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Peripheral Tissue Homing Receptors Enable T Cell Entry into Lymph Nodes and Affect the Anatomical Distribution of Memory Cells

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Peripheral tissue homing receptors enable T cells to access inflamed nonlymphoid tissues. In this study, we show that two such molecules, E-selectin ligand and α4β1 integrin, enable activated and memory T cells to enter lymph nodes (LN) as well. This affects the quantitative and qualitative distribution of these cells among regional LN beds. CD8 memory T cells in LN that express these molecules were mostly CD62Llo and would normally be classified as effector memory cells. However, similar to central memory cells, they expanded upon Ag re-encounter. This led to differences in the magnitude of the recall response that depended on the route of immunization. These novel cell shares properties of both central and effector memory cells and reside in LN based on previously undescribed mechanisms of entry. The Journal of Immunology, 2013, 191: 2412–2425.
CD62L and whose properties include those associated with both T<sub>CM</sub> and T<sub>EM</sub>.

**Materials and Methods**

**Mice**

Mice were maintained in pathogen-free facilities at the University of Virginia, and all animal protocols were approved by the University of Virginia Institutional Animal Care and Use Committee. C57BL/6 (B6) mice were obtained from Charles River Laboratories or National Cancer Institute (Bethesda, MD) OT-1 RAG<sup>1</sup> mice were from The Jackson Laboratory. OT-1 Thy.1.1 mice were first-generation crosses of OT-1 RAG<sup>1</sup>& 7/7, and B6 Thy.1-1 mice. CD8<sup>+</sup> T cells in OT-1 Thy.1.1 mice are almost entirely cells specific for ov<a><sub>a257–264</sub></a> restricted by H-<sub>2</sub>K<sub>d</sub>, and these cells were used for most adoptive transfers. AAD mice were generated previously in the laboratory (34). AAD mice were crossed with B6 Thy.1-1 mice to generate mice that were AAD<sup>+</sup>Thy.1<sup>+</sup>Thy.1<sup>+</sup>. Mice with a transgenic TCR specific for Thy<sup>1.1</sup> (FH) were generated in the laboratory (34).

**Viruses**

Recombinant vaccinia virus-expressing OVA (vac-OVA) was a gift from Dr. J. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Recombinant vaccinia virus expressing murine tyrosinase was a gift from Dr. N. Restifo (National Cancer Institute). Viruses were administered by indicated route at 1 <sup>×</sup> 10<sup>8</sup> PFU/mouse.

**T cell adoptive transfers**

Spleen and LN were harvested from OT-1 RAG<sup>1</sup> mice and OT-1 Thy.1.1 mice. Single-cell suspensions were prepared, and cells were positively enriched for CD8<sup>+</sup> T cells with MACS reagents and columns (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Cells were consistently 97–99% CD8<sup>+</sup> by flow cytometry (C. Brinkman, unpublished observations). In some cases, unenriched OT-1 Thy.1.1 T cells were used in which case injection numbers were extrapolated from the percentage of total cellularity shown to be CD8<sup>+</sup> ov<a><sub>a257</sub></a> specific (~20%; unpublished observations). In some experiments, OT-1 T cells were labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) or 1 μM CellTrace Violet (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. For injection, T cells were resuspended in sterile saline, and unless otherwise noted, 10,000 CD8<sup>+</sup> OT-I cells were injected in 200 μl into the dorsal tail vein. FH cells were prepared using the same methodology. Unenriched FH cells (2 <sup>×</sup> 10<sup>5</sup>) were injected into the dorsal tail vein in 200 μl, which was ~1 <sup>×</sup> 10<sup>8</sup> CD8<sup>+</sup> cells.

**Dendritic cell culture and immunization**

Bone marrow-derived dendritic cells (BMDC) were generated from bone marrow as described previously (35). BMDC were activated by coculture for 10–12 h with NIH 3T3 cells expressing CD40L. Before immunization, BMDC were pulsed with 10 μM ov<a><sub>a257–264</sub></a> (Biomolecular Core Facility, University of Virginia) for 1 h at 37°C with 10 μg/ml human β2-microglobulin (Calbiochem, San Diego, CA). BMDC were washed twice with warm 1X PBS and resuspended in sterile saline for injection. Mice were immunized with 1 <sup>×</sup> 10<sup>5</sup> BMDC in 200 μl either i.v., i.p., or s.c.

**Cell lines**

NIH 3T3 fibroblasts expressing CD40L were maintained at 37°C with 5% CO<sub>2</sub> in RPMI 1640 supplemented with 5% FCS (Sigma-Aldrich, St. Louis, MO), 7.5 mM HEPES, and 2 mM l-glutamine (Life Technologies).

**Flow cytometric analysis and cell sorting**

Lymphoid tissues were homogenized into single-cell suspensions. Most lymphoid samples were stained for surface markers following 10-min incubation with purified anti-CD16/32 (2.4G2; BioXCell, Lebanon, NH) to block FcRs. OT-1 T cells were identified by staining with anti-CD8 (53-67; eBioscience and BD Biosciences), anti-Thy-1.2 (H1S51; eBioscience), and/or H-2K<sup>b</sup>-ov<a><sub>a257</sub></a>-tetramer–allophycocyanin generated in-house. Activation status was determined by staining with anti-CD44 (IM7; E Bioscience). Integer expression was evaluated with anti-αβ<sub>2</sub>D<sub>3</sub>K<sub>2</sub>; eBioscience) anti-αβ<sub>1</sub> (R1-2; eBioscence and BioLegend), sometimes followed by streptavidin–PerCP-Cy5.5 or streptavidin–PE-Cy7 (eBioscience). For experiments, samples were stained with anti-CD8 (MEL14; eBioscience). Recombinant murine E- and P-selectin-Human IgG Fc fusion proteins (R&D Systems) were also used to detect ESL and P-selectin ligand (PSEL). Staining was at 4°C in HBSS with 2 mM Ca<sup>2+</sup>, 1 mM HEPES buffer, and 5% FCS (Sigma-Aldrich). These reagents were detected with goat anti-human-PE (Jackson ImmunoResearch Laboratories, West Grove, PA) when stained separately. When co-incubating with both fusion proteins, either E-selectin–IgG or P-selectin–IgG was biotinylated using a One-Step Ab Biotinylation kit (Miltenyi Biotech) and detected using strepavidin–allophycocyanin (eBioscience). Samples were collected on FACSCanto I and II instruments (BD Biosciences) and analyzed using FlowJo software (version 8.8.7; Tree Star). Electronic sorting was done on a BD FACSSelect SE Turbo Sorter with DIVA Option.

**FTY720 treatment**

FTY720 was a gift from Dr. V. Brinkmann (Novartis Pharma, Basel, Switzerland). For injection, FTY720 was diluted in sterile saline, and mice were given 1 mg/kg FTY720 in 200 μl i.p. FTY720 treatment was initiated at indicated times and repeated every 24 h until harvest.

**In vivo Ab blockade of transferred T cells**

Function-blocking anti–E-selectin (9A9), anti–P-selectin (RB40.34), and anti–CD62L (ME-L-14) were purified from supernatants provided by the Lymphocyte Culture Center (University of Virginia). Supernatants purified with protein G–Sepharose (Amersham Biosciences, Piscataway, NJ) columns. Anti–VCAM-1 (MK2.7) was purchased commercially (BioXCell). B6 mice were injected with 5 <sup>×</sup> 10<sup>7</sup> CD8<sup>+</sup>Thy.1.1 OT-I cells and immunized s.c. with BMD. Seven days later, CD8<sup>+</sup> T cells were enriched from LN and spleen, labeled with CellTrace Violet, and incubated with anti–CD62L for 30 min at 4°C. The percentage OT-I of CD8<sup>+</sup> T cells was determined by flow cytometry, and 4 <sup>×</sup> 10<sup>7</sup> activated OT-I (along with bulk CD8<sup>+</sup> T cells and 100 μl ME-L-14) and 1500 IU recombinant human IL-2 (Chiron, Emeryville, CA) were injected into naive Thy.1<sup>+</sup> mice pretreated with Abs. Blocking Abs or Rat IgG (Sigma-Aldrich) were administered i.p. at 100 μg in 200 μl/mouse 8 h before T cell injection. Sixteen hours after transfer, mice were euthanized, and lymphoid organs were processed for flow cytometry.

**Lymphocyte recovery from peripheral tissues**

Lymphocytes were recovered from lung using a protocol from Dr. T. Braciale (University of Virginia) with modifications (3). Lymphocytes were purified using lymphocyte M (Cedarlane Laboratories, Burlington, ON, Canada). T cells were recovered from the ear using a protocol provided by Dr. E. Tewalt (University of Virginia). Ears were split manually into dorsal and lateral sheets and minced with scissors and were incubated in HBSS with 1 mg/ml Collagenase Type XI (Sigma-Aldrich) and 60 U/ml Dounce I for 30 min at 37°C. These preparations were then homogenized and filtered through nylon mesh, and lymphocytes were purified as above using lymphocyte M.

**Isolation of stromal cells**

Tissues were harvested, placed in ice-cold DMEM with 10% FCS (Sigma-Aldrich), minced with scissors, and digested with 0.42 U/ml Liberase TM (Roche, Indianapolis, IN) and 60 U/ml Dounce I (Sigma-Aldrich) in EBM-2 medium (Lonza) for 40 min at 37°C. Stromal cells were negatively selected using CD45 beads and the autoMACS system (Miltenyi). CD4<sup>+</sup>CD<sub>8</sub><sup>+</sup> cells were stained with mAbs against gp38–allophycocyanin (BioLegend) and CD31–FITC (eBioscience), DX5–PerCPCy5.5 (BioLegend), and E- and P-selectin or VCAM-1 as above. DAPI (Sigma-Aldrich) was used for live/dead discrimination. Buffers were supplemented with 2 mM l-glutamine and MEM amino acids (Life Technologies) and MEM nonessential amino acids (Corning, Manassas, VA).

**Microscopy**

Tissues were flash frozen on liquid nitrogen, embedded, blocked, and cut into 6-μm sections on Superfrost/Plus slides (Thermo Fisher Scientific, Rockville, MD) in the Research Histology Core at the University of Virginia. Sections were fixed with acetone and incubated with anti-CD16/32 (2.4G2; BioXCell) and stained with FITC anti–P-selectin (RB40.34), anti–E-selectin (9A9), or anti–VCAM-1 (MK2.7) and biotinylated anti-CD31 (390), followed by DyLight 549 streptavidin (Jackson ImmunoResearch Laboratories). Slides were mounted in Vectashield with DAPI (Vector Laboratories). For FITC signal amplification, sections were blocked with PBS containing 3% H<sub>2</sub>O<sub>2</sub> and 0.1% NaN<sub>3</sub> and a Biotin–Avidin Blocking kit (Vector Laboratories), and Fluorescein tyramide signal amplification (PerkinElmer) was performed as per the manufacturer’s instructions. Images were acquired on a Nikon Microphot FXA system using Adobe Photoshop 5.5.
Statistical analysis

Two-tailed Student t tests and one-way ANOVA with Tukey posttests were done with GraphPad software (Prism version 5.0). A p value < 0.05 was considered significant.

Results

Activated CD8 T cells that traffic into Ag-free LN are not reprogrammed to express new homing receptors

We first determined whether effector and memory CD8 T cells that traffic from sites of Ag encounter into Ag-free LN are reprogrammed to express different peripheral tissue HR. Mice that had received OT-I cells were immunized by different routes with OVA257 peptide-pulsed BMDC, and activated T cells were evaluated in Ag-draining and Ag-free LN 3 d later. We reasoned that HR reprogramming in Ag-free LN, if it occurred, would be even more evident if the cells were sequestered for additional time. Therefore, some mice were treated for 4 additional days with FTY720, which interferes with T cell exit from LN (36). Long-term FTY720 treatment has been associated with apoptosis of lymphocytes (37). However, we saw no reduction in the fraction or number of OT-I or endogenous CD8 lymphocytes in FTY720-treated animals compared with saline-treated controls using this protocol (C. Brinkman, unpublished observations). Intravenous immunization with BMDC results in T cell activation restricted to the mediastinal LN and spleen (2–4). Activated OT-I cells that remained in these organs 7 d after i.v. immunization uniformly expressed the α4 integrin subunit but not α4β7, indicating that they were α4β1+. CD8 T cells activated in mesenteric LN normally upregulate α4β7, whereas those activated in skin-draining LN upregulate ESL (3, 4). However, cells activated in mediastinal LN and spleen that subsequently trafficked to mesenteric LN did not (Fig. 1A; C. Brinkman, unpublished observations). This was true regardless of whether FTY720 was used to sequester them for additional time. Although there was a small increase in the ESL+ fraction among cells that redistributed into the axillary/brachial LN in the experiment shown (Fig. 1A), this was not elevated in FTY720-treated mice compared with untreated mice and was not reproducible in other experiments.

To test whether HR reprogramming could be induced by inflammation, mice transferred with OT-I cells and immunized i.v. with OVA257-pulsed BMDC on day 0 were subsequently infected with vaccinia virus by i.p. or s.c. routes on day 7, treated with FTY720 from day 8, and harvested on day 11. To ensure that an antiviral immune response was induced in appropriate LN, the vaccinia virus used encoded tyrosinase (mTyr-Vac), and mice also received tyrosinase-specific TCR transgenic FH T cells (34) 1 d before infection. Inoculation with mTyr-Vac s.c. in the scruff of the neck induced strong proliferation of FH cells in skin-draining axillary/brachial and cervical LN (C. Brinkman, unpublished observations). THY1.1+ THY1.2+ OT-I cells and were immunized i.v. with BMDC 24 h later. 1 × 10⁶ CFSE”Thy1.1” OT-I cells and were immunized i.v. with BMDC. FTY720 or vehicle treatment began 72 h later and was repeated every 24 h until harvest at 7 d. Histograms are gated on divided CD8”Thy1.1” cells and positive staining thresholds were set with FMO controls. Results are representative of five mice for each condition from two experiments. Albino AAD+ Thy 1.1+ mice received 1 × 10⁶ CFSE+ Thy1.2+ OT-I cells and were immunized i.v. with BMDC 24 h later. 1 × 10⁶ CFSE”Thy1.1” OT-I cells were immunized i.v. with BMDC. FTY720 or vehicle treatment began 72 h later and was repeated every 24 h until harvest at 7 d. Histograms are gated on divided CD8”Thy1.1” cells and positive staining thresholds were set with FMO controls. Results are representative of five mice for each condition from two experiments. Albino AAD+ Thy 1.1+ mice received 1 × 10⁶ CFSE+ Thy1.2+ OT-I cells and were immunized i.v. with BMDC 24 h later. 1 × 10⁶ CFSE”Thy1.1” OT-I cells were immunized i.v. with BMDC. FTY720 or vehicle treatment began 72 h later and was repeated every 24 h until harvest at 7 d. Histograms are gated on divided CD8”Thy1.1” cells and positive staining thresholds were set with FMO controls. Results are representative of five mice for each condition from two experiments. Albino AAD+ Thy 1.1+ mice received 1 × 10⁶ CFSE+ Thy1.2+ OT-I cells and were immunized i.v. with BMDC 24 h later. 1 × 10⁶ CFSE”Thy1.1” OT-I cells were immunized i.v. with BMDC. FTY720 or vehicle treatment began 72 h later and was repeated every 24 h until harvest at 7 d. Histograms are gated on divided CD8”Thy1.1” cells and positive staining thresholds were set with FMO controls. Results are representative of five mice for each condition from two experiments.
lished observations) and divided FH T cells upregulated ESL (Fig. 1B). However, activated OT-I cells that had trafficked from mediastinal LN and spleen into axillary/brachial and cervical LN remained ESL<sup>−/−</sup>. Similarly, i.p. mTyr-Vac induced strong proliferation of FH cells in mesenteric LN but activated OT-I that had trafficked into the mesenteric LN from mediastinal LN and spleen did not upregulate α<sub>4</sub>β<sub>7</sub> (Fig. 1C). Thus, CD8 T cells activated in mediastinal LN and spleen do not upregulate ESL and α<sub>4</sub>β<sub>7</sub> when they traffic into Ag-free skin and gut-draining LN. This is true even if those LN are inflamed and capable of inducing expression of these molecules on cells that are activated there.

We also addressed whether peripheral tissue HR expression on cells activated in skin-draining LN could be reprogrammed. s.c. immunization with BMDC results in T cell activation restricted to skin-draining LN (2, 4). Over 80% of activated OT-I cells that remained in skin-draining axillary/brachial LN draining the im-

unization site after 7 d expressed ESL, whereas 25–30% were α<sub>4</sub>β<sub>7</sub><sup>+</sup> (Fig. 2A). Although most ESL<sup>+</sup> cells were α<sub>4</sub>β<sub>7</sub><sup>neg</sup> (ESL single-positive or SP), most α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells did express ESL (double-positive or DP) (Fig. 2B). The proportions of ESL SP cells were identical in the priming axillary/brachial LN and the inguinal LN, which is also skin draining but Ag-free in this immunization scheme (Fig. 2A). Most cells that trafficked from axillary/brachial LN into mesenteric and mediastinal LN were still ESL<sup>+</sup> but failed to upregulate α<sub>4</sub>β<sub>7</sub> (Fig. 2A). However, 45–65% were α<sub>4</sub>β<sub>7</sub><sup>+</sup>, twice the proportion in both skin-draining LN (Fig. 2A, 2B). This is consistent with the possibility that ESL SP cells that had traf-

ficked to mesenteric and mediastinal LN had been reprogrammed to become DP cells. However, the α<sub>4</sub>β<sub>7</sub><sup>+</sup> proportion was not fur-
ter increased when cells were sequestered for 4 additional days after redistribution using FTY720 (Fig. 2A, 2B). These findings suggested that the enhanced representation of α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells in mesenteric and mediastinal LN relative to skin-draining LN was due to differential accumulation of activated T cell subpopulations and not reprogramming.

α<sub>4</sub>β<sub>7</sub> and ESL can mediate CD62L-independent entry of activated CD8 T cells into LN

To directly test the hypothesis that activated T cells expressing peripheral tissue HR selectively traffic to different LN, OT-I cells that had been activated in skin-draining LN were transferred into Ag-free recipients, and their distribution assessed 16 h later. In vivo blockade of CD62L was used to remove it as a means of LN entry. Anti-CD62L reduced the number of cotransferred naive (CD44<sup>lo</sup>) CD8 T cells that entered skin-draining and mediastinal LN by over 99% (Fig. 3A). However, the number of activated OT-I cells that entered LN in the presence of anti-CD62L was still 16–62% of the preinjection fraction, whereas those that entered skin-draining LN did not upregulate ESL and α<sub>4</sub>β<sub>7</sub> (Fig. 3B). OT-I and CD44<sup>hi</sup> cotransferred en-
genous CD8 T cells that entered skin-draining LN in the pres-
ence of anti-CD62L were significantly enriched for ESL SP cells relative to the preinjection fraction, whereas those that entered mesenteric and mediastinal LN and spleen were not (Fig. 3C, 3D).

In contrast, although cotransferred CD44<sup>hi</sup> DP CD8 T cells were enriched in mesenteric and mediastinal LN, there was no statis-
tical enrichment of either the α<sub>4</sub>β<sub>7</sub> SP or DP OT-I populations. This analysis was complicated by the possibility that different subpopulations might also home to peripheral tissue. However, the fact that these cells entered LN in the presence of anti-CD62L suggested that ESL, α<sub>4</sub>β<sub>7</sub><sup>+</sup>, or both might play a role.

To test this possibility directly, we treated mice with blocking Abs directed against the ligands for ESL or α<sub>4</sub>β<sub>7</sub> just prior to adoptive transfer of OT-I cells that had been activated in skin-draining LN. Treatment of mice with blocking Ab against VCAM-1, the ligand of α<sub>4</sub>β<sub>1</sub>, significantly reduced the infiltration of both DP and α<sub>4</sub>β<sub>1</sub>-enriched OT-I cells into mesenteric LN, whereas ESL SP and DN cells were unaffected (Fig. 4A). Similarly, VCAM-1 blockade reduced the infiltration of α<sub>4</sub>β<sub>7</sub> SP OT-I cells into skin-draining LN but not of ESL SP cells (Fig. 4B). In contrast to the results for mesenteric LN, VCAM-1 blockade failed to reduce infiltration of DP cells into skin-draining LN, indicating that they had another means of entry. In keeping with this, blockade of E-selectin, the receptor for ESL, also did not block the infiltration of DP cells into skin-draining LN but did substantially reduce the proportion and the number of both OT-I ESL SP and cotransferred ESL SP CD44<sup>hi</sup> T<sub>CD8</sub> (Fig. 4C–E).

However, E-selectin blockade had no effect on the entry of any subpopulations of either CD44<sup>hi</sup> T<sub>CD8</sub> or OT-I into mesenteric LN, mediastinal LN, or spleen (Fig. 4F, 4G; C. Brinkman, unpublished observations). Leukocyte rolling on vascular endothelium can de-
pend on both E-selectin and P-selectin (38–40). Although blockade of P-selectin alone had no effect on infiltration of either OT-I or cotransferred CD44hi CD8 T cells into any LN, infiltration of ESL SP cells into skin-draining LN was consistently inhibited to an even greater extent when both E and P-selectin were blocked (Fig. 4D, 4E; C. Brinkman, unpublished observations). However, this difference fell short of statistical significance. These results demonstrate that \( \alpha_4 \beta_1 \) integrin can mediate CD62L-independent entry of CD8 T cells into both skin-draining and mucosal LN, whereas ESL can mediate CD62L-independent entry of CD8 T cells into skin-draining LN. Collectively, these results show that activated CD8 T cells distribute nonuniformly among different LN using molecules that are normally associated with homing to peripheral tissues.

Vascular expression of homing receptor ligands suggests alternative routes of T cell entry into skin draining and mucosal LN

To understand how ESL and \( \alpha_4 \beta_1 \) integrin enable activated T cells to enter LN, we assessed expression of their ligands in LN HEV and peripheral tissue vasculature. E-selectin was expressed on a subset of CD31+ blood vessels in skin but was not detected on HEV in either skin-draining LN (Fig. 5A), or mesenteric and mediastinal LN, or the CD31+ vessels of small intestine, lung, or spleen (C. Brinkman, unpublished observations). Flow cytometry of CD31+ stromal cells demonstrated that E-selectin was expressed only on CD31+gp38neg blood endothelial cells (BEC) from skin vessels and not skin lymphatic endothelial cells (LEC) (Fig. 5B) or stromal cells from any LN or other organs (Fig. 5D; C. Brinkman, unpublished observations). In contrast, VCAM-1 and P-selectin were expressed on CD31+ HEV in all LN as well as in skin, lung, and small intestine CD31+ vessels (Fig. 5A; C. Brinkman, unpublished observations). P-selectin was highly expressed on a similar proportion of skin BEC as E-selectin (Fig. 5B), and a substantial fraction of these cells expressed both molecules (Fig. 5C). It was also expressed on BEC from all other LN, as well as those from lung and small intestine, but with the exception of lung, was absent from LEC (Fig. 5B, 5D; C. Brinkman, unpublished observations). VCAM-1 was expressed on 13–23% of BEC from different LN, and 3–12% of BEC from associated peripheral tissues (Fig. 5B; C. Brinkman, unpublished observations). On the basis of these data, \( \alpha_4 \beta_1 \) CD8 T cells could enter LN directly via VCAM-1+ HEV or indirectly via VCAM-1+ blood vessels in skin, intestine, and lung and then move into LN via afferent lymph. However, the restricted expression of E-selectin expression on skin microvasculature suggests that ESL SP and DP CD8 T cells most likely enter skin-draining LN via the skin vasculature and afferent lymph.

Expression of homing receptors influences the distribution of LN-resident memory cells independent of CD62L

We next evaluated whether memory OT-I cells that continued to express peripheral tissue HR would also distribute nonuniformly among different LN. Six to eight weeks after i.v. BMDC immunization, 50–75% of OT-I memory cells in all LN and spleen expressed \( \alpha_4 \beta_1 \) integrin, whereas fewer than 5% expressed ESL or...
FIGURE 4. α4β1 and ESL can mediate CD62L-independent entry of activated OT-I T cells into LN. (A–G) CD8 T cells were enriched from LN and spleen of s.c. BMDC immunized mice, labeled with CellTrace Violet, treated with MEL-14 for 30 min and injected into naive B6 mice. Recipients were treated i.p. with 100 μg 9A9, RB40, MK2-7, or Rat IgG control Abs 8 h prior to T cell transfer, and processed for flow (Figure legend continues)
αβ₂ (Fig. 6A). Similarly, ~70% of memory OT-I cells after s.c. BMDC immunization expressed ESL and αβ₁ either alone or together but not αβ₂ (Fig. 6B, 6C). Importantly, OT-I ESL SP cells remained significantly enriched in skin-draining LN, whereas αβ₁ SP OT-I cells remained enriched in mesenteric and mediastinal LN and spleens (Fig. 6B, 6C). This was true in the priming auxillary and brachial LN and in skin-draining but Ag-free cervical and inguinal LN (C. Brinkman, unpublished observations). Endogenous CD44^hi ESL SP CD8 T cells were also enriched in skin-draining LN, whereas endogenous CD44^lo αβ₁ SP cells were enriched in other LN and spleens (Fig. 6D, 6E). Importantly, the fraction of resting memory OT-I cells resident in skin LN, out of the total in all LN, lung, spleen, and skin, was substantially higher in animals that had been s.c. immunized than those immunized i.v. (Fig. 6F). Subcutaneous immunization also resulted in a higher fraction of resting memory OT-I cells in skin as extrapolated from the ears, although this did not reach statistical significance (Fig. 6F). In contrast, the fraction of resting memory OT-I cells in mesenteric and mediastinal LN, out of the total in all LN, lung, spleen, and skin, was equivalent in animals immunized by either route (Fig. 6F). However, i.v. immunization more efficiently seeded the spleen and lung with memory OT-I T cells (Fig. 6F).

These data indicate that the expression of peripheral tissue HR on resting memory T cells results in quantitative differences in their distribution among different LN beds.

The ability of ESL and αβ₁ to mediate entry of effector T cells into LN in a CD62L-independent manner led us to hypothesize that memory OT-I expressing these same receptors were not classical TCM. Indeed, only about a quarter to a third of OT-I resting memory cells in skin-draining LN expressed CD62L after either s.c. or i.v. immunization (Fig. 7A, 7B). However, ~90% of the CD62L^hi OT-I cells expressed αβ₁, PSL, or ESL after s.c. immunization (Fig. 7A, 7C), and a comparable fraction expressed either αβ₁ or PSL after i.v. immunization (Fig. 7B, 7C). CD62L^lo cells were more likely to express ESL and PSL than were CD44^hi CD62L^lo cells (Fig. 7D). However, a substantial fraction of these CD62L^lo cells in LN still expressed CCR7, whereas only a small fraction of those in spleen did so (Fig. 7E). In keeping with earlier studies, this is consistent with a role for CCR7 in enabling entry of these cells into LN either via HEV or afferent lymph, although other molecules such as CXCR4 and its ligand CXCL12 could also be involved (41). Taken together with the results in Fig. 4, these data emphasize that peripheral tissue HR enable CD62L^lo CD8 T cells, many of which are CCR7⁺, to be a significant component of LN resident memory.

**Influence of tissue-homing molecule expression on the recall response**

We next determined whether the nonuniform distribution of resting memory cells among LN influenced the magnitude of the recall response and the HR expression on recall effectors. αβ₁ SP memory OT-I cells purified from LN and spleen of i.v. immunized mice and ESL SP memory OT-I cells purified from LN and spleen of s.c. immunized mice were transferred into new hosts that were immunized 5 d later with vac-OVA i.v. or s.c. Immunization with vaccinia virus via the i.v. route results in T cell activation in all LN and spleen, while s.c. immunization results in T cell activation in skin-draining LN (C. Brinkman, unpublished observations). Both populations expanded upon re-encounter with Ag (Fig. 8A), consistent with previously established properties of TCM (28, 42, 43). Importantly, transferred ESL SP OT-I memory cells produced significantly larger recall responses to s.c. vaccinia virus challenge than did αβ₁ SP cells, whereas there was no statistical difference in their recall responses to i.v. immunization (Fig. 8A, 8B). This is consistent with the preferential homing of ESL SP cells to skin-draining LN and their enhanced representation there. Intravenous immunization did result in a trend to a higher response among ESL SP memory cells, but this was not significant. This could suggest that ESL SP cells are more proliferative than αβ₁ SP cells. However, these populations expressed comparable levels of CD62L, and thus, any potential differences in proliferation cannot be ascribed to differences in the representation of classically defined TCM and TEM subpopulations (Fig. 8C). Thus, these data indicate that memory cell localization under the influence of peripheral tissue HR can determine the size of the recall response to localized immunization.

Because the HR expression patterns of activated CD8 T cells were fixed upon redistribution to Ag-free LN (Figs. 1, 2), we evaluated the ability of “polarized” resting memory T cells to upregulate new peripheral tissue HR during a recall response. Vac-OVA induced uniform αβ₁ expression on both primary and recall effectors, regardless of immunization route (Fig. 8D). After s.c. immunization with vac-OVA, naive and sorted αβ₁ SP OT-I memory cells substantially upregulated ESL in skin-draining LN, whereas sorted ESP SP cells maintained it (Fig. 8D, 8E). Conversely, i.v. immunization with vac-OVA downregulated ESL on recall effectors derived from sorted ESp SP cells (Fig. 8D, 8F). Thus, memory cells can both downregulate previously programmed peripheral tissue HR and upregulate new HR, depending on the LN in which they encounter Ag during a recall response.

**Discussion**

Several studies have demonstrated the importance of peripheral tissue HR in enabling activated and memory T cells to access inflamed nonlymphoid tissues, and have shown that expression of these receptors is programmed by distinct LN microenvironments (2–8, 15, 44). We have discovered that peripheral tissue HR also determine the distribution of recently activated effectors and resting memory CD8 T cells among different LN. Activated and memory CD8 T cells enter Ag-free LN via CD62L-independent pathways involving either ESL or αβ₁ integrin. These results highlight a previously undescribed role for peripheral tissue HR in controlling the migration of memory CD8 T cells to LN. Importantly, LN-resident resting memory CD8 T cells expressing these molecules were mostly CD62L⁺ and as such could be classified as TEM. However, in addition to their residence in a location characteristic of central memory, they also expanded upon Ag re-encounter, leading to differences in the magnitude of the recall response that depended on the route of immunization. That these memory cells responded to viral challenge in LN also indicates that they entered the LN parenchyma and were not merely passing through the subcapsular space, as has been described for effector cells that enter nonreactive LN via lymphatic drainage from upstream LN draining sites of viral infection (45). Thus, these LN-
residential resting memory cells exhibit characteristics previously ascribed to both T*EM and T*CM.

Our results demonstrate conclusively that entry into resting LN is not limited to CD62L⁺ T cells and that the peripheral tissue HR α₄β₁ integrin and ESL, which are known to mediate initial rolling on vascular endothelium (9, 10), can determine the ability of activated and memory CD8 T cells to home to and reside in Ag-free LN under steady-state, noninflammatory conditions. It was previously shown that CD62L⁺ CD8 effectors could enter inflamed but not resting LN using CXCR3, but the molecule(s) responsible

FIGURE 5. E-selectin is expressed in skin vasculature but not skin-draining LN or other organs. (A) Organs were removed from naive B6 mice and processed for immunofluorescent microscopy. Magnification is ×20 objective in skin, ×10 in LN. Scale bar, 100 μm in skin and 200 μm in LN. Results represent samples from three mice. (B–D) Organs were removed from naive B6 mice, digested to liberate endothelial cells, and stained for flow cytometry. Plots are gated on live, singlet, CD45⁺,ter119⁻,DX5⁻,CD31⁺ cells, and positive staining thresholds were set with FMO controls. Cells pooled from 10–14 mice representative of two such experiments.
Expression of peripheral tissue HR influences the distribution of LN-resident memory cells independent of CD62L Thy 1.2+ mice received CFSE Thy 1.1+ OT-I cells and were immunized i.v. (A and F) or s.c. (B–F) with BMDC. Mice were harvested 6–8 wk later. Plots are gated on CD44hiCD8+Thy1.1+ cells in (A) and (B) and positive staining thresholds were set with FMO controls. (D and E) CD44hiCD8+Thy1.1neg (non OT-I) T cells were gated on and the fraction expressing the indicated peripheral tissue HR was determined. Skin LN composed of pooled axillary, brachial, cervical, and inguinal LN. (A and B) Results representative of 6–10 mice of each condition from three to four experiments. (C) Results are pooled data of five to six mice from two experiments. (D and E) Results are pooled data of eight mice from two experiments. Bars show mean values (± SEM). *p < 0.05, **p < 0.01, ***p < 0.001 paired Student t test compared with skin LN in (C–E), unpaired in (F). In (F), total skin T cell cellularity estimated by extrapolating cell counts from two ears (≈2 cm²) to the entire surface area of a 20-g mouse (≈36 cm²).
for rolling and sticking on HEV was not defined (46). There have been disparate reports concerning the role of \( \alpha_4 \beta_1 \) integrin in LN entry. Early work suggested a minimal role for entry by resting lymphocytes (47, 48), in keeping with a study that failed to detect VCAM-1 on HEV (49, 50). Later studies showed VCAM-1 expression on both mouse and rat HEV (51, 52). In keeping with this, \( \alpha_4 \beta_1 \) integrin has previously been shown to substitute for LFA-1 in T cell adhesion to and transmigration across ex vivo cultures of rat high endothelial venule cells (53) and to overlap with LFA-1 in supporting the entry of lymphocytes into peripheral LN (52). LFA-1 and \( \alpha_4 \beta_1 \) integrin also were shown to play overlapping roles in plasmacytoid dendritic cell adhesion to and transmigration across inflamed HEV (54). However, none of these reports have suggested a role for \( \alpha_4 \beta_1 \) integrin in substituting for CD62L to mediate the adhesion and diapedesis steps of lymphocyte entry. They also did not associate the use of \( \alpha_4 \beta_1 \) integrin with functional characteristics of the lymphocytes. We found that, VCAM-1 is expressed on a substantial fraction of HEV in resting LN and to a more limited extent on blood vessels in skin, intestine, and lung. Thus, \( \alpha_4 \beta_1 ^+ \) CD8 T cells could enter LN directly via VCAM-1+ HEV or indirectly via VCAM-1+ peripheral tissue vasculature and subsequent movement into LN via afferent lymph (25, 26).

We also showed that CD8 T cells that coexpress ESL and PSL enter skin-draining LN via interactions with E-selectin, whereas P-selectin plays at most a secondary role in this process. It was previously shown that P-selectin enabled entry of CD62LnegPSL+ memory CD4 T cells into inflamed but not resting LN (55). One explanation for the disparity is the elevated level of P-selectin on the HEV of inflamed compared with resting LN (55). In keeping with previous work (19), we found E-selectin expression in the resting vasculature of the skin but not in skin-draining LN, other LN, or other peripheral tissues. This strongly suggests that ESL+ activated and memory CD8 T cells can enter resting skin-draining LN via afferent lymph drainage from the skin. Our results are

**FIGURE 7.** A large fraction of LN-resident memory CD8 T cells express tissue-homing molecules and are CD62LnegThy1.1+ mice received Thy1.1+ OT-I cells and were immunized s.c. (A, C, D, and E) or i.v. (B) with BMDC. Mice were harvested 6–8 wk later. (A and B) Representative plots gated on CD44^hiCD8+Thy1.1+ cells. (E) Representative plots gated on CD44^hiCD8+Thy1.1+CD62Lneg cells. Open histograms show FMO negative control and shaded histograms show CCR7 staining. Results are pooled data of eight mice from two experiments in (C), four mice from one experiment representative of three experiments in (D), representative of three experiments with three mice each in (E). Bars show mean values (± SEM). *p < 0.05, **p < 0.01, ***p < 0.001 unpaired Student t test in (C) and (D).
FIGURE 8. Influence of tissue-homing molecule expression on the recall response. LN and spleen from mice that had received Thy1.1+ OT-I cells were harvested 28 d after s.c. or i.v. BMDC immunization. Cells were flow sorted to select for CD44hi CD8+ Thy1.1+ cells. α4+ESLneg and α4negESL+ subsets were collected separately. A total of 1 × 10^4 sorted cells were transferred into naive Thy1.2+ mice together with 3 × 10^6 CFSE+ B6 splenocytes. These were used as an internal control for differences in injection efficiency and cellular distribution among mice. Five days later, mice were challenged with 1 × 10^6 PFU vac-OVA i.v. or s.c. or left unchallenged. (A and B) Summary data from rechallenged mice. (C) Summary of CD62L expression by α4 SP and ESL SP OT-I from mice immunized i.v. or s.c. with BMDC. Skin LN comprised of pooled axillary, brachial, cervical, and inguinal LN. Column labels indicate sorted populations and row labels indicate route of vac-OVA and site of analysis. (D) Representative two-dimensional FACS (Figure legend continues)
consistent with an early lymphatic cannulation study that showed a bias for memory T cells in afferent lymph (56) and led to the hypothesis that memory T cells enter LN from peripheral tissue and via afferent lymph (57). However, this study did not distinguish whether the cells were central or effector memory phenotype, and it did not examine the mechanism of entry into afferent lymph. Our work is also consistent with more recent work showing that CCR7 enables naive, effector, and memory CD4 T cells, and effector or unfractionated CD8 T cells in peripheral tissues to enter LN via the afferent lymph (25–27). However, these also did not address the role of adhesion molecules in cell entry into peripheral tissues or afferent lymph.

It is well established that the site of immunization controls the distribution of effector CD8 T cells into peripheral tissues (3, 5, 6, 8). This is also true of resident effector memory cells, which remain in nonlymphoid tissues and do not recirculate (15, 21, 58–60). However, it is unclear if the site of immunization controls the distribution of naive T cells and T CM isolated from different peripheral tissues demonstrate a modest level of homing preference back to those tissues (24). However, neither the molecules involved nor the influence of the site of immunization has been determined. On the basis of the relative size of recall responses after localized BMDC priming, we had previously suggested that the site of immunization influenced the quantitative distribution of memory T cells in different lymphoid organs (61, 62). In the current study, we found that the peripheral tissue HR expressed by resting memory cells, determined by the site of original Ag encounter, dictate the steady-state phenotypic and numerical distribution of memory cells among LN. This was evident even though CD62L-mediated entry was also occurred, demonstrating its fundamental importance. Importantly, LN-resident memory CD8 T cells expressing peripheral tissue HR proliferate upon rechallenge. On the one hand, ESL SP memory cells were disproportionately represented in skin-draining LN, resulting in an enhanced recall response after s.c. immunization. On the other hand, αβ1 integrin did not selectively influence the entry of memory CD8 T cells into particular LN or the overall size of the recall immune response to i.v. immunization. Thus, LN-resident memory is both systemic as well as quantitatively and qualitatively regional, enabling recall responses that are numerically larger and prefocused to home to appropriate peripheral tissues.

We previously showed that BMDC immunization by different routes constrained T cell activation to different LN beds, and programmed activated CD8 T cells to express distinct peripheral tissue HR (3, 4). We also demonstrated that activated CD8 T cells traffick to Ag-free LN, and these redistributed cells contain memory precursors (2). In this study, we show that entry into Ag-free LN does not change peripheral tissue HR expression, regardless of the sites of activation and trafficking, even when those LN are draining sites of viral infection. This contrasts with another study in which it was concluded that CD8 T cells primed by vaccinia virus in the inguinal LN expressed αβ2 integrin after trafficking to Ag-free mesenteric LN (32). Expression of αβ2 was eliminated by using FTY720 beginning 24 h prior viral infection to prevent inguinal LN egress. However, we have found that when FTY720 treatment is delayed until 24 h postinfection, cells activated in inguinal LN directly upregulate αβ2 (C. Brinkman, unpublished observations). This is consistent with the idea that FTY720 control of DC trafficking (63) may alter the infiltration of DC subsets that have distinct HR programming capabilities. Nonetheless, in vivo–generated memory T cells expressing one peripheral tissue HR can be reprogrammed to express a new one when rechallenged in vivo, whereas either retaining or downregulating previously programmed HR. This supports and extends previous in vitro studies (5, 7). Thus, recall effector T cells can acquire a multipotential homing phenotype after priming and reactivation in disparate sites. This enables cells to return to the original site of infection but also to home to new sites of infection and inflammation. These results suggest that appropriately constructed prime-boost vaccine regimens could generate memory cells and recall effectors with multipotential tissue homing abilities.

Our results provide a cautionary note concerning the ways in which memory T cells have been subdivided. In keeping with expectations about their localization, T CM have been defined as CD62L+, whereas T EM have been defined as CD62L− (13). However, these characterizations have most often been based on cells isolated from spleen, blood, and nonlymphoid tissues rather than from LN (13, 14, 28, 31, 64, 65). It is implicit in this classification that T CM cells use CD62L to enter LN via HEV in the same manner as naive cells and that T EM would potentially be excluded from LN altogether. However, we showed that most memory OT-I cells in LN were CD62L−, and peripheral tissue HR can mediate T cell entry into LN in lieu of CD62L. These cells also mounted a robust recall response. There have also been several reports of CD62L+ memory CD4 and CD8 T cells in peripheral tissues (15, 19, 29, 30). These are found in appreciable numbers (19) and appear to be migratory in the case of CD4 memory T cells (15). A recent study by Bromley et al. (27) identified a subset of CD4 memory T cells that constitutively drain from skin to LN in a CCR7-dependent manner and recirculate between blood, skin, and LN. These cells do not fit neatly into either T CM or T EM categorizations. Although they expressed intermediate levels of CD62L and were ESL+, the role of these molecules in the migration of these cells, and degree to which their HR expression and migration patterns were fixed or flexible, was not evaluated. Our findings stand in contrast to another study that suggested that CD8 memory T cells do not recirculate from skin to LN (15). Instead, they complement the work of Bromley et al. (27) and extend the concept of peripheral tissue recirculating memory to CD8 T cells. We think it likely that the functional roles for ESL and αβ1 integrin in CD62L-independent recruitment to uninflamed LN, which we have described in this paper, will also apply to the memory CD4 cells described by Bromley et al. (27).

Collectively, this indicates that the exclusive use of CD62L and CCR7 to distinguish T CM and T EM is insufficient, particularly when investigating cells in LN. If the essential features of T CM are resident in LN and expansion after Ag encounter, then the definition of T CM must also account for cells that are CD62L−, enter LN via alternate mechanisms, and may also be represented in peripheral tissue.

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*Plots from rechallenged mice gated on divided CD44hiCD8+Thy1.1+ cells and positive staining thresholds were set with FMO controls. (A, B, C, E and F) Pooled data from four to eight mice per group from two experiments. Bars show mean values (± SEM). *p < 0.05, **p < 0.01, ***p < 0.001 by unpaired Student t test."
Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In the abbreviations footnote, there is a prefix (mTyr) that was placed incorrectly. The abbreviations in question are currently written: mTyr-PSL, P-selectin ligand; Vac, vaccinia virus used encoded tyrosinase. They should read: PSL, P-selectin ligand; mTyr-Vac, vaccinia virus expressing tyrosinase. We apologize for any confusion this may have caused.

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Corrections


Several small errors appear in figure labeling. In Figs. 4E and 4G, the y-axis label should read “# of CD44[^hi] CD8 Cotransfer,” not “% of CD44[^ hi] CD8 Cotransfer;” in Fig. 6E, the y-axis label should read “α4 SP of CD44[^hi] CD8[^−],” not “ESL SP of CD44[^ hi] CD8[^−];” and in Fig. 8A, the x-axis label for the rightmost black bar should read “s.c. Vac-OVA,” not “s.v. Vac-OVA.” We apologize for any confusion this may have caused. The corrected figures are shown below. The legends were correct as published and are shown below for reference.

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FIGURE 4. αβ1 and ESL can mediate CD62L-independent entry of activated OT-I T cells into LN. (A–G) CD8 T cells were enriched from LN and spleen of s.c. BMDC immunized mice, labeled with CellTrace Violet, treated with MEL-14 for 30 min and injected into naive B6 mice. Recipients were treated i.p. with 100 μg 9A9, RB40, MK2-7, or Rat IgG control Abs 8 h prior to T cell transfer, and processed for flow cytometry 16 h later. Skin LN composed of pooled axillary, brachial, cervical, and inguinal LN. (A and B) Results are from at least three mice per group from one experiment representative of two independent experiments. (C) Plots are gated on labeled Thy1.1\(^+\)CD8\(^+\) OT-I or Thy1.1\(^-\)CD44\(^+\)CD8\(^-\) endogenous T cells and positive staining thresholds were set with FMO controls. (C–G) Results are from at least three mice per group from one experiment representative of three independent experiments. Bars show mean values (± SEM). *p < 0.05, **p < 0.01, ***p < 0.001 unpaired Student t test in (A and B), Tukey posttests of one-way ANOVA in (D–G).
FIGURE 6. Expression of peripheral tissue HR influences the distribution of LN-resident memory cells independent of CD62L Thy 1.2 mice received CFSE Thy 1.1 OT-I cells and were immunized i.v. (A and F) or s.c. (B–E) with BMDC. Mice were harvested 6–8 wk later. Plots are gated on CD44 CD8 Thy1.1 cells in (A) and (B) and positive staining thresholds were set with FMO controls. (D and E) CD44 CD8 Thy1.1 (non OT-I) T cells were gated on and the fraction expressing the indicated peripheral tissue HR was determined. Skin LN composed of pooled axillary, brachial, cervical, and inguinal LN. (A and B) Results representative of 6–10 mice of each condition from three to four experiments. (C) Results are pooled data of five to six mice from two experiments. (D and E) Results are pooled data of eight mice from two experiments. Bars show mean values (± SEM). * p < 0.05, ** p < 0.01, *** p < 0.001 paired Student t test compared with skin LN in (C–E), unpaired in (F). In (F), total skin T cell cellularity estimated by extrapolating cell counts from two ears (~2 cm2) to the entire surface area of a 20-g mouse (~36 cm2).
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