Helper-Independent, L-Selectin^low^ CD8^+^ T Cells with Broad Anti-Tumor Efficacy Are Naturally Sensitized During Tumor Progression

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Helper-Independent, L-Selectin<sup>low</sup> CD8<sup>+</sup> T Cells with Broad Anti-Tumor Efficacy Are Naturally Sensitized During Tumor Progression<sup>1</sup>

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We recently reported that the CD4<sup>+</sup> T cell subset with low L-selectin expression (CD62L<sup>low</sup>) in tumor-draining lymph nodes (TDLN) can be culture activated and adoptively transferred to eradicate established pulmonary and intracranial tumors in syngeneic mice, even without coadministration of IL-2. We have extended these studies to characterize the small subset of L-selectin<sup>low</sup> CD8<sup>+</sup> T cells naturally present in TDLN of mice bearing weakly immunogenic tumors. Isolated L-selectin<sup>low</sup> CD8<sup>+</sup> T cells displayed the functional phenotype of helper-independent T cells, and when adoptively transferred could consistently eradicate, like L-selectin<sup>low</sup> CD4<sup>+</sup> T cells, both established pulmonary and intracranial tumors without coadministration of exogenous IL-2. Whereas adoptively transferred L-selectin<sup>low</sup> CD4<sup>+</sup> T cells were more potent on a cell number basis for eradicating 3-day intracranial and s.c. tumors, L-selectin<sup>low</sup> CD8<sup>+</sup> T cells were more potent against advanced (10-day) pulmonary metastases. Although the presence of CD4<sup>+</sup> T cells enhanced generation of L-selectin<sup>low</sup> CD8<sup>+</sup> effector T cells, the latter could also be obtained from CD4 knockout mice or normal mice in vivo depleted of CD4<sup>+</sup> T cells before tumor sensitization. Culture-activated L-selectin<sup>low</sup> CD8<sup>+</sup> T cells did not lyse relevant tumor targets in vitro, but secreted IFN-γ and GM-CSF when specifically stimulated with relevant tumor preparations. These data indicate that even without specific vaccine maneuvers, progressive tumor growth leads to independent sensitization of both CD4<sup>+</sup> and CD8<sup>+</sup> anti-tumor T cells in TDLN, phenotypically L-selectin<sup>low</sup> at the time of harvest, each of which requires only culture activation to unmask highly potent stand-alone effector function. The Journal of Immunology, 2000, 165: 5738–5749.

Adoptive immunotherapy of established mouse tumors is commonly performed by transferring T cells obtained from the tumor-draining lymph nodes (TDLN) of tumor-infiltrating lymphocytes of syngeneic tumor-bearing mice. Before such adoptive transfer, T cells are typically culture activated to correct tumor-induced signaling abnormalities as well as to provide numeric expansion (1–6). Many culture techniques historically employed for this purpose have yielded anti-tumor T cells that displayed limited or no therapeutic efficacy against weakly immunogenic tumors unless exogenous rIL-2 was coadministered during treatment. Furthermore, such cultured T cells often lacked therapeutic efficacy against established extrapulmonary tumors even when exogenous rIL-2 was coadministered (7–13). In contrast, the inclusion of anti-CD3 or bacterial superantigen during culture activation of TDLN T cells has yielded anti-tumor T cells that can be adoptively transferred to cure syngeneic mice of tumors established at all tested anatomic locations, including pulmonary, s.c., intracranial, and i.p. (14–16, 59). Furthermore, their efficacy is highly evident even without coadministration of exogenous IL-2 (14–16).

The improved ability to unmask and preserve potent anti-tumor effector T cells in vitro has enabled more precise phenotypic characterization of those T cell subpopulations naturally sensitized as a consequence of tumor growth. For example, highly potent, naturally sensitized, pre-effector T cells are concentrated within the TDLN T cell subset displaying low or absent surface expression of L-selectin (CD62L), a peripheral lymph node-homing receptor whose down-regulation during sensitization may promote T cell trafficking to other locations (17, 18). Anti-CD3 culture activation and adoptive transfer of the isolated L-selectin<sup>low</sup> T cell subset has proved therapeutically far superior to adoptive transfer of L-selectin<sup>high</sup> (L-selectin<sup>high</sup>) or unfractionated TDLN T cells, illustrating the potential value of eliminating irrelevant and/or suppressor T subpopulations during culture activation and adoptive therapy (14, 15).

To optimize adoptive immunotherapy with L-selectin<sup>low</sup> TDLN-derived T cells, it is essential to characterize the relative contributions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to tumor rejection. The observed contributions of each subset are highly dose dependent and are furthermore influenced by the relative proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells present during culture activation and adoptive transfer. For example, TDLN CD4<sup>+</sup> T cells appear to play mainly a helper role in tumor rejection when numerically dominated by TDLN CD8<sup>+</sup> T cells (19). However, the small L-selectin<sup>low</sup> subset of CD4<sup>+</sup> TDLN T cells can be isolated, culture activated, and
adoptively transferred to reject established pulmonary and intracranial tumors even without coadministered CD8\(^+\) T cells or exogenous IL-2 (14). Given the remarkable “stand-alone” therapeutic potential of purified L-selectin\(^{low}\) CD4\(^+\) TDLN T cells, we have endeavored to better characterize the even smaller subpopulation of L-selectin\(^{low}\) CD8\(^+\) T cells that is also detected in freshly harvested TDLN. Our initial testing of the L-selectin\(^{low}\) CD8\(^+\) subpopulation failed to reveal therapeutic potency against intracranial tumors (14), leading us to investigate alternative isolation techniques to improve recoveries for more thorough analysis and therapeutic dose escalations. We present the first evidence that the L-selectin\(^{low}\) CD8\(^+\) T cell subset possesses a stand-alone curative potency with several similarities to that displayed by the CD4\(^+\) subset. Because such therapeutically potent L-selectin\(^{low}\) CD8\(^+\) T cells can be obtained from CD4 knockout mice or from normal mice variously depleted of CD4\(^+\) T cells, it appears that their initial sensitization as well as their effector action has a capacity for true helper independence.

Materials and Methods

**Mice**

Female C57BL/6N (B6) and BALB/c 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 8–10 wk. In addition, B6 background CD4 knockout (CD4\(^{KO}\)) and CD8a knockout (CD8\(^{ KO}\)) mice (JR269 and JR265, respectively) were obtained from The Jackson Laboratory (Bar Harbor, ME).

**Tumors**

The MCA 205 and 203 fibrosarcomas, syngeneic to B6 mice, were originally induced with 3-methylcholanthrene (14). The tumors have been maintained in vivo by serial s.c. transplantation of thawed cryopreserved mince in B6 mice and were used within the eighth 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 2 h at room temperature as previously described (14). The CT-26 colon adenocarcinoma, syngeneic to BALB/c mice (20, 21), was provided by Gary Nabel (Vaccine Research Center, National Institutes of Health, Bethesda, MD) and was similarly maintained by serial s.c. transplantation in BALB/c mice.

**mAbs and flow cytometry**

Hybridomas producing mAb against murine CD4 (GK1.5), CD8 (2.43), and L-selectin (MEL-14) were obtained from the American Type Culture Collection (Manassas, VA) and were used to prepare Ab-rich ascites fluid (15). PE- and or FITC-conjugated rat anti-mouse (RoM) reagents to CD3, CD4, CD6, CD45RB (B220), MAC3, NK1.1, and L-selectin (CD62L) as well as sublass-matched control Ab and FITC-conjugated goat anti-rat (GnR) and mouse anti-rat (MoR) Ab were purchased from PharMingen (San Diego, CA). Cells to be analyzed by direct immunofluorescence were FcR blocked at 4°C for 20 min with 1 \(\mu\)g of unconjugated RoM-CD32 (24G2, PharMingen) and 10 \(\mu\)g of unconjugated normal mouse IgG (Jackson ImmunoResearch, West Grove, PA) in FACS buffer (Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS containing 5% FCS and 0.02% sodium azide), then exposed to Fc-conjugated Ab. Cells to be analyzed by indirect immunofluorescence because of their prior incubation with unconjugated RoM-CD62L. RoM-CD4, and/or RoM-CD8 were, depending on the assay, Fc blocked with 10 \(\mu\)g of unconjugated mouse IgG and/or exposed to additional unconjugated RoM-CD62L, -CD4, and/or -CD8. Samples were washed and counterstained either with FITC-Grn IgG or FITC-MoR mAb, then washed in FACS buffer. In some cases cells were counterblocked with 1 \(\mu\)g of unconjugated RoM-CD32 to block FcR and saturate cell-bound FITC-Grn Ab, then stained with PE-RoM-CD4 or -CD8, washed in FACS buffer, and resuspended for analysis. Cells were finally washed and resuspended in 0.5 ml of FACS buffer with 0.8 \(\mu\)g/ml PI for immediate analysis or were fixed in FACS buffer with 1% added paraformaldehyde for deferred analysis. Fixed or unfixed samples were subjected to three- or two-color analysis on a FACScan flow microfluorometer (Becton Dickinson, Sunnyvale, CA).

**Sensitization and fractionation of syngeneic tumor-draining lymph node cells**

B6 mice or BALB/c mice were inoculated s.c. with 1.5 \(\times\) 10\(^6\) MCA-205 or CT-26 tumor cells, respectively, in both flanks. Twelve (MCA-205) or 9 (CT-26) days later, inguinal TDLN 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 (15). Mouse T cell enrichment columns containing GoR-Ig Ab-coated glass beads (R&D Systems, Minneapolis, MN) and Cytovac Technologies (Edmonton, Canada) were used to isolate individual TDLN subpopulations. The manufacturers’ protocols were followed, except that TDLN cells were preincubated for 20 min at 4°C in concentrations of anti-L-selectin (CD62L), anti-CD4 and/or anti-CD8 ascites pretitrated for efficacy (1/10,000, 1/1,000, and 1/100, respectively), washed in Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS, then applied to the columns to isolate L-selectin\(^{low}\) T cells (unfractionated for CD4\(^+\) and CD8\(^+\)), L-selectin\(^{high}\) CD4\(^+\) T cells, or L-selectin\(^{low}\) CD8\(^+\) T cells. Ninety to 95% of effluent cells were strongly CD3\(^{+}\) by direct fluorescence analysis, and effluent cells that had been preincubated with anti-CD4 or anti-CD8 were quantitatively depleted of these populations (see Fig. 1). In some experiments, T cells were negatively immunoselected for L-selectin at the beginning of culture, then negatively immunoselected for CD4 at the end of 5 days of culture.

**Anti-CD3/IL-2 activation of TDLN**

Effluent TDLN cells were activated on 24-well plates precoated with the anti-CD3 mAb as described previously (14). Each well contained 4 \(\times\) 10\(^6\) cells in 2 ml of complete medium (CM), as well as 2 \(\times\) 10\(^6\) irradiated (3000 rad) freshly harvested splenocytes from normal syngeneic mice. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mm 2-mercaptoethanol, 1 mm sodium pyruvate, 2.5 mm L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, 0.5 mg/ml fungizone (all from Life Technologies, Grand Island, NY), and 5 \(\times\) 10\(^3\) M 2-ME (Sigma). After 2 days of incubation at 37°C in 5% CO\(_2\), activated cells were suspended in 4 U/ml of human rIL-2 (Chiron, Emeryville, CA) at 1–2 \(\times\) 10\(^6\) 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 or \(^{31}\)Cr release assays (14).

**Adaptive immunotherapy**

B6 mice were inoculated intracranially in the right hemisphere with 1 \(\times\) 10\(^6\) syngeneic tumor cells in 10 \(\mu\)l of HBSS to establish brain metastases (14) or with 1 \(\times\) 10\(^5\) tumor cells in 50 \(\mu\)l of HBSS under the midline ventral skin to establish s.c. tumors (16). Three days after tumor inoculation, mice received sublethal total body irradiation (500 rad), followed by infusion of anti-CD3-activated syngenic effector T cells suspended in 1.0 ml of HBSS through the tail vein. Mice followed for evidence of intracerebral tumor progression were monitored for survival with an end point of cure or preterminal neurologic symptoms (14). Mice with established s.c. tumors were evaluated by serial caliper measurements and euthanized when the product of two perpendicular dimensions was \(\geq 300\) mm\(^2\) (16). The 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\(^5\) tumor cells suspended in 1.0 ml of HBSS to establish pulmonary metastases. Three or 10 days later, mice received 500 rad, then anti-CD3-activated syngenic effector T cells were given i.v. in 1.0 ml of HBSS through the tail vein. On day 18 (3-day model) or day 11 (10-day model) after T cell inoculation, all mice were sacrificed for enumeration of tumor nodules on the surface of the lung, as previously described (14).

In some experiments, on days 2 and 7 of adoptive transfer, mice received 1 ml of a 1/4 dilution of GK1.5 ascites or a 1/10 dilution of 2.43 ascites i.v. to deplete CD4 or CD8 T cells, respectively. Quantitative in vivo depletion was confirmed by splenocyte analysis of sacrificed sentinel mice, as described previously (16).

**Cytokine assays**

A total of 2 \(\times\) 10\(^6\) culture-activated T cells derived from MCA-205 TDLN were exposed to 5 \(\times\) 10\(^6\) irradiated (5000 rad) stimulator cells, the latter consisting of s.c. passaged, freshly harvested, and enzymatically digested tumors (MCA-205 or MCA-203); in vitro passed stroma-free tumor cell line (H-12 derivitization of MCA-205); or tumor-associated macrophages (TAM). To generate TAM, single-cell suspensions of s.c. passaged MCA-205 or MCA-203 were plated onto glass petri dishes at a density of 80 \(\times\) 10\(^5\) cells/10-cm plate in CM. The cell suspension was incubated for 45 min at 37°C, then nonadherent cells were vigorously washed away and discarded. The adherent cells were harvested by trypsinization.
FIGURE 1. Representative FACS analysis demonstrating efficacy of negative immunoselection purifications. TDLN cells were freshly harvested from B6 mice bearing syngeneic MCA-205 tumors, then were unprocessed (undepleted), preincubated with rat anti-mouse L-selectin (Rm-M-CD62L) Ab alone (L-selectin depleted), preincubated with Rm-M-CD8 and Rm-M-CD62L (CD8/L-selectin depleted), or preincubated with Rm-M-CD4 and Rm-M-CD62L (CD4/L-selectin depleted). Each Ab-preincubated group was then applied to glass bead columns precoated with GoR-Ig Ab (see Materials and Methods), and effluents were further processed in parallel with the undepleted group. Aliquots of each group were variously stained with Rm-M-CD62L, Rm-M-CD4, Rm-M-CD8, Rm-M-CD3, or control, as shown), counterstained with FITC-M-CD4, Rm-M-CD8, Rm-M-CD4 and Rm-M-CD3, or control, as shown), and evaluated by FACS analyses. A fixed marker (M1) was set for each group to enable quantitation of positively staining subpopulations as well as background staining in the control groups. Displayed (percentage) values in the control histograms indicate the percentage of background staining within the M1 region. Displayed (percentage) values in the other histograms indicate the percentage of cells staining positively for anti-CD4, anti-CD8, or anti-CD3, corrected for background staining. Percentages were not determined for anti-CD62L staining, because a continuum of CD62L expression, rather than positive or negative expression, was observed.

with 0.25% trypsin for 15 min at 37°C, then washed in CM. T cells were exposed to these various stimulators or to immobilized anti-CD3 mAb for 24 h in 2 ml of CM in 24-well plates at 37°C (14). Each stimulator group was also cultured in the absence of added T cells to enable subsequent correction for background (nonspecific) cytokine production. Supernatants were harvested, and the concentrations of IFN-γ, GM-CSF, IL-2, IL-4, and IL-10 were measured by ELISA using paired mAb and standards purchased from PharMingen as described previously (14).

**In vitro cytotoxicity assay**

Four-hour 51Cr release assays, and preparation of lymphokine-activated killer (LAK) control cells from syngeneic B6 normal splenocytes were performed as described previously (14). The MCA-205 tumor cells (1 × 106) were labeled with 51Cr (Na 51 CrO 4, 100 mCi; DuPont, Wilmington, DE) at 37°C for 1 h and washed three times in CM. Target cells (1 × 105) 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 (Tiers-Tek 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) × 100. Nonspecific LAK cells were generated from B6 normal spleen cells by incubating 2 × 106 cells/ml in CM containing 1000 U/ml IL-2 for 3 days and were used as a cytotoxic effector cell-positive control.

**Statistical analysis**

The significance of differences in numbers of pulmonary metastases between groups and the survival of mice with intracranial tumors were analyzed by the exact rank modification of the Wilcoxon rank-sum test. A two-tailed p < 0.05 (p1 = 0.025) was considered significant.

**Results**

**Isolation of L-selectinlow TDNL T cell subsets**

Our previous studies had combined positive and negative immunoselection techniques (e.g., nylon wool passage, panning, and magnetic beading) to purify both L-selectinhigh and L-selectinlow TDNL T cell subpopulations, followed by subfractionation of CD4+ and CD8+ subsets (14). Such multistep purifications established the L-selectinhigh subpopulation’s lack of therapeutic potency, but also subjected L-selectinlow subpopulations to prolonged processing before culture. Therefore, in the present studies we used a single-step negative immunoselection procedure to remove nontherapeutic L-selectinhigh T cells while hastening isolation and culture of highly enriched L-selectinlow T cells.

Freshly harvested TDNL cells were preincubated with pretitrated concentrations of 1) rat anti-mouse L-selectin (Rm-M-CD62L) Ab alone, 2) Rm-M-CD62L plus Rm-M-CD4 Ab, or 3) Rm-M-CD62L plus Rm-M-CD4 Ab, then were applied to glass bead columns precoated with GoR-Ig Ab. Depending on the preincubation cocktail, fractionated subsets emerging in the column effluents were 1) purified L-selectinlow T cells (both CD4+ and CD8+), 2) purified L-selectinlow CD4+ T cells, or 3) purified L-selectinlow CD8+ T cells. Depletion of L-selectinhigh cells was confirmed by FACS analyses. Rm-M-CD8 or Rm-M-CD4 coated T cells were effectively depleted to ≤ 0.5% final contamination in the column effluent (Fig. 1). In addition, FcR+ and/or adherent subpopulations such as B cells, myeloid cells, and NK cells were efficiently removed by the GoR-Ig Ab-coated glass beads, as evidenced by a < 1% contamination of cells staining positively for non-T-lineage markers anti-B220 (CD45RB), anti-MAC-3, and anti-NK1.1, respectively (not shown). Column effluent cells were >90% CD3+, with < 10% of CD3+ cells possessing a CD4+ CD8+ phenotype (Fig. 1). This minor CD3+ CD4+ CD8+ subpopulation was also evident in TDNL not subjected to mAb exposure and column fractionation.

The observed total content of L-selectinlow CD8+ T cells in TDNL was typically half that observed for L-selectinlow CD4+ T cells due variously to a smaller total CD8+ T cell content and/or a smaller proportion of CD8+ T cells displaying low L-selectin expression (not shown). Nonetheless, adequate numbers of highly enriched L-selectinlow cells of either the CD4+ or the CD8+ subset...
could be obtained for study purposes by processing sufficient numbers of TDLN. Preparations depleted of CD4\(^+\) or CD8\(^+\) T cells before culture (Fig. 1) remained consistently depleted when analyzed by FACS at the end of 5-day culture (not shown). Isolated effluent subpopulations displayed only a modest proliferative response in anti-CD3/IL-2 culture (typically 3-fold expansion in 5 days), but a consistently remarkable anti-tumor effector activity (see below).

Either CD4\(^+\) or CD8\(^+\) sensitized L-selectin\(^{low}\) TDLN T cells can eradicate 3-day established MCA-205 pulmonary and intracranial tumors

Previous experiments demonstrated a dose-dependent ability of L-selectin\(^{low}\) CD4\(^+\) TDLN T cells to eradicate established intracranial tumors when 1–2 x 10\(^6\) cells were administered as adoptive therapy after culture activation (14). The same ability was consistently demonstrated in the present experiments by culture-activated L-selectin\(^{low}\) CD4\(^+\) TDLN T cells enriched by single-step negative immunoselection columns (Fig. 2A). The therapeutic effects of similarly prepared L-selectin\(^{low}\) CD8\(^+\) T cells also proved dose dependent, and administration of 5 x 10\(^6\) culture-activated L-selectin\(^{low}\) CD8\(^+\) T cells proved sufficient to eradicate established 3-day intracranial tumors (Fig. 2A). This was a considerably higher dose of L-selectin\(^{low}\) CD8\(^+\) T cells than had previously been available for study (14). Therefore, although culture-activated L-selectin\(^{low}\) CD8\(^+\) T cells displayed lesser therapeutic potency on a cell number basis than the L-selectin\(^{low}\) CD4\(^+\) subset, both subsets possessed a dose-dependent capacity to eradicate intracranial and pulmonary tumors as single-agent therapy without administration of exogenous rIL-2. In addition, adoptive transfer of either 1 x 10\(^6\) L-selectin\(^{low}\) CD4\(^+\) or 1 x 10\(^5\) L-selectin\(^{low}\) CD8\(^+\) TDLN T cells was sufficient to eradicate established 3-day pulmonary metastases (Fig. 2B).

As shown previously for L-selectin\(^{low}\) CD4\(^+\) T cells (14), tumor rejection by L-selectin\(^{low}\) CD8\(^+\) T cells was restricted to the relevant sensitizing tumor (Fig. 3), consistent with an Ag-dependent, rather than an LAK-mediated or otherwise Ag-unrestricted mechanism of rejection.

**FIGURE 2.** Treatment of day 3 intracranial and pulmonary metastases with L-selectin\(^{low}\) TDLN T cell subsets. L-selectin\(^{low}\) T cells unfractionated for CD4 and CD8, L-selectin\(^{low}\) CD4\(^+\) T cells, and L-selectin\(^{low}\) CD8\(^+\) T cells were each prepared from freshly harvested TDLN cells of B6 mice bearing syngeneic 12-day MCA-205 tumors as described in Materials and Methods. Following 5-day culture activation with anti-CD3/IL-2, each T cell group was harvested and administered to sublethally irradiated (500 rad) B6 mice bearing day 3 intracranial (A) or pulmonary (B) metastases as described in Materials and Methods. A, Long term survival of mice with intracranial MCA-205 treated with 1 x 10\(^6\) (1E6) L-selectin\(^{low}\) T cells unfractionated for CD4 and CD8 (L-Sel- All), 1E6 L-selectin\(^{low}\) CD4\(^+\) T cells (L-Sel- CD4), or 1E6, 2E6, or 5E6 L-selectin\(^{low}\) CD8\(^+\) T cells (L-Sel- CD8). Historically, mice surviving symptom free at day 60 are cured in this model. B, Enumeration of pulmonary metastases (each dot represents a mouse) in mice sacrificed 18 days after treatment with 1E6 L-selectin\(^{low}\) T cells unfractionated for CD4 and CD8 (all), L-selectin\(^{low}\) CD4\(^+\) T cells, or L-selectin\(^{low}\) CD8\(^+\) T cells.
the most potent on a cell number basis for eradicating established 10-day pulmonary metastases, purified L-selectinlow CD8+ T cells were significantly more potent than purified L-selectinlow CD4+ T cells and, moreover, were effective as single-component therapy without coadministration of exogenous rIL-2 (Fig. 4). L-selectinlow CD8+ T cells remained therapeutically more potent than L-selectinlow CD4+ T cells regardless of whether each subset was isolated before or after culture activation (not shown).

Adoptively transferred purified L-selectinlow CD4+ TDLN CD8+ T cells are more effective than purified L-selectinlow CD4+ TDLN CD8+ T cells for the eradication of established MCA-205 s.c. tumors, but are CD8+ dependent

Established s.c. tumors display a relatively low susceptibility to adoptive immunotherapy (16), and trafficking studies have demonstrated the low initial accumulation of T cells in s.c. tumors compared with pulmonary or intracranial tumors (17). Nonetheless, 3-day established MCA-205 s.c. tumors were completely rejected within 3–4 wk following adoptive transfer of 5 × 10^6 L-selectinlow T cells culture-activated from either day 9 or day 12 MCA-205 sensitized TDLN (Fig. 5 and not shown). Such L-selectinlow T cells were unfractionated with regard to CD4+ and CD8+, with each mouse receiving ~1 × 10^6 CD4+ and 4 × 10^6 CD8+ T cells at adoptive transfer. The observed rejection consisted of an initial 1- to 2-wk phase of attenuated tumor growth, followed by a 1- to 2-wk phase of objective tumor regression (Fig. 5, A–C). In vivo mAb depletion studies demonstrated that either CD4+ or CD8+ L-selectinlow T cells alone were competent to sustain the initial phase of growth attenuation, whereas subsequent tumor regression failed to occur with either CD4+ or CD8+ cell depletion (Fig. 5A).

In the absence of such in vivo mAb depletion, complete rejection of established 3-day s.c. tumors was also eventually achieved by adoptive transfer of as few as 1 × 10^6 L-selectinlow CD4+ T cells alone (Fig. 5B). However, a protracted initial phase of attenuated tumor growth delayed objective regression by 1–3 wk, depending on the L-selectinlow CD4+ T cell dose, compared with therapy with combined (CD4+ plus CD8+) L-selectinlow T cells. Such delayed rejection appeared to require the late recruitment of host CD8+ T cells, because, as shown in Fig. 5A, mice depleted of CD8+ cells failed to achieve such rejection despite receiving an otherwise curative dose of L-selectinlow CD4+ T cells.

Mice receiving purified L-selectinlow CD8+ T cells alone experienced an initial phase of tumor growth attenuation that was not significantly different from that observed following treatment with combined (CD4+ plus CD8+) L-selectinlow T cells (Fig. 5, A and C). In addition, several mice receiving the highest dose of purified L-selectinlow CD8+ T cells experienced early (rather than delayed) complete tumor regression (Fig. 5D). Except for these infrequent cures, no objective tumor regression, early or delayed, was observed in mice receiving purified L-selectinlow CD8+ T cells alone, although all such treated mice experienced significant delays in tumor progression compared with untreated mice (Fig. 5, A and C).

Anti-tumor sensitization of CD4+ and CD8+ TDLN T cells can occur in the complete absence of either subset

Huang et al. demonstrated that CD8+ as well as CD4+ T cell sensitization to tumor Ag is initially mediated by cross-priming host APC rather than by direct contact with tumor cells (22). Host APC are capable of processing and presenting exogenous Ag in an MHC class I-restricted context to CD8+ T cells, but such CD8+ sensitization may require or can be enhanced by temporally linked
interactions between APC and CD4⁺ T cells (23–25). We there-
fore examined whether sensitization of L-selectin low CD8⁺ pre-
effector T cells in tumor-bearing mice required the presence of host
CD4⁺ T cells. We inoculated syngeneic CD4 KO and CD8 KO mice with MC-
205 sarcoma cells s.c., then 12 days later harvested TDLN T cells
for a single immunoselection procedure to remove L-selectinhigh T
cells (see Materials and Methods). Cells were culture activated by
anti-CD3/IL-2 treatment and were evaluated in adoptive therapy
experiments. Adoptive transfer of either 2 × 10⁶ L-selectinhlow CD4⁺ or L-
selectinhlow CD8⁺ T cells sensitized in MCA-205-bearing syngeneic
knockout mice eradicated established pulmonary or intracranial
MCA-205 tumors when adoptively transferred into tumor-bearing
normal B6 mice (Figs. 6), demonstrating that either subset was
effectively sensitized in the absence of the other.

Nonetheless, it remained possible that sensitization and/or acti-
vation of L-selectinhlow CD8⁺ effector function could be enhanced
by the copresence of TD LN CD4⁺ T cells (23–25). In studies
performed primarily in normal syngeneic B6 mice, CD8⁺ T cells
were depleted of CD4⁺ T cells at various experimental stages (ei-
ther by in vivo depletion with anti-CD4 during sensitization or
adoptive therapy or by in vitro CD4⁺ depletion using negative
immunoselection immediately before or after culture activation, see
Materials and Methods); in parallel, TDLN T cells were prepared
from syngeneic CD4KO mice. In each instance, effectiveness of CD4
depletion was confirmed by FACS analyses (see Materials and
Methods). Sufficient cells were prepared under each experimental
condition to permit treatment of individual B6 tumor-bearing mice
with 1 × 10⁶ T cells. For the treatment of 3-day pulmonary metastases, 1 × 10⁶ adop-
tively transferred CD4-depleted L-selectinhlow CD8⁺ T cells were
as effective as 1 × 10⁶ CD4-intact L-selectinhlow T cells (81%
CD8⁺ T cells and 19% CD4⁺ T cells), except in the case of L-
selectinhlow CD8⁺ T cells prepared from knockout mice (Fig. 7A).
As previously observed and anticipated (Fig. 2A) (14), adoptive

![A. Adoptive therapy with Ab depletion](image)

**FIGURE 5.** Treatment of 3-day s.c. metastases with L-selectinhlow TDLN T cell subsets. Syngeneic C57BL/6 (B6) mice were injected with viable MCA-205 cells, and TDLN was harvested on day 9 to prepare purified L-selectinhlow T cells, unfractionated for CD4 and CD8, purified L-selectinhlow CD4⁺ T cells, and/or purified L-selectinhlow CD8⁺ T cells. On day 5 after anti-CD3/IL-2 culture activation, T cell groups were harvested and adoptively transferred to treat syngeneic mice with 3-day established s.c. tumors (see Materials and Methods). Recipient mice received 500 rad before adoptive transfer. Subsequent growth of the s.c. tumors was serially evaluated. The ordinate displays the tumor area determined by two perpendicular caliper measurements; the abscissa displays the day following tumor inoculation. Symbols on each line display the average tumor measurement of each treatment group at particular time points (five mice per treatment group, with the SD displayed, except for D, where each line represents an individual mouse). Data are shown in the log scale to facilitate visualization of differences between groups at early time points. A. Adoptive therapy with Ab depletion. Mice received no adoptive therapy or 5 × 10⁵ (5E6) L-selectinhlow T cells unfractionated for CD4 and CD8; in addition, groups receiving T cells received no in vivo mAb depletion (L-selectinhlow), concomitant in vivo CD4 mAb depletion (L-selectinhlow & anti-CD4), or concomitant in vivo CD8 mAb depletion (L-selectinhlow & anti-CD8). On day 12, group A1 vs A2: *p* = 0.0002; A2 vs A3: *p* = 0.0779; A3 vs A4: *p* = 0.222. B and C, CD4⁺ and CD8⁺ dose titrations. Each treatment group received adoptive transfer of L-selectinhlow T cells unfractionated for CD4 and CD8 (L-selectinhlow (CD4⁺CD8⁺)), purified L-selectinhlow CD4⁺ T cells (L-selectinhlow CD4), and/or purified L-selectinhlow CD8⁺ T cells (L-selectinhlow CD8) at the doses shown in the legends. No in vivo Ab depletions were performed. On day 12, group C1 vs C2: *p* < 0.0001; C2 vs C3: *p* = 0.7404; C2 vs C4: *p* = 0.2598. On day 38, C1 vs C3: *p* = 0.0021; C1 vs C4: *p* = 0.0001. D, Divergent treatment outcomes for individual group C3 mice displayed; each line represents a single mouse.
concentrations of IFN-γ not shown). Specific IL-2 production was usually not demonstrable in vivo during sensitization as well as when TDLN CD4+ following in vitro exposure to the sensitizing tumor (Fig. 8A). L-selectin low CD8+ T cells isolated from TDLN each displayed a similar capacity to produce cytokines in vitro. Both L-selectin low CD4+ and L-selectin low CD8+ T cells consistently produced high concentrations of IFN-γ during in vitro exposure to the sensitizing tumor, and such IFN-γ production was specific for the sensitizing tumor (Fig. 8A), corresponding to the anti-tumor specificity also demonstrated during adoptive therapy (Fig. 3). In addition, specific GM-CSF, but not TNF-α, IL-4, or IL-10 production was observed following in vitro exposure to the sensitizing tumor (Fig. 8B and not shown). Specific IL-2 production was usually not demonstrable when these T cells were stimulated with sensitizing tumor cells, even though stimulation with anti-CD3 resulted in IL-2 production (not shown) (14). Furthermore, L-selectin low CD8+ T cells did not lyse the relevant tumor targets in standard 4-h 51Cr release assays (Fig. 9).

In additional experiments, the reactivity of MCA-205 sensitized TDLN T cells was assayed using either purified MHC class II+ tumor cells or enriched MHC class II+ TAM as stimulators. Culture-activated L-selectin low CD4+ T cells released IFN-γ upon contact with MHC class II+ TAM, but not upon contact with the MHC class II-nonexpressing tumor cells. In contrast, culture activated L-selectin low CD8+ T cells released IFN-γ upon contact with either relevant TAM or MHC class I-expressing tumor cells (Fig. 8C).

**Generation of L-selectin low CD8+ TDLN T cells with highly potent effector activity is not tumor or mouse strain restricted**

To confirm that the sensitization of highly potent pre-effector L-selectin low CD8+ T cells was not confined to a single tumor model or mouse strain, L-selectin down-regulated CD8+ T cells were isolated and culture activated from the TDLN of syngeneic BALB/c mice bearing the colonic adenocarcinoma CT-26. Isolated L-selectin low CD8+ T cells or L-selectin low CD4+ T cells activated by anti-CD3/IL-2 treatment displayed marked therapeutic potency against CT-26 in adoptive transfer experiments even without coadministration of exogenous IL-2 or the absent T cell subset (Fig. 10).

**Discussion**

Previous characterization of the highly potent effector T cells activated from TDLN T cells by anti-CD3 or superantigen treatment revealed that their therapeutic activity involved participation of both CD8+ T cells and CD4+ T cells (16, 19). It was subsequently demonstrated that the culture-activated subset of L-selectin low CD4+ T cells could even be adoptively transferred as single agent therapy to achieve ablative tumor rejections regardless of whether the sensitizing tumor cells themselves expressed MHC class II molecules (14). The dramatic stand-alone therapeutic potency of this L-selectin low CD4+ subset has tentatively been ascribed to the superior ability of L-selectin-down-regulated T cells to traffic into tumors at all tested anatomic locations (17) coupled with the CD4+ T cell’s capacity to recognize tumor Ag presented by MHC class II+ host APC resident within tumors (9, 14, 17, 26, 27). The mechanisms by which adoptively transferred L-selectin low CD4+ T cells mediate successful rejection of MHC class II-nonexpressing tumors are under study and may variously depend upon cytokine-induced apoptosis or anti-angiogenic effects, contact-mediated (e.g., FAS/APO-1-ligand) apoptosis, recruitment of CD8+ CTL, and/or recruitment of Ag-unrestricted accessory cells such as tumoricidal macrophages and LAK cells (reviewed in Ref. 9).

We hypothesized that L-selectin low CD8+ T cells might also possess potent anti-tumor activity, coupling the superior trafficking capacity of L-selectin low T cells (17) with a capacity for direct interactions with MHC class I-expressing tumor cells (Fig. 8). The present report confirms that isolated L-selectin low CD8+ TDLN T cells display a remarkable therapeutic potential following anti-CD3/IL-2 activation, which is far superior in potency to anti-tumor CD8+ T cells derived from TDLN T cells or tumor-infiltrating lymphocytes by previous culture techniques. Optimally prepared, anti-CD3/IL-2-activated L-selectin low CD8 provided consistently curative stand-alone adoptive therapy against relevant tumors implanted in either lung or brain and significantly attenuated s.c. tumor progression, with no requirement for coadministration of exogenous IL-2. Culture-activated L-selectin low CD8+ T cells from TDLN developed greater effector activity when L-selectin low...
CD4⁺ T cells were also present during sensitization and/or culture activation (Fig. 7). Nonetheless, even when L-selectinlow CD8⁺ T cells were sensitized, culture activated, and adoptively transferred in the absence of TDLN CD4⁺ T cells, they could consistently eradicate both established pulmonary and intracranial tumors when administered in sufficient numbers (Figs. 6 and 7).
It is therefore apparent that L-selectin low CD8 T cells display therapeutic characteristics of helper-independent T (HIT) cells during both effector and effector limbs of the anti-tumor immune response (4, 28, 29). Although CD8 HIT cells have long been recognized to represent a naturally occurring subset of CD8 T cells (4, 28 –34), before the present study they had been identified only in rare animal tumor models in which the tumors expressed very strong viral Ag (4, 28, 29) and in experimentally contrived transgenic models (35, 36). In contrast, the therapeutically potent L-selectin low CD8 HIT cells described in the present study were spontaneously sensitized in syngeneic mice bearing progressive, weakly immunogenic tumors. The failure to identify CD8 HIT cells previously in these long-studied tumor models was probably a consequence of their typically minimal presence in TDLN. In addition, because culture activation of CD8 HIT cells appears to be enhanced by the copresence of CD4 T cells (Fig. 6), it is possible that earlier culture methods that routinely failed to sustain CD4 T cells alone was below the limits of assay detection (<25 pg).

The mechanism(s) by which L-selectin low anti-tumor CD8 T cells achieve helper independence are under study and may include their ability to achieve high expression of CD40 ligand even in the presence of tumor (our manuscript in preparation), leading to superior APC conditioning (37). L-selectin low anti-tumor CD8 T cells share this property and additional characteristics with the L-selectin low anti-tumor CD4 T cells subset. Each subset is therapeutically active against tumors at multiple anatomic locations as stand-alone adoptive therapy without IL-2 coadministration. Thus, each appears to possess the anatomically unrestricted, broad trafficking capacity that is the signature property of activated L-selectin low TDLN T cells (9, 14, 17, 26). Each subpopulation produces IFN-γ and GM-CSF in vitro following contact with the relevant sensitizing tumor target (Fig. 8), but with a typical absence of specific IL-2, TNF-α, IL-4, and IL-10 production or direct target lysis (Fig. 9). Despite this highly circumscribed in vitro response to tumor contact, both CD4 and CD8 L-selectin low T cells retain the capacity for more diverse cytokine production, including IL-2, as evidenced with anti-CD3 restimulation at the end

![Figure 8](http://www.jimmunol.org/)
of culture (14). It is also likely that L-selectin low CD8 T cells implement CTL activity following adoptive transfer, because culture-activated TDLN T cells obtained from syngeneic perforin knockout mice display severe anatomic restrictions in their tumor rejection capacity when adaptively transferred into normal tumor-bearing hosts (59).

It is nonetheless apparent that L-selectin low CD4 and CD8 anti-tumor T cells possess significant functional differences, beginning with different practical requirements for recognizing tumor Ag. L-selectin low CD8 T cells sensitized to MCA-205 produced IFN-γ when exposed to either MHC class I-expressing MCA-205 tumor cells or enriched MCA-205 derived TAM (Fig. 8C). In contrast, L-selectin low CD4 T cells sensitized to MCA-205 did not produce IFN-γ when exposed to MHC class II MCA-205 tumor cells, but did produce IFN-γ when exposed to MHC class II MAC-205-derived TAM. It is therefore likely that tumor rejection may be triggered either through direct T cell contact with tumor cells or, alternatively, by T cell contact with cross-stimulating host APC present within the tumor bed (26, 38, 39). In the MCA-205 tumor model, either option is theoretically available for anti-tumor CD8 T cells, but only the second option is available to anti-tumor CD4 T cells, because MCA-205 tumor cells in situ do not express MHC class II molecules (27). Although it is possible that the reactivity of CD8 T cells to TAM involves an element of triggering by contaminant tumor cells, the triggering of anti-tumor CD8 T cells by cross-priming host APC that process exogenous tumor Ag is a well-accepted phenomenon (22).

Our studies have repeatedly demonstrated that purified L-selectin low CD8 and CD4 TDLN T cell subsets are not simply interchangeable as therapy. For example, adoptively transferred L-selectin low CD4 T cells were relatively more potent on a cell number basis for eradicating 3-day intracranial tumors, whereas L-selectin low CD8 T cells proved more effective against 10-day pulmonary metastases (see Figs. 2 and 4). The most extreme differences in therapeutic performance were observed for established s.c. tumors, probably reflecting the lower trafficking efficiency of even L-selectin low T cells into tumors at this anatomic site (17). Although purified CD4 and CD8 L-selectin low subsets were each therapeutically active against MCA-205 s.c. tumors, only purified L-selectin low CD4 T cells provided consistently curative adoptive therapy (Fig. 5). Nonetheless, such L-selectin low CD4 T cell-mediated tumor rejection was a delayed process with apparent dependence upon recruitment of host CD8 T cells (Fig. 5, A and B). Furthermore, undelayed rejection of established MCA-205 s.c. tumors was only observed when CD8 L-selectin low T cells were included as a component of adoptive therapy (Fig. 5, A and D).

These results suggest that CD8 and CD4 L-selectin low T cell subsets can play distinctive and complimentary roles during adoptive therapy. Whereas the therapeutic efficacy of purified L-selectin low CD8 T cells varies strongly in proportion to the observed accumulation efficiencies of T cells at these sites (pulmonary tumors > intracranial tumors ≥ s.c. tumors) (17), the therapeutic efficacy of purified L-selectin low CD4 T cells appears to be largely independent of such trafficking variances. This may reflect superior abilities of L-selectin low CD4 T cells to proliferate intratumorally, sustain APC conditioning, and/or recruit additional host effector elements, including CD8 T cells (37, 40–44) (our manuscript in preparation). Nonetheless, purified L-selectin low CD8 T cells displayed greater efficacy than purified L-selectin low CD4 T cells in eradicating advanced (day 10) pulmonary tumors even without coadministration of exogenous rIL-2 (Fig. 4) and also were essential for achieving rapid rejection of MCA-205 s.c. tumors with adoptive therapy (Fig. 5, A and D). It remains to be determined whether such therapeutic distinctions reflect the L-selectin low CD8 T cell’s superior capacity to interact directly with MHC class I+, MHC class II+ tumor cells (Fig. 8C).

Given the relative insensitivity of L-selectin low CD4 T cells to trafficking variances and the effector impact of L-selectin low CD8 T cells even against advanced tumors, it is not surprising that these subsets are often therapeutically superior and even synergistic when administered together (Figs. 4 and 5A). mAb depletion experiments furthermore suggest that purified L-selectin low CD4 T cells can eventually replicate such synergy during adoptive therapy of both s.c. and intracranial tumors by recruiting host anti-tumor CD8 T cells (Fig. 5A; H. Kagamu and S. Shu, unpublished observations). In these experimental models such CD8 recruitment appears to be appropriately delayed by the host’s exposure to immunosensitizing sublethal irradiation before adoptive transfer (Fig. 5, A and B). Because both CD8-dependent and CD8-independent therapeutic effects are observed during subsequent tumor rejection (Fig. 5A), it is reasonable to postulate a dual helper and effector...
role for L-selectinlow CD8+ T cells. For example, L-selectinlow CD8+ T cell adoptive therapy of s.c. tumors required CD8+ T cells to achieve objective tumor regression, but long-term tumor growth arrest was nonetheless achieved even in the absence of CD8+ T cells (Fig. 5A).

In contrast, although adoptive transfer of purified L-selectinlow CD8+ HIT cells could initially attenuate s.c. tumor growth as well as purified CD4+ or even combined (CD4+ plus CD8+) L-selectinlow T cells, the former’s therapeutic effect was usually unsustained beyond 2 wk. These results suggest that the helper independence of CD8+ HIT cells may be less easily sustained at tumor sites where T cell trafficking is relatively inefficient, as epitomized in murine models by established s.c. challenges (17). However, because Ag availability often causes vaccination strategies to favor CD8+ T cell sensitization (3, 45–47), it is desirable to identify adjunct treatments that promote sustained effector activity of CD8+ HIT cells when they must be adoptively transferred without CD4+ T cells. The adjunct administration of exogenous rIL-2 for this purpose is well preconditioned. In fact, previously characterized cultured CD8+ HIT cells with specificity for the FBL3 lymphoma were therapeutically effective as adoptive therapy only when exogenous rIL-2 was coadministered (29, 48). More recently, Shrin- kant and Mescher demonstrated that adoptively transferred OVA-specific CD8+ HIT cells from transgenic OT-1 mice could traffic successfully to a peritoneal challenge of EL4-OVA tumor and transiently control tumor growth, but spontaneously left the site of tumor and developed elements of split anergy unless rIL-2 was coadministered (35, 36). Such previous reports demonstrate the capacity of adjunct cytokine treatment to provide a satisfactory surrogate for CD4+ participation during CD8+ HIT cell adoptive therapy. However, because simultaneous coadministration of rIL-2 with activated TDLN T cells can paradoxically inhibit adoptive therapy, especially when T cells already exhibit therapeutic competence without adjunct rIL-2 (9, 49, 50), the optimal schedule and treatment, especially when T cells already exhibit therapeutic competence without adjunct rIL-2 (9, 49, 50), the optimal schedule and therapy are challenging even in mouse experiments.

The ability to detect L-selectinlow anti-tumor CD8+ HIT cells even in weakly immunogenic tumor models such as MCA-205 and CT-26 suggests that similar naturally occurring pre-effector T cells may also be detectable in cancer patients. Nonetheless, strategies to isolate L-selectinlow CD4+ and CD8+ T cells for culture activation and adoptive therapy are challenged even in mouse experiments by the small numbers of L-selectinlow T cells currently obtainable from TDLN. The reduced percentage of L-selectinlow CD8+ T cells present within TDLN compared with L-selectinlow CD4+ T cells may reflect additional physiological constraints, such as less efficient host APC-mediated sensitization of CD8+ T cells to exogenous tumor Ag (22, 54). Nonetheless, the natural existence of these anti-tumor pre-effector T cell subpopulations, even in limited numbers, underscores the host’s ability to implement highly efficient sensitization to tumor Ag even in the suboptimal malignant environment, including the routine sensitization of CD8+ HIT cells. Efforts are ongoing to define vaccine maneuvers and other treatments that will enhance sensitization and resultant L-selectin down-regulation and proliferation of both CD4+ and CD8+ T cells within TDLN. Recent work by Tanaka et al. demonstrated that vaccination with tumor cells modified by B7.1 and IFN-γ gene transfection markedly boosted the proportion and absolute numbers of L-selectinlow pre-effector T cells in TDLN, even for tumors classically characterized as nonimmunogenic (55). Such vaccine strategies therefore appear promising for increasing the availability of highly potent L-selectinlow pre-effector T cells in TDLN for purposes of culture activation and adoptive therapy. Furthermore, improved yields may allow better delineation of functional differences in the L-selectin- and L-selectinlow subsets of L-selectinlow populations. Finally, the heightened stability of CD8+ HIT cells in culture compared with non-HIT cells (28, 29) may permit their long term numerical expansion with retained function in vivo despite marginal initial yields and sluggish proliferation during brief anti-CD3/IL-2 culture activation. Efforts are ongoing to define the optimal TCR-stimulating and costimulatory stimuli to enhance propagation of isolated L-selectinlow TDLN T cells, including repeated coculture with tumor Ag-pulsed dendritic cells (49, 56–58).

References


