Tumor Infiltration by Adoptively Transferred T Cells Is Independent of Immunologic Specificity but Requires Down-Regulation of L-Selectin Expression

Jørgen Kjaergaard and Suyu Shu

*J Immunol* 1999; 163:751-759;
http://www.jimmunol.org/content/163/2/751

---

**References**  This article *cites* 26 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/163/2/751.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Tumor Infiltration by Adoptively Transferred T Cells Is Independent of Immunologic Specificity but Requires Down-Regulation of L-Selectin Expression

Jørgen Kjaergaard and Suyu Shu

Adoptive immunotherapy with anti-CD3/IL-2 activated tumor-draining lymph node (LN) T cells is capable of eradicating tumor established at various histological sites. Tumor-specific effector lymphocytes have recently been identified to be LN T cells with down-regulated L-selectin (L-selectin	extsuperscript{−}). Using fluorochrome labeling, the present study determined the early trafficking pattern of systemically transferred cells. In mice with 10-day established pulmonary 3-methylcholanthrene (MCA) 205 metastases, accumulation of cells in tumors was evident as early as 2 h after i.v. cell transfer, and, by 24 h, >50-fold higher numbers of cells were seen in metastases than in normal tissues. Similarly, transferred cells selectively infiltrated s.c. tumors, albeit at a lower rate. Analysis of the transferred cells isolated from recipient mice revealed that tumor-infiltrating cells were mostly L-selectin	extsuperscript{−} (>95%). By contrast, only 24% and 58% L-selectin	extsuperscript{−} cells were found in the LN and spleen, respectively. The ability of L-selectin	extsuperscript{−} cells to accumulate at tumor sites was confirmed by the transfer of purified cell populations. Despite this selective tumor infiltration, the trafficking pattern did not reflect antigenic specificity, and tumor regression occurred only after the transfer of tumor-specific effector cells. These results, thus, suggest that there are two distinct mechanisms operative in successful adoptive immunotherapy. Early infiltration of tumors by transferred cells is dictated by the physiological properties of cells and is independent on their immunologic specificity. Tumor regression, however, requires immunologically specific interactions at the site of tumor.

accumulation, selective retention, or proliferation of adoptively transferred murine tumor-infiltrating lymphocytes (TILs) at the tumor site. They hypothesized that a minute number of antitumor effector cells might be sufficient to initiate the regression of an established metastases. Because of the heterogeneity in function and specificity of the effector cell populations used in most studies, it must be presumed that only a small fraction of the transferred cells has the antitumor reactivity and the potential to mediate tumor regression in vivo. It is, therefore, difficult to establish a correlation between cells infiltrating tumor tissue and cells possessing immunologic specificity toward the tumor. In our previous study, several cloned T cell lines were established from the anti-CD3/IL-2 activated tumor-draining LN T cells by repeated stimulation with tumor cells (16). These long-term cultured T cells demonstrated tumor accumulation that correlated with their specificity of antitumor effects when tested against advanced pulmonary metastases. In the clinical setting, however, cloning is not always feasible and practical because culture duration and manipulation often results in the generation of cells with altered physiologic characteristics. To gain insights into the mechanism of tumor eradication by the systemic transfer of short-term cultured cells, we conducted an investigation with a focus on the early trafficking pattern and the distribution of transferred effector T cells.

T cells from tumor-draining LNs display a heterogeneous expression of the homing molecule, L-selectin (CD62L). Recent experimental results identified a small population (~20%) of LN T cells with down-regulated L-selectin (L-selectin ~) to be the sole immune effector cells mediating the regression of established tumors (17, 18). In the current study, migration and tumor infiltration of the transferred cells were also analyzed with particular reference to the expression of L-selectin. Our results provide unequivocal evidence indicating that tumor infiltration by transferred cells reflects the physiologic characteristics, but not the immunologic specificity, of the activated draining LN T cells. However, the presence of specifically sensitized T cells in the transferred cell population is required for initiating tumor eradication.

Materials and Methods

Animals

Female C57BL/6N (B6) mice, 6–8 wk old, were purchased from the Biologic Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). They were maintained in a specific pathogen-free environment according to National Institutes of Health guideline and were used for experiments at the age of 8–12 wk.

Tumors

The MCA 205 fibrosarcoma is a 3-methylcholanthrene (MCA)-induced tumor of B6 origin (9). The tumor has been routinely passed in vivo by serial s.c. transplantation in syngeneic mice and was used within the fifth tumor of B6 origin (9). The tumor has been routinely passed in vivo by health guideline and were used for experiments at the age of 8–12 wk. Tumors

Tumor-draining LN cells

B6 mice were inoculated s.c. with 1 × 10^6 MCA 205 tumor cells on both flanks. Twelve days later, tumor-draining inguinal LNs were harvested, and single cell suspensions were prepared mechanically as described previously (3). LN cells were activated with anti-CD3 mAb (145-2C11) immobilized on 24-well tissue culture plates at 4 × 10^6 cells/ml of complete medium (CM) for 2 days. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh t-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone (all from Life Technologies, Grand Island, NY), and 5 × 10^-5 M 2-ME (Sigma). After anti-CD3 activation, cells were harvested, washed, and further cultured in gas-permeable culture bags (Baxter Healthcare, Deerfield, IL) at 2 × 10^6 cells/ml of CM supplemented with 4 U IL-2/ml. Three days later, cells were harvested, washed, and resuspended in HBSS for adoptive immunotherapy. In some experiments, splenic T cells from normal B6 mice were used as control cells. T cells in the spleen cell suspensions were concentrated by passing through nylon wool columns (Robbins Scientific, Sunnyvale, CA) as previously described (18). These T cells were activated and expanded identically to that of tumor-draining LN cells.

Adoptive immunotherapy

B6 mice were injected i.v. with 3 × 10^5 MCA 205 or MCA 207 tumor cells suspended in 1 ml of HBSS to initiate pulmonary metastases. Ten days after tumor inoculation, all mice received sublethal WBI (500 cGy) delivered from a 106Cs irradiator (J.C. Shepard & Associates, Glendale, CA). Activated T cells were then given i.v. to each mouse at numbers indicated. On day 21, mice were killed and metastatic tumor nodules on the surface of lungs enumerated after counterstaining with India ink (19). Lungs with >250 metastatic nodules were assigned ≥250, as this was the maximum number of metastases that can be reliably counted.

Therapeutic efficacy of transferred cells was also assessed for the treatment of tumors. In this system, mice were inoculated s.c. with 6 × 10^6 MCA 205 tumor cells in 100 μl HBSS. Three days later, mice received sublethal WBI (500 cGy) followed by i.v. transfer of cells. The diameters of s.c. tumors were measured twice weekly with a vernier caliper, and size was recorded as an average of perpendicular measurements and presented in mm as the mean of a group.

Trafﬁcking of tetramethylrhodamine isothiocyanate (TRITC)-labeled cells

For ﬂuorochrome labeling, cells were washed and resuspended at 1 × 10^6/ml in RPMI 1640 containing 0.5 μg TRITC/ml (Sigma). Following incubation at 37°C in a 5% CO2 atmosphere for 30 min, the cells were washed twice in RPMI 1640 and resuspended in HBSS before adoptive transfer. At a different time point after i.v. transfer of TRITC-labeled cells into tumor-bearing mice, samples of organs and tissues, including the lung, liver, kidney, spleen, brain, skin with s.c. tumors, and LN, were harvested and ﬁxed in 4% formalin for 24 h, before being placed in 30% sucrose for an additional 24 h. These tissues were snap frozen in n-Hexane at −70°C, and 8-μm cryosections were prepared from 10–20 different cutting surfaces. TRITC-labeled cells were identiﬁed and counted using a ﬂuorescence microscope (Olympus, New Hyde Park, NY) equipped with a filter combination of BP545 for rhodamine detection. In lungs, the area of metastases was estimated, and the number of TRITC-labeled cells in the tumor, as well as in the surrounding normal tissue, were counted using a 40× objective and a reticule containing 100 squares. The number of TRITC-labeled cells in 20–30 metastases from each lung was averaged and presented as the number of cells per 0.024 mm² because this was the average size of 10-day pulmonary metastases. Afterward, the sections were counterstained with Meyer’s hematoxylin to conﬁrm the presence of metastases by light microscopy.

For estimation of labeled cells in blood, mice were anesthetized with 0.8 mg pentobarbital i.p. before collecting 100 μl blood from the left ventricle of the heart. RBC were lysed with ammonium chloride-potassium buffer at room temperature for 1 min, washed twice in RPMI 1640, and ﬁxed in 1% paraformaldehyde. The percentage of TRITC-labeled cells was estimated by FACS analysis, and the average number of labeled cells/ml blood was calculated.

Isolation and characterization of adoptively transferred cells in tumors and lymphoid organs

Twenty-four hours after i.v. transfer of TRITC-labeled cells, lungs containing metastatic tumors or skin with s.c. tumor nodules were collected, and single cell suspensions were prepared by digesting minced tissues in 40 ml of HBSS containing 0.1% collagenase, 0.01% DNase, and 2.5 U/ml hyaluronidase (Sigma) for 1 h at room temperature. Preliminary experimental results indicated that treatment of LN cells with a mixture of these three nonproteolytic enzymes did not interfere with the detection of L-selectin by immunofluorescence staining (data not shown). Therefore, in routine analyses, single cell suspensions from LNs or spleens were prepared mechanically by teasing organs with needles followed by pressing tissue fragments with the blunt end of a plastic syringe. The cells were ﬁltered through a layer of no. 100 nylon mesh, washed twice in RPMI 1640...
L-selectin and 2–3 bridoma (mel-14) ascites fluid at 1:3000 dilution. The cells were washed

containing 5% FCS, and leukocytes were separated by centrifugation through a ficoll-hypaque gradient (Histopaque-1119; Sigma). Cells were stained by indirect immunofluorescence for the expression of L-selectin. The membrane fluorescence (FITC) and intracellular staining (TRITC) were analyzed by two-color flow cytometry to characterize L-selectin expression on the transferred cells.

Fractionation of activated LN cells based on L-selectin expression

Activated tumor-draining LN cells were separated into L-selectin+ and L-selectin− cells with the use of Rat T-cell immunocolumns that contained glass beads coated with goat anti-rat Ig (Biotex Laboratories, Edmonton, Canada). Cells were incubated for 20 min at 4°C with the L-selectin hybridoma (mel-14) ascites fluid at 1:3000 dilution. The cells were washed and 2–3 × 10³ in 4 ml of HBSS was loaded onto each immunocolumn, according to the manufacturer’s instructions. After 30 min of incubation, the first 15-ml pass-through fraction containing highly purified (>95%) L-selectin+ cells was collected. After rinsing the column with 30 ml HBSS containing 2% FCS, the glass beads were transferred to a 50-ml tube, along with 30 ml HBSS. The L-selectin+ cells were dislodged from the beads by vigorous pipetting. In some experiments, immunocolumn was incubated with 2 ml of L-selectin hybridoma ascites fluid at 1:3000 dilution for 1 h. The column was then washed with 30 ml HBSS before 2–3 × 10³ uncoated cells in 4 ml were allowed to run through the column bed. Using this method of purification, the recovered L-selectin− cells had little bound mAb on their surface.

Statistical analysis

The significance of differences in numbers of pulmonary metastases between groups was analyzed by the Wilcoxon rank-sum test. Differences of numbers of cells infiltrating tumor tissues were analyzed by the Student’s t test. A two-tailed p value of ≤0.05 was considered significant.

Results

Therapeutic efficacy of activated tumor-draining LN T cells against advanced pulmonary metastases

Previous studies have shown that freshly harvested LN cells, draining the progressively growing MCA 205 sarcoma, contained ~35% of CD3+ T cells with equal proportions of CD4+ and CD8+ cells (20). After in vitro stimulation with anti-CD3 for 2 days, followed by culture in 4 U/ml of IL-2 for 3 days, these activated cells were virtually all T cells (>95% Thy1.2 and TCRαβ) with predominately CD8 (~70%) and some CD4 (~20%) T cells. The in vivo antitumor efficacy of anti-CD3/IL-2 activated tumor-draining LN T cells has been well documented for the treatment of 3-day tumors established in the lung and skin, as well as in the brain (5–9). To facilitate the study of cell trafficking, we attempted to treat 10-day established MCA 205 pulmonary metastases. At the time of treatment, metastatic nodules were macroscopic and clearly visible. In addition to the systemic transfer of activated cells, mice were also pretreated with WBI (500 cGy) to facilitate homing and detection of the transferred cells. This dose of WBI might eliminate tumor-induced immunosuppression, which at times interfered with the function of transferred immune cells, especially in the case of therapy against s.c. tumors (7, 10). In three independent experiments, a dose-response relation between the number of tumor-draining LN T cells transferred, and the antitumor efficacy was established (Table I). Complete tumor regression was seen in all experiments after the transfer of 50 × 10⁶ activated LN cells. Significant antitumor effects were also seen in animals receiving as few as 20 × 10⁶ cells. Mice treated with 50 × 10⁶ activated naive spleen T cells failed to mediate antitumor effects. Adoptive immunotherapy mediated by tumor-draining LN T cells was also found to be immunologically specific. As demonstrated in a criss-cross experiment (Table II), cells derived from LNs draining the MCA 205 sarcoma were therapeutically effective against the MCA 205 tumor, but not against the antigenically distinct MCA 207 tumor. Similarly, activated MCA 207 tumor-draining LN T cells were only effective against the MCA 207, but not the MCA 205, tumor. These results demonstrate that despite the polyclonal T cell activation induced by the anti-CD3, draining LN cells mediated tumor-specific effects, and the specificity was determined during the in vivo sensitization of the tumor-draining LN. Apparently, the in vitro activation stimulated the committed cells to functionally mature into effector cells, which does not require antigenically specific stimulation.

Table I. Adoptive immunotherapy of 10-day established MCA 205 pulmonary metastases with anti-CD3/IL-2 activated MCA 205 tumor-draining LN cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. Cells Transferred</th>
<th>Mean No. Pulmonary Metastases (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>—</td>
<td>0</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Normal splenic T cells</td>
<td>50 × 10⁶</td>
<td>&gt;250</td>
</tr>
<tr>
<td>MCA 205 tumor-draining LN</td>
<td>50 × 10⁶</td>
<td>0</td>
</tr>
<tr>
<td>cells</td>
<td>40 × 10⁶</td>
<td>11 (8)†</td>
</tr>
<tr>
<td></td>
<td>20 × 10⁶</td>
<td>72 (17)†</td>
</tr>
<tr>
<td></td>
<td>10 × 10⁶</td>
<td>205 (25)</td>
</tr>
</tbody>
</table>

* Cells from inguinal LNs draining progressively growing MCA 205 for 12 days were activated by the anti-CD3/IL-2 method and given i.v. to mice bearing 10-day established pulmonary MCA 205 metastases. All mice were WBI (500 cGy) before cell transfer.

† Lungs were harvested on day 21. Each experimental group consisted of five mice.

Table II. Specificity of adoptive immunotherapy mediated by anti-CD3/IL-2 activated tumor-draining LN cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. Cells Transferred</th>
<th>Mean No. Pulmonary Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MCA 205</td>
</tr>
<tr>
<td>—</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MCA 205 tumor-draining LN</td>
<td>50 × 10⁶</td>
<td>&gt;250</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td>0†</td>
</tr>
<tr>
<td>MCA 207 tumor-draining LN</td>
<td>50 × 10⁶</td>
<td>&gt;250</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The adoptive immunotherapy procedure is the same as that in Table I. Each experimental group consisted of five mice.

† Significantly different from other groups.
FIGURE 1. Kinetics of infiltration by adoptively transferred anti-CD3/IL-2 activated tumor-draining LN T cells. Mice with 10-day established pulmonary MCA 205 metastases were given i.v. transfer of $50 \times 10^6$ TRITC-labeled cells. Twenty metastases from each lung (three mice/group) were examined and the average numbers ± SD of fluorescent cells per 0.024 mm$^2$ were calculated. Traffic patterns of fresh naive splenic T cells (a), anti-CD3/IL-2 activated naive splenic T cells (b), anti-CD3/IL-2 activated MCA 207 tumor-draining LN cells (c), and anti-CD3/IL-2 activated MCA 205 tumor-draining LN cells (d) were examined. *, Significantly different ($p < 0.01$) between the two groups.

Trafficking of systemically transferred T cells in metastatic nodules

It was assumed that because of the therapeutic efficacy and specificity of adoptive immunotherapy, only immunologically specific T cells were able to penetrate and localize in the tumor. To seek direct evidence to support or dispute this assumption, cells labeled with TRITC were used to study their in vivo distribution after systemic transfer. Preliminary experiments indicated that the current labeling method did not affect cells’ functions, including in vivo antitumor activity and specificity (data not shown). It was therefore assumed that the trafficking pattern of the functional cells would be minimally affected by TRITC labeling.

After the transfer of labeled cells, tissue was harvested at different time points, and quantitative analysis was performed. As shown in Fig. 1d, as early as 2 h after i.v. transfer of a therapeutic number ($50 \times 10^6$) of MCA 205 tumor-draining LN cells, accumulation of fluorescent cells in MCA 205 metastatic nodules was significantly higher than that in surrounding normal lung parenchyma ($p < 0.01$). Within the first 24 h, the number of tumor-draining LN cells visualized within metastases increased, with concurrent decrease in numbers of labeled cells in the surrounding normal tissue. Unexpectedly, this accumulation within MCA 205 metastases of activated tumor-draining LN cells was not immunologically specific because a similar number of activated MCA 207 tumor-draining LN cells, as well as activated splenic T cells from naive mice, were found within the tumors (Fig. 1, b and c). Twenty-four hours after transfer, the accumulation of naive splenic T cells was, however, significantly lower ($p < 0.01$) than that found for MCA 205 tumor-draining LN T cells. Fig. 2 shows examples of cell distribution in metastatic nodules and normal lung tissues after the transfer of TRITC-labeled cells. Another control used for trafficking studies was freshly isolated, noncultured splenic T cells from normal mice. These fresh T cells failed to localize within pulmonary metastases (Fig. 1a), indicating that the anti-CD3/IL-2 activation culture procedure conditioned T cells to infiltrate into metastatic tumors following systemic adoptive transfer. These results demonstrated a tumor infiltration pattern independent of immunologic specificity, and suggest that the mere presence of an excess of effector cells in the tumor tissue does not always result in eradication of the tumor.

In nonlymphoid organs, only a few of the transferred cells were seen, mostly in the liver, kidney, ovary, and cortex of the adrenal gland. No labeled cells were found in the brain and muscle. However, in lymphoid organs, such as spleens and LNs, large numbers of transferred T cells were detected around the white pulp and paracortical areas, respectively. The i.v. transferred cells rapidly exited the bloodstream after they were no longer detectable 8 h after transfer (data not shown).

Therapeutic efficacy and trafficking of tumor-draining LN T cells into s.c. tumors

After i.v. transfer of cells, the lung represents the first capillary bed at which cells will travel through, thus the model system may not reflect a systemic migration pattern. To examine the ability of cells traveling beyond the lung, we used another model system in which mice bearing s.c. tumors were treated with the systemic adoptive immunotherapy. In this situation, a large number ($6 \times 10^6$) of MCA 205 tumor cells were used to induce a macroscopic tumor rapidly. Three days later, mice were treated with WBI followed by i.v. transfer of $50 \times 10^6$ activated MCA 205 tumor-draining LN cells. Similar to previous observations (7), treatment with tumor-draining LN cells completely suppressed the growth of s.c. tumors (Fig. 3a). Mice treated with the control anti-CD3/IL-2 activated normal spleen cells had progressively growing tumors, which were not different from control mice treated with irradiation only.

Studies with TRITC-labeled cells at the s.c. tumor site confirmed that the early trafficking pattern of the transferred cells was not immunologically specific, since both anti-CD3/IL-2 activated tumor-draining LN and activated normal spleen cells were able to penetrate into the tumor mass at a similar level, but tumor regression occurred only after the transfer of activated MCA 205 tumor-draining LN cells (Fig. 3b). Of note is that the number of labeled cells in s.c. tumors is substantially lower than that found in pulmonary metastatic nodules (Fig. 2, b and d). These results indicate that a small number of effector cells were capable of circulating...
and penetrating into s.c. tumors, initiating an immunologically specific antitumor reaction.

**L-selectin expression and migration pattern of transferred T cells to tumor tissues and lymphoid organs**

The homing molecule, L-selectin, is highly expressed on naive T cells in circulation, and its primary function is to facilitate lymphocyte binding to specialized high endothelial venules (HEV) in LN and Peyer’s patches (21). In the inguinal LN draining a progressive tumor, there was an increase from 5–10% to 15–25% of T cells with down-regulated L-selectin expression. After in vitro stimulation with anti-CD3 and IL-2, activated cells contained 40% L-selectin⁺ T cells (17). In previous studies, the L-selectin⁺ cell population was found to be attributed to the entire antitumor reactivity of the tumor-draining LNs, while L-selectin⁻ cells did not show any detectable antitumor effects (17, 18). To determine whether early migration of transferred T cells to tumor tissues was regulated by subpopulation differences in the expression of L-selectin, we took a different approach to study cell trafficking. Mononuclear cell suspensions were prepared from tumor-bearing lungs, solid s.c. tumors, and lymphoid organs after adoptive immunotherapy. These cells were stained for L-selectin.

Preliminary experiments indicated that the detection of L-selectin on T cells was not affected by enzymatic digestion during cell preparation. By two-color flow cytometric analyses, transferred cells, gated on the basis of their TRITC staining, could be analyzed for L-selectin expression. Twenty-four hours after adoptive transfer, the majority (>95%) of transferred cells from MCA 205 tumor-draining LN cells were L-selectin⁻ (Fig. 4A). In mice with 3-day established s.c. MCA 205 tumors, infiltrating cells were clearly seen 16 h after the transfer of 5 × 10⁶ TRITC-labeled MCA 205 tumor-draining LN cells. Fluorescence micrograph and histology of MCA 205 pulmonary tissues 16 h after the transfer of 5 × 10⁶ L-selectin⁻ (e) or L-selectin⁺ (f) T cells derived from activated MCA 205 tumor-draining LN cells. Numerous L-selectin⁺, but not L-selectin⁻, T cells were found within MCA 205 pulmonary metastatic nodules (200×).
Therapeutic efficacy and trafficking pattern of L-selectin<sup>low</sup> cells purified from activated tumor-draining LN cells

During an acute neutrophil inflammation, there is a requisite role for L-selectin in the initial attachment of neutrophils to endothelium, but this binding is followed by a rapid endoproteolytic release of L-selectin molecules from the cell surface (22). To exclude the possibility of a similar loss of L-selectin expression due to in vivo modulation, activated tumor-draining LN cells were separated into L-selectin<sup>low</sup> and L-selectin<sup>high</sup> populations before adoptive immunotherapy. The resulting cells were >95% pure, as confirmed by the flow cytometric analysis. In two independent experiments, the transfer of as few as 2 × 10<sup>6</sup> L-selectin<sup>low</sup> T cells derived from activated MCA 205 tumor-draining LN T cells resulted in nearly complete eradication of 10-day established pulmonary MCA 205 metastases, whereas the transfer of 20 × 10<sup>6</sup> L-selectin<sup>high</sup> T cells did not demonstrate therapeutic effects (Table III). Analysis of the T cell phenotype of these two cell populations revealed no significant difference that could contribute to their antitumor reactivities. Purified L-selectin<sup>low</sup> cells were composed of 23 ± 3.6% CD4 and 59.8 ± 2% CD8 cells, whereas L-selectin<sup>high</sup> cells contained 32.8 ± 2.5% CD4 and 56.2 ± 7.2% CD8 cells. In a criss-cross experiment (Table IV), L-selectin<sup>low</sup> T cells mediated antitumor effects against the MCA 205, but not the MCA 207, tumor, whereas the L-selectin<sup>high</sup> T cells from MCA 207 tumor-draining LNs demonstrated antitumor activity against MCA 207, but not MCA 205, metastases.

Analysis of tumor localization using TRITC-labeled cells 24 h after transfer demonstrated that there were ~10 times more L-selectin<sup>low</sup> cells than L-selectin<sup>high</sup> cells in the tumors (Table IV, and Fig. 2, e and f). Again, the infiltration of effector cells did not reflect the immunological specificity of the transferred cells because the numbers of L-selectin<sup>low</sup> cells from both MCA 205 and

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Therapeutic efficacy of anti-CD3/IL-2 activated MCA 205 tumor-draining LN T cells against MCA 205 s.c. tumors. B6 mice were injected s.c. with 6 × 10<sup>6</sup> MCA 205 tumor cells. Three days later, they were treated with WBI (500 cGy) followed by i.v. transfer of 50 × 10<sup>6</sup> anti-CD3/IL-2 activated tumor-draining LN or activated normal spleen cells (a). Numbers of fluorescent cells per mm<sup>2</sup> s.c. tumor tissue at different time points following the transfer of 50 × 10<sup>6</sup> activated cells as indicated (b). Twenty random sections of tumor from three mice were used to calculate average numbers ± SD of fluorescent cells per mm<sup>2</sup> metastatic tissue.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** A. FACS analysis of L-selectin expression on i.v. transferred TRITC-labeled MCA 205 tumor-draining LN cells (50 × 10<sup>6</sup>) recovered from different tissues at 24 h after transfer. Mononuclear cell suspensions prepared from lungs with 10-day metastases, 3-day s.c. tumors, LNs, and spleens were stained for L-selectin and analyzed by two-color flow cytometry. Samples were gated on TRITC-positive cells to obtain histograms displaying the expression of L-selectin of the transferred cells. Broken lines depict isotype control staining. Representative results of three independent experiments. B, Phenotype analysis of transferred cells isolated from lungs with pulmonary MCA 205 metastases 24 h after adoptive immunotherapy. Experimental design is similar to that of A.
Table III. Adoptive immunotherapy of 10-day established MCA 205 pulmonary metastases with L-selectin\(^{+}\) and L-selectin\(^{-}\) T cells derived from activated MCA 205 tumor-draining LN cells

<table>
<thead>
<tr>
<th>Cells(^{a})</th>
<th>No. Cells Transferred</th>
<th>Mean No. Pulmonary Metastases (SEM)</th>
<th>Mean No. Pulmonary Metastases (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>50 × 10(^6)</td>
<td>61 (19)(^{b})</td>
<td>85 (24)(^{b})</td>
</tr>
<tr>
<td>L-selectin(^{+}) T cells</td>
<td>20 × 10(^6)</td>
<td>243 (7)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>L-selectin(^{-}) T cells</td>
<td>5 × 10(^6)</td>
<td>0(^{b})</td>
<td>0(^{b})</td>
</tr>
<tr>
<td>L-selectin(^{-}) T cells</td>
<td>2 × 10(^6)</td>
<td>0(^{b})</td>
<td>4 (4)(^{b})</td>
</tr>
</tbody>
</table>

\(^{a}\) The adoptive immunotherapy procedure is the same as that in Table I. L-selectin\(^{+}\) and L-selectin\(^{-}\) T cells were derived from anti-CD3/IL-2 activated MCA 205 tumor-draining LN cells shortly before adoptive transfer. Each experimental group consisted of five mice.

\(^{b}\) Significantly different from no-treatment groups.

Discussion

Although direct contact between immune effector cells and their tumor target cells is believed to be a prerequisite for initiating biological reactions leading to tumor eradication, experimental results have not provided an unequivocal proof of its significance. Previous studies of the trafficking pattern of adoptively transferred nonspecific adherent NK cells in syngeneic as well as xenogeneic mouse tumor models did not reveal specific interactions (11, 12). Since A-NK cells are large, rigid, and less deformable than other lymphoid cells (23) and lack the immunologic specificity to interact with tumor cells, these results suggest that their in vivo distribution after i.v. transfer more likely reflects their physiological characteristics than immunological properties. However, it does appear that tumor tissues have the ability to capture and retain those cells destined to circulate through.

In the current study, we analyzed the trafficking pattern of systemically transferred, tumor-specific effector T cells. Unlike many previously described antitumor effector cell populations, the anti-CD3/IL-2 activated LN cells are generated from tumor-bearing mice. These cells, thus, are closely analogous to the cells one would isolate from cancer patients for the development of clinically applicable adoptive immunotherapy. In laboratory studies, we found that, despite their potent in vivo antitumor effects, the anti-CD3/IL-2 activated tumor-draining LN cells lack demonstrable cytotoxic activity against their specific tumor target cells in vitro (20). Also different from previous studies, the current adoptive immunotherapy experiments were conducted in the absence of systemic IL-2. In the past, systemic administration of IL-2 has been an integral and necessary part of the treatment when in vitro cultured cells, such as lymphokine-activated killer and TILs, were used for therapy (24, 25). Therefore, our study is unique in that the influence of the presence of IL-2 in vivo on the trafficking and antitumor efficacy is excluded. The absence of IL-2 administration in cellular therapy has drastically reduced the severe toxicity associated with clinical adoptive immunotherapy (26).

Using the fluorescent dye TRITC to label effector cells, we demonstrated that the short-term cultured LN T cells rapidly accumulated in tumor metastases located in the lung in much higher numbers than in the surrounding normal parenchyma. However, this accumulation alone did not appear to reflect immunologic specificity of the infiltrating cells, since there was an equal extent of infiltration by the nonspecific effector cells as well as normal spleen T cells activated identically. The ability of these cells to infiltrate into tumor tissues appeared to be a characteristic acquired during the in vitro activation because a similar localization of noncultured normal splenic T cells was not observed. Intravenous transfer of cells into mice with lung metastases may be regarded as regional delivery of the cells to the tumor. Using a different model system, preferential infiltration by the anti-CD3/IL-2 activated cells was also evident in the s.c. growing tumors, although the number of fluorescent cells was low. This finding indicates that the transferred cells were capable of circulating systemically through the lung capillaries and redistributing to other tissues. The present results differ considerably from our previously reported findings where long-term T cell lines and clones were found to accumulate in the lung metastases in an immunologically specific manner (16). The difference may also be attributed to the differences in culture conditions. The long-term established, tumor-specific clones were exclusively of CD8 phenotype and displayed in vitro cytotoxic effects parallel to the in vivo therapeutic effects, characteristics not shared by the 5-day anti-CD3/IL-2 activated effector cells used for the current study.

More recently, we have identified a small population of T cells with down-regulated expression of L-selectin in the tumor-draining LN s to be the specifically sensitized tumor-immune cells (17, 18). Consistent with these observations, transferred T cells isolated from both pulmonary metastases and s.c. tumor nodules were

Table IV. Specificity and tumor localization of L-selectin\(^{+}\) and L-selectin\(^{-}\) T cells derived from anti-CD3/IL-2 activated tumor-draining LN cells

<table>
<thead>
<tr>
<th>Source of Draining LNs</th>
<th>Cells</th>
<th>Mean No. Metastases (SEM)*</th>
<th>Mean No. Labeled Cells (SEM)/0.024 mm(^2)</th>
<th>Mean No. Labeled Cells (SEM)/0.024 mm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MCA 205</td>
<td>—</td>
<td>0</td>
<td>34.3 (2.6)(^{d})</td>
<td>31.6 (3.1)(^{d})</td>
</tr>
<tr>
<td>MCA 207</td>
<td>L-selectin(^{-}) T cells</td>
<td>5 × 10(^6)</td>
<td>31.7 (4.5)(^{d})</td>
<td>30.1 (4.5)(^{d})</td>
</tr>
<tr>
<td>MCA 205</td>
<td>L-selectin(^{-}) T cells</td>
<td>5 × 10(^6)</td>
<td>3.1 (1.4)</td>
<td>3.3 (1.4)</td>
</tr>
<tr>
<td>MCA 205</td>
<td>L-selectin(^{-}) T cells</td>
<td>20 × 10(^6)</td>
<td>10.6 (2.0)(^{d})</td>
<td>9.0 (1.0)</td>
</tr>
</tbody>
</table>

\(^{a}\) The adoptive immunotherapy procedure is the same as that in Table I. L-selectin\(^{+}\) and L-selectin\(^{-}\) T cells were derived from anti-CD3/IL-2 activated tumor-draining LN cells shortly before adoptive transfer. Each experimental group consisted of five mice.

\(^{b}\) Cells were labeled with TRITC prior to adoptive transfer into B6 mice with 10-day established pulmonary metastases (three mice/group). Twenty-four hours later, lungs were harvested, and numbers of fluorescent cells in metastatic tumor and normal surrounding tissues were counted as described in Materials and Methods.

\(^{c}\) Significantly different from the no-treatment groups.

\(^{d}\) Significantly different from no. cells in normal tissues.
largely L-selectin− cells. In contrast, the majority of transferred cells recovered from LNs of the recipient mice were L-selectin+, suggesting that L-selectin+ cells were capable of recirculating through peripheral LNs by interaction with HEV (27). In the spleen, the migration of lymphocytes involves sinusoidal endothelia, and the molecular mechanisms are distinct and not yet characterized. In our study, both L-selectin+ and L-selectin− transferred cells in equal numbers were found in the spleen of treated animals. To further rule out the possibility of in vivo down-regulation of L-selectin expression due to a variety of factors, including interactions with tumor cells, we fractionated activated cells into L-selectin+ and L-selectin− cells before adoptive immunotherapy. This experimental approach with purified cells clearly demonstrated the correlation between L-selectin down-regulation and the increased ability of T cells to penetrate to and localize in the tumor mass. Since the accumulation of L-selectin− cells in the tumor is independent of the immunologic specificity of the effector cells, the physiological properties of activated L-selectin− cells must have played a dominant role. Compared with L-selectin+ cells, the activated L-selectin− LN T cells expressed a high level of cell adhesion molecules, including LFA-1, CD44, and the αβ integrin (data not shown). While the precise role of these molecules remains to be determined, preliminary results suggest the involvement of G protein-coupled chemokine receptors because in vitro treatment of effector cells with Pertussis toxin abrogated the therapeutic efficacy of both unfractionated and L-selectin− tumor-draining LN cells (S. Mukai, unpublished observations). This suggests that G protein-coupled receptors of the αβ class are required for L-selectin− cell emigration through tumor tissues (28).

The demonstration of selective, but not immunologically specific, migration of systemically transferred effector cells to the malignant tissue suggests that the early trafficking of activated LN cells is governed by mechanisms independent of specific Ag recognition. Once the effector cells established a close contact with tumor cells or Ag-presenting cells in the tumor bed, specific interactions involving TCRs must have played a pivotal role in initiating tumor regression. In addition to serving as targets in adoptive immunotherapy models, tumors provide a source of Ags to further stimulate the expansion of immune effector cells. In previous experiments, we transferred immune cells into normal and tumor-bearing recipients, and, 9 days later, spleen cells were harvested and tested for their therapeutic efficacy in adoptive immunotherapy (29). Therapeutic effector cells could only be retrieved from tumor-bearing recipients, indicating that the maintenance and proliferation of L-selectin− cells may facilitate their homing to the tumor tissue while avoiding the expression and the abundance of adhesion molecules on their surface. These observations support our hypothesis that the infiltration into tumors by the systemically transferred cells is a phenomenon independent of immunologic specificity, although tumor response will additionally require the presence of T cells with tumor specificity.

In conclusion, the present study demonstrates that tumor eradication by the systemic adoptive transfer of specifically sensitized T cells requires two distinct but sequential biological mechanisms. Apparently, the anti-CD3/IL-2 culture system has conditioned T cells to acquire a distinct circulation pattern irrespective of their immunologic specificity. Although migratory properties of activated LN cells are poorly understood, the lack of L-selectin expression and the abundance of adhesion molecules on their surface may facilitate their homing to the tumor tissue while avoiding the normal route of lymphocyte recirculation through secondary lymphoid organs, such as the LNs and Peyer’s patches. Once the cells have infiltrated the tumor tissue, interactions with specific tumor Ags result in clonal expansion of tumor-specific effector cells and triggering lytic mechanisms, leading to the eradication of the tumor.

Acknowledgments

We thank the Chiron Corporation for kindly providing human rIL-2 and Laura Hogan for her assistance in the preparation of the manuscript.

References


Downloaded from http://www.jimmunol.org/ by guest on April 14, 2017


