 42.1, 43.3, and 52.3% cells stained positively for IFN- $\gamma$ , IL-2, and

IL-4, respectively. These results collaborate well with ELISA data and support the conclusion that the CD4 cells are likely a mixture of Th1 and Th2 cells. However, the possible presence of Th0 cells cannot be excluded. Although the division of cytokine-producing Th cells into Th1 and Th2 has provided a valuable framework to investigate the regulatory role of T cells, analyses of T cell clones in different immune responses suggest that classification into discrete subsets may not be always realistic (28). In our study, it remains unclear which CD4 cells are responsible for the observed in vivo antitumor effects, although we favor the hypothesis that cells with Th1-type characteristics may play a dominant role because of their ability to induce macrophage activation and delayed-type hypersensitivity responses. Additional experiments are necessary to elucidate the precise function of each Th subpopulation.

In summary, our study demonstrated that a minor subpopulation of CD4 T cells in LNs responding to a progressively growing tumor became sensitized to the tumor-specific Ags with concurrent down-regulation of L-selectin expression. These CD4 cells, when purified in the L-selectin<sup>-</sup> fraction, responded to anti-CD3/IL-2 stimulation, resulting in the generation of potent therapeutic cells. The L-selectin<sup>-</sup> CD4 cells secreted both Th1- and Th2-type lymphokines after exposure to specific tumor cells, which correlate with their in vivo antitumor reactivity. In our clinical adoptive immunotherapy protocols for recurrent cancer, LNs draining the site of autologous tumor immunization were used to generate immune effector cells. Such LN cells contained various proportions of L-selectin<sup>-</sup> T cells (10 to 50%). The isolation and in vitro activation of these cells may allow the generation of highly potent effector cells for cancer therapy. The ability to specifically secrete cytokines by the activated cells, if confirmed, may facilitate the identification of therapeutically effective cells in vitro, the lack of which has been a major impediment to the development of adoptive immunotherapy of cancer in humans.

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