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Activated CD8 T Cells Redistribute to Antigen-Free Lymph Nodes and Exhibit Effector and Memory Characteristics


Exogenous dendritic cells display restricted trafficking when injected in vivo and stimulate CD8 T cell responses that are localized to a small number of lymphoid compartments. By examining these responses in the presence and absence of FTY720, a drug that causes sequestration of T cells in lymph nodes, we demonstrate that a significant fraction of divided CD8 T cells redistribute into Ag-free lymph nodes within 3 days of activation. Despite variation in the level of expression of CD62L, redistribution of these cells is CD62L-dependent. Redistributed CD8 T cells exhibit characteristics of differentiated effectors. However, when re-isolated from Ag-free lymph nodes 3 days after activation and transferred into naive mice, they persist for at least 3 wk and expand upon Ag challenge. Thus, CD8 T cells that redistribute to Ag-free lymph nodes 3 days after immunization contain memory precursors. We suggest that this redistribution process represents an important mechanism for establishment of lymph node resident central memory, and that redistribution to Ag-free nodes is an additional characteristic to be added to those that distinguish memory precursors from terminal effectors. The Journal of Immunology, 2008, 181: 1814–1824.

Over the last several years, much has been done to elucidate the processes of CD8 T cell activation and differentiation in vivo. The interactions between Ag-specific T cells and Ag-presenting dendritic cells (DC) during the early stages of T cell activation have been nicely detailed using two-photon microscopy (1–3). Similarly, tracking the entry of soluble Ag into individual lymph nodes (LN) after s.c. injection has provided insight into multiple stages of Ag presentation by LN-resident and skin-derived DC (4). In addition, the distribution of activated effectors into peripheral nonlymphoid tissues at later times during the primary response has also been evaluated in detail (5–9). However, the understanding of intermediate stages of T cell differentiation, and their progression within individual lymphoid compartments is incomplete. Many previous studies have used pathogens that activate systemic responses, and the ensuing T cell responses have been examined only in the spleen (10–14). Because the spleen is both a T cell activation site and a repository for responses have been examined only in the spleen (10–14). Be-

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5 Abbreviations used in this paper: DC, dendritic cell; LN, lymph nodes; BMDC, bone marrow-derived dendritic cell; TCM, central T memory.

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Unlike pathogens, exogenous bone marrow-derived DC (BMDC) localization is highly restricted in vivo, and depends on the injection route (32–39). Thus, BMDC enable immune responses in individual lymphoid compartments to be studied without the uncertainty of Ag localization and persistence. To date, several studies have examined the characteristics of CD8 immune responses that develop after BMDC immunization and have noted differences based on route of injection (32, 37–42). However, few have examined the early temporal phases and spatial distributions of these responses, and how these relate to the characteristics of the resulting memory cells.

In the present work, we have used the localized trafficking of BMDC to examine the activation and differentiation of CD8 T cells in individual lymphoid compartments over time. Furthermore, we have confined these activated cells to lymphoid tissue by using the drug FTY720 (43). Using these techniques, we have identified a population of extensively divided cells that rapidly redistribute into demonstrably Ag-free LN in a CD62L-dependent manner. These redistributed cells exhibit effector characteristics, and also contain memory precursors. The existence of this population is not currently appreciated in models of primary immune responses or memory establishment. We suggest that redistribution of activated cells to Ag-free LN early during the primary response represents an important mechanism for establishment of LN resident T_{CM} cells, and an important characteristic of central memory precursor cells that is not included in current models.

Materials and Methods

Mice, viruses, and viral infection

C57BL/6, OT-I RAG1−/−, and C57BL/6 Thy-1.1 mice were obtained from Charles River Laboratories, Taconic Farms, and The Jackson Laboratory, respectively. OT-I Thy1.1 mice were first generation crosses of OT-I RAG1−/− and C57BL/6 Thy-1.1 mice. All animals were maintained in pathogen-free facilities. Recombinant vaccinia virus expressing OVA (vacinia-OVA) was a gift from Dr. J. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Mice were immunized with 1 × 10^6 PFU virus i.v. via the dorsal tail vein. All protocols were approved by the Institutional Animal Care and Use Committee.

Adoptive transfer of OT-I cells

Single cell suspensions from the spleen and pooled LN of OT-I RAG1−/− and OT-I Thy1.1 mice were enriched for CD8 T cells by negative selection (Stem Cell Technologies). Preparations were consistently 97–99% CD8+ and OT-I Thy1.1 mice were enriched for CD8 T cells by negative selection using the drug FTY720 (43). Using these techniques, we have identified a population of extensively divided cells that rapidly redistribute into demonstrably Ag-free LN early during the primary response represents an important mechanism for establishment of LN resident T_{CM} cells, and an important characteristic of central memory precursor cells that is not included in current models.

FIGURE 1. BMDC immunization via different routes results in T cell priming in distinct and limited lymphoid compartments. Mice received 4 × 10^6 CD8−enriched, CFSE-labeled OT-I cells and were immunized 18–36 h later with CD40L-activated OVA257-pulsed BMDC or no BMDC (adoptive transfer (AT) only) via the indicated route. Lymphoid organs were harvested 24 h later. Dot plots are gated on CD8+ H-2Kb-OVA257-tetramer+ lymphocytes. Data is representative of three independent experiments.

Flow cytometric analysis of surface markers and sorting

Single cell suspensions were incubated with anti-CD16/32 (93, eBioScience) to block Fc receptors. PerCP anti-CD8a (53–67.6), PE anti-α4 integrin (R1–2), and PE anti-αβ7 (DATK32) were from BD Biosciences, allophycocyanin anti-IFN-γ (XMG1.2), allophycocyanin anti-Thy1.2 (53–2.1) and anti-Thy1.1 (HIS51), PE anti-CD69 (H1.2F3), FITC and PE anti-CD4 (IM7), PE and PE-Cy7 anti-CD62L (MEL-14), and anti-CD127-PE (ATR34) were from eBioscience. Allophycocyanin-conjugated H-2Kb-OVA257 tetramer was generated in-house. Cells from mice treated with anti-CD62L in vivo were blocked with 10% normal goat serum before incubation with PE goat F(ab)2 anti-rat IgG (Jackson ImmunoResearch), followed by purified rat IgG (Jackson ImmunoResearch) and then PerCP-Cy5.5 anti-CD8α and allophycocyanin anti-Thy1.2. Samples were analyzed on FACSCalibur and FACSCanto instruments (Becton Dickinson) using FlowJo software (TreeStar). Electronic sorting was conducted on a Becton Dickinson FACSVantage SE Turbo Sorter with DIVA Option.

Analysis of intracellular IFN-γ production

Lymphoid cells from mice 5 days after i.v. immunization were assessed for cytokine production by incubation for 5 h at a ratio of 1:1 with LB15.13 stimulator cells that had been pulsed with 100 μM OVA257. Medium was supplemented with 50 U/ml IL-2 (Chiron) and 10 μg/ml brefeldin A (Sigma-Aldrich). Lymphoid cells were pretreated with 100 μM TAPI-2 (PepTides International) for 1 h and during the assay to prevent CD62L down-regulation as described (46). Cells were fixed and permeabilized using Cytofix/Cytperm (BD Biosciences) and stained for intracellular IFN-γ.

Results

BMDC immunization leads to localized activation of CD8 T cells followed by redistribution into noncontiguous LN

We previously demonstrated that peptide-pulsed BMDC infiltrate a limited subset of lymphoid organs based on injection route, resulting in localized T cell activation in specific lymphoid compartments (39). CD8+ OT-I TCR transgenic T cells labeled with CFSE were adoptively transferred into mice, and OVA257 peptide-pulsed CD40L-activated BMDC were injected by either i.p., i.v., or s.c. routes 18–36 h later. Twenty-two hours after BMDC injection, activated OT-I cells were identified based on expression of CD69. CD69+ OT-I cells were localized to spleen and mediastinal LN after i.v. immunization, to mesenteric LN and mediastinal LN after i.p. immunization, and to axillary/brachial LN after s.c. immunization (Fig. 1 and data not shown). S.c. immunization also led to...
activation of cells in spleen, although this was highly variable in different experiments (data not shown). By 72–96 h after immunization, CFSE dilution revealed a substantial number of divided OT-I cells in these sites of CD69 up-regulation, or “priming compartments” (indicated by asterisks in Fig. 2). However, extensively divided OT-I cells were also present in all other LN examined at this time. Greater than 90% of OT-1 cells remained undivided in the same compartments of mice that had received either no BMDC or unpulsed BMDC (data not shown), demonstrating that divided cells in these other LN had been activated by Ag.

The presence of divided OT-I cells in LN other than those in which initial BMDC priming occurred was not due to delayed migration of BMDC to these sites: we previously demonstrated that OT-I cells injected up to 48 h after BMDC still underwent activation only in the previously identified priming compartments (39). Thus, the presence of divided OT-I cells in LN noncontiguous with the priming compartments appeared to represent the dissemination of activated T cells. To directly test this hypothesis, we confined OT-I cells to the compartment in which they were activated by treating mice with FTY720, a
pharmacological analog of sphingosine 1-phosphate that inhibits T lymphocyte egress from LN by binding to S1P1 receptors (43). In the priming compartments of immunized mice treated with FTY720, the CFSE dilution profiles showed an accentuated accumulation of more extensively divided cells compared with controls (Fig. 2), and a 3–4-fold increase in the average number of activated OT-I cells in priming LN (data not shown). However, FTY720 treatment reduced the presence of divided OT-I cells in noncontiguous LN of these same mice by 60–95% in different experiments. These data demonstrate that divided OT-I cells exited priming compartments and redistributed to Ag-free LN by 3 days after immunization.

**FIGURE 3.** Redistribution of divided T cells occurs at physiological T cell precursor frequencies. A and D, Thy1.1⁺ mice received the indicated number (A) or 4 × 10⁶ (D) CD8-enriched, CFSE-labeled Thy1.2⁺ OT-I cells and were immunized i.v. 24 h later with BMDC. Lymphoid organs were harvested 7 (A) or 3 days (D) after immunization. Dot plots are gated on CD8⁺ Thy1.2⁺ lymphocytes. Values in large type indicate the percentage of OT-I cells that had divided one or more times and were CD62L⁺. B and C, Endogenous responses were evaluated 7 days after immunization of normal C57BL/6 mice with CD40L-activated OVA₂₅₇-pulsed BMDC. Dot plots are gated on live (B) or CD8⁺ H-2K⁺-OVA₂₅₇-tetramer⁺ (C) lymphocytes. Values in bold indicate the percentage of CD8⁺ cells that were H-2K⁺-OVA₂₅₇-tetramer⁺ (B) or the percentage of OT-I cells that had divided one or more times and were CD62L⁺ (C). Data is representative of two to four mice per condition in at least three independent experiments. Asterisks indicate priming compartments as defined by CD69 up-regulation in Fig. 1 and in the text.
Redistribution of divided T cells occurs at physiological T cell precursor frequencies

It was possible that the redistribution of activated T cells from priming to Ag-free lymphoid compartments is due to the adoptive transfer of large numbers (4 × 10^6) of T cells, resulting in the “spillover” of activated T cells into other LN. Thus, we compared responses elicited by i.v. immunization with BMDC in mice transferred with as few as 4 × 10^2 OT-I T cells. We also examined the endogenous CD8 T cell response in mice that did not receive any transferred cells. By day 7 after immunization, redistribution of CFSE-diluted cells into axillary/brachial LN had occurred at all adoptive transfer numbers tested (Fig. 3A). Similar data were also obtained after immunization via the i.p. and s.c routes (data not shown). Redistribution of OVA257-H-2Kb tetramer positive CD8 cells was also evident in the endogenous response (Fig. 3, B and C). These results demonstrate that the rapid redistribution of activated T cells into Ag-free LN is not simply due to “spillover” from priming compartments based on limitations of space and large numbers of activated cells, but is also characteristic of immune responses based on relatively low numbers of T cells.

Activated CD62L^{high} OT-I cells redistribute into noncontiguous LN predominantly via a CD62L-dependent mechanism

After immunization by different routes, a significant fraction of divided OT-I cells in priming compartments of mice that received 4 × 10^6 OT-I cells had down-regulated CD62L expression by 72 h, consistent with a pre-effector or effector phenotype (Fig. 3D and data not shown). However, ~1/3 of these divided cells remained CD62L^{high} despite having undergone as many as 6–7 cell divisions. Although the divided cells in Ag-free LN had undergone a comparable number of cell divisions, the CD62L^{high} fraction was even greater: 62–76% (Fig. 3D and data not shown). Similar data were also obtained after immunization via the i.p. and s.c routes (data not shown). In keeping with previous work (24, 47), we found that the percentage of cells that remained CD62L^{high} after activation was directly correlated with the number of adoptively transferred cells (Fig. 3, A and C and data not shown). However, in all cases, as well as in the endogenous response, the representation of CD62L^{high} divided cells was enhanced in Ag-free axillary LN (Fig. 3, A and C). This again demonstrates that redistribution is not due to the elevated percentage of activated CD62L^{high} cells that develop in animals transferred with large numbers of OT-I cells.

The above results suggested that CD62L was responsible for the entry of divided OT-I cells into Ag-free LN. To test this, i.v. immunized animals were treated with anti-CD62L Ab beginning 6 h after BMDC injection, and continuing every 24 h until harvest 3 days postimmunization. This resulted in the accumulation of more extensively divided OT-I cells in mediastinal LN priming compartment (Fig. 4A), and the ratio of divided to undivided cells increased from ~5:1 to roughly 150:1. This likely reflects anti-CD62L blockade of entry of naïve cells coupled with continued division of activated OT-I cells already in the LN. In contrast, CD62L blockade substantially inhibited the redistribution of

**FIGURE 4.** Activated CD62L^{high} OT-I cells redistribute into noncontiguous LN predominantly via a CD62L-dependent mechanism. Mice received 4 × 10^6 CD8-enriched, CFSE-labeled OT-I cells and were immunized i.v. with BMDC 18–36 h later. A, B, and D, Anti-CD62L or Rat IgG was injected 6 h later and every 24 h thereafter until harvest at 72 h. Dot plots and histogram are gated on CD8^{+} Thy1.2^{+} lymphocytes and are representative of four to six mice in two to three independent experiments. CD62L expression on cells from anti-CD62L treated was measured as described in Materials and Methods. B, Numbers in large type indicate percentage of divided cells expressing α4β7. A and B are from independent experiments. Asterisks indicate priming compartments as defined by CD69 up-regulation in Fig. 1 and in the text. C, CD62L expression on divided CD62L^{high} (black fill) and CD62L^{low} (solid line) cells in axillary/brachial LN. Dashed line is negative staining control lacking only anti-CD62L Ab. D, Summary data showing mean values (±SEM) from three mice in one experiment representative of two independent experiments.
divided OT-I cells into Ag-free peripheral LN (Fig. 4A). Although there was seemingly little effect of anti-CD62L treatment on the redistribution of divided OT-I cells into mesenteric LN, the majority of these cells in anti-CD62L-treated mice expressed CD62Lhigh integrin, while those in control mice were predominantly negative (Fig. 4B). No such enrichment for CD62Lhigh OT-I cells was evident in other Ag-free LN. Expression of this integrin is known to suffice for entry of naive or memory T cells into mesenteric LN (48). Thus, our results show that activated cells that redistribute into most Ag-free LN do so by a CD62L-dependent mechanism.

The CD62L-dependent redistribution of CD62Lhigh divided OT-I cells was not surprising. Importantly, however, the level of CD62L expression on CD62Llow cells was significantly above background (Fig. 4C). Thus, we were interested to know whether CD62Llow cells, which predominated at lower adoptive transfer numbers and in the endogenous response, redistributed by this same mechanism. To test whether CD62Llow cells were enriched among the divided OT-I cells that continued to redistribute in anti-CD62L treated mice, we used fluorescently labeled anti-rat IgG to detect bound anti-CD62L Ab. Using this approach, we could clearly distinguish CD62Lhigh and CD62Llow populations in all lymphoid organs. We found that there was no enrichment of CD62Llow divided OT-I cells in Ag-free axillary/brachial or cervical LN in anti-CD62L treated mice relative to the populations in untreated mice (Fig. 4, A and D). Thus, the presence of divided CD62Lhigh and CD62Llow cells in Ag-free peripheral LN is equally dependent on CD62L. This also establishes that, in contrast to redistribution into mesenteric LN, CD62Llow cells have
no method to enter peripheral LN independent of CD62L. This result suggests that either the level of expression on CD62L<sub>low</sub> cells suffices for LN entry or these cells enter LN as CD62L<sub>high</sub> cells and down-regulate it thereafter.

**Stability of CD62L<sup>high</sup> and CD62L<sup>low</sup> cells in Ag-free LN after redistribution**

To determine whether CD62L<sup>high</sup> cells down-regulated this molecule after redistribution into peripheral LN, we treated i.v. immunized mice with FTY720 starting on day 3 and every 24 h thereafter until harvest on day 9. By delaying FTY720 treatment, we allowed initial redistribution to occur, but then trapped redistributed cells and prevented movement of cells between compartments. Using mice transferred with either 4 x 10<sup>6</sup> or 4 x 10<sup>4</sup> OT-I cells, we found that the ratios of divided CD62L<sup>high</sup> to CD62L<sup>low</sup> OT-I cells in Ag-free LN of FTY720 treated mice were not different from the ratios in untreated mice (Fig. 5, A and B). This result demonstrates that neither population expands or contracts in relation to the other during the 6 days after redistribution occurs. Consistent with this, in the absence of FTY720 treatment the relative percentages of CD62L<sup>high</sup> and CD62L<sup>low</sup> cells in Ag-free LN of i.v immunized animals do not change between 7 and 40 days after immunization (Fig. 5C). Similar data were also obtained after immunization via the i.p. route (data not shown). We cannot rigorously exclude the possibility that conversion of CD62L<sup>high</sup> cells occurs, but is balanced by an equivalent conversion of CD62L<sup>low</sup> cells to a CD62L<sup>high</sup> phenotype. Nonetheless, these data collectively indicate that both CD62L<sup>high</sup> and CD62L<sup>low</sup> CD8 T cells are long-term residents of LN.

**Redistributed OT-I cells have a well-differentiated effector cell phenotype**

Most models of CD8 T cell differentiation envision that cells with effector activity will migrate from lymphoid sites to peripheral nonlymphoid tissues, blood, and spleen. Thus, it was of interest to determine the effector status of the divided CD62L<sup>high</sup> and CD62L<sup>low</sup> OT-I cells that had redistributed into Ag-free LN. Therefore, we assessed their ability to produce IFN-γ after a short ex vivo peptide restimulation. To avoid the short-term reduction in CD62L expression that follows Ag stimulation, we incubated cells ex vivo peptide restimulation. To avoid the short-term reduction in CD62L expression that follows Ag stimulation, we incubated cells after a short-term incubation with TAP1-2, an inhibitor of the TNF-α-converting enzyme shedding (46), during stimulation. TAP1-2 treatment had no effect on IFN-γ production (data not shown). Compared with divided cells in spleen as representative of fully differentiated effectors, we found no significant difference in the percentage of divided cells in Ag-free LN making IFN-γ, or in the mean fluorescence intensity of IFN-γ expression (Fig. 6, A–F). Interestingly, in both Ag-free LN and spleen, the representation of effector cells in the CD62L<sup>high</sup> subset was equivalent to or higher than that of the CD62L<sup>low</sup> subset, depending on the number of cells that were adoptively transferred. Divided cells in Ag-free LN and spleen also uniformly down-regulated CD127 × 72 h postimmunization (Fig. 6G), and up-regulated CD44 and CD62L<sup>high</sup> expression (Fig. 6C). Interestingly, in both Ag-free LN and spleen, the representation of effector cells in the CD62L<sup>high</sup> subset was equivalent to or higher than that of the CD62L<sup>low</sup> subset, depending on the number of cells that were adoptively transferred. Divided cells in Ag-free LN and spleen also uniformly down-regulated CD127 × 72 h postimmunization (Fig. 6G), and up-regulated CD44 and CD62L<sup>high</sup> expression (Fig. 6C). Simulated data were also obtained after immunization via the i.p. and s.c routes (data not shown). Thus, divided OT-I cells that had redistributed to peripheral LN were well differentiated.

**Redistributed OT-I cells contain memory cell precursors**

The foregoing results established that the divided OT-I cells that had redistributed to peripheral LN persisted for at least 9 days, but also exhibited characteristics of well-differentiated effector cells. Thus, we were interested in whether these cells were terminal, or were also a source of memory. In keeping with the latter possibil-
ity, we found a significant increase in the percentage of divided OT-I cells in Ag-free LN that were CD127/H11001 by 7 days after immunization (Fig. 7A). Even larger percentages of these cells were CD127/H11001 on day 7 when the adoptive transfer number was reduced (Fig. 7B). Although these results were consistent with the idea that the redistributed T cells re-expressed CD127, they did not exclude the possibility that CD127/H11001 cells entered Ag-free LN at a later time point. However, when we trapped redistributed cells in Ag-free LN by initiating FTY720 treatment on day 3 and continuing until day 7, we found that over two-thirds of the divided T cells in Ag-free axillary/brachial and cervical LN were CD127/H11001 (Fig. 7C). This result indicates that the divided OT-I cells that redistribute on day 3 possess the ability to up-regulate CD127 by day 7, or that a minor subset of redistributed cells that are initially CD127/H11001 expand significantly over this time period. Either mechanism is consistent with the idea that the redistributed cells contain memory CD8 T cell precursors.

To directly establish the potential of redistributed cells to seed memory, we sorted divided CD62L<sup>high</sup> and CD62L<sup>low</sup> Thy1.1<sup>+</sup> OT-I cells from Ag-free LN 3 days after i.v. immunization. The purity of each of these populations was 90% as assessed by flow cytometry (data not shown). Twenty thousand cells of each phenotype were transferred into naive Thy1.2<sup>+</sup> mice together with 3 x 10<sup>6</sup> CFSE-labeled Thy1.2<sup>+</sup> TCR transgenic T cells with an irrelevant specificity (49). These were used as an internal control for differences in injection efficiency and cellular distribution among mice. Three weeks later, mice were challenged with vaccinia-OVA i.v. or left unchallenged and lymphoid organs were harvested on day 7 after infection. Three to four million events were collected in each sample. Numbers indicate gated populations as a percentage of total lymphocytes. Asterisks indicate priming compartments as defined by CD69 up-regulation in Fig. 1 and in the text.

**FIGURE 7.** Redistributed OT-I cells contain memory cell precursors. Thy 1.1<sup>+</sup> mice were adoptively transferred with 4 x 10<sup>6</sup> (A and C), or the indicated number of CFSE-labeled Thy 1.2<sup>+</sup> OT-I cells and immunized i.v. with BMDC 18–36 h later. Lymphoid organs were harvested at the indicated times after immunization (A), or at 7 days (B and C). A, Mean values from two to six mice (±SEM) pooled from seven independent experiments. B, Mean values (±SEM) from three mice per group in one experiment. C, Mice received FTY720 or vehicle beginning 3 days after immunization and then every 24 h until harvest on day 7. Dot plots are gated on CD8<sup>+</sup> Thy1.2<sup>+</sup> lymphocytes. Large type numbers in upper left indicate percentage of divided cells expressing CD127. D, Pooled Ag-free LN from Thy 1.2<sup>+</sup> mice that had received Thy1.1<sup>+</sup> OT-I cells were harvested 72 h after i.v. immunization. Cells were electronically sorted to select for CFSE dilution (more than one division), 7-AAD<sup>neg</sup>, CD8<sup>+</sup>, and Thy1.1<sup>+</sup>. CD62L<sup>high</sup> and CD62L<sup>low</sup> subsets were collected separately. Sorted cells (2 x 10<sup>5</sup>) were transferred into naive Thy1.2<sup>+</sup> mice together with 3 x 10<sup>6</sup> CFSE-labeled Thy1.2<sup>+</sup> TCR transgenic T cells with an irrelevant specificity (49). These were used as an internal control for differences in injection efficiency and cellular distribution among mice. Three weeks later, mice were challenged with vaccinia-OVA i.v. or left unchallenged and lymphoid organs were harvested on day 7 after infection. Three to four million events were collected in each sample. Numbers indicate gated populations as a percentage of total lymphocytes. Asterisks indicate priming compartments as defined by CD69 up-regulation in Fig. 1 and in the text.
OT-I cells were virtually undetectable in either peripheral LN or spleen of unimmunized animals (Fig. 6D). However, they were readily detectable in both compartments of vaccinia-challenged animals. Relative to the cotransferred CFSE-labeled control cells, recall OT-I cells were more evident in spleen that LN, as would be expected for day 7 effectors. Nonetheless, both CD62L<sup>high</sup> and CD62L<sup>low</sup> transferred cells gave rise to significant numbers of recall cells in LN. Interestingly, the number of recall cells derived from transferred CD62L<sup>high</sup> precursors was ~5-fold higher than from the same number of CD62L<sup>low</sup> precursors. Collectively, our results suggest that the activated cells that redistribute into Ag-free LN within 72 h after activation contain precursors of memory CD8 cells, and these are enriched in the subset of redistributed cells that are CD62L<sup>high</sup>.

Discussion

In the current work, we have used local immunization with peptide-pulsed, CD40L-activated BMDC to characterize the differentiation of CD8 T cells in individual LN and their migration among LN. Peptide-pulsed BMDC traffic rapidly into lymphoid tissue in a highly restricted pattern after injection (37, 38), and remain localized and active for at least 48 h (39). Thus, the use of this immunogen avoids the uncertainty about location and amount of Ag that is inherent in the use of viruses, bacteria, or peptides in an adjuvant to study T cell activation in vivo. Using this system, we have demonstrated the existence of a subset of activated CD8 T cells that: 1) redistribute into Ag-free LN within 72 h via a CD62L-dependent mechanism, 2) exhibit the characteristics of fully differentiated effector cells; and 3) contain precursors of memory cells.

An important observation in this study is that CD8 T cells activated in one LN redistribute within 3 days into other LN that are demonstrably free of APC-bearing cognate Ag. Most models of CD8 T cell differentiation emphasize its occurrence in lymphoid compartments in which Ag is encountered, followed by dissemination to blood, spleen, and nonlymphoid tissues (5–14). However, models for the differentiation of CD8 T cells in LN specifically have evolved from studies in which differentiated effector cells were identified primarily or exclusively in draining LN or spleen, and presumptive nondraining LN were not evaluated (6, 15–18). It was suggested in one previous study (20) that CD8 T cells activated after intranasal administration of influenza virus redistributed to nondraining LN. However, no conclusive data to support this suggestion was provided, and it was also acknowledged that these results might be due instead to delayed Ag presentation in these initially nondraining compartments. Indeed, using the same virus and route of administration, another group made similar observations and concluded that the response was “broadly systemic” (19). This is in keeping with the demonstration that Ag presentation after viral infection is prolonged (21, 22) and occurs in LN where virus is not detected (15). The suggestion that activated CD8 T cells had redistributed from priming to Ag-free LN was also made in one prior study using BMDC as an immunogen (40). Again, however, no direct demonstration of this phenomenon was provided, and the authors acknowledged the possibility of disseminated Ag presentation due to transfer of peptide-MHC complexes to endogenous DC. More recently, Liu et al. (50) used FTY720 administration to provide the first direct demonstration that virus-specific cells in skin-draining LN could redistribute to other LN. However, this study did not investigate the mechanism of redistribution, nor the relationship of these cells to either conventional effector cells or memory precursors. In this study, we circumvented uncertainty concerning Ag localization by using peptide-pulsed BMDC, and used FTY720 to directly demonstrate that activated OT-I cells redistribute from priming compartments to Ag-free LN. We have extended the observations of Liu et al. (50) by showing that redistribution occurs regardless of the number of OT-I cells adoptively transferred, and also during the endogenous response, indicating that it is not simply a consequence of activating large numbers of Ag-specific T cells in spatially limited lymphoid compartments. We have also observed FTY720-inhibitable redistribution of activated T cells to LN using vaccinia virus as an immunogen (our unpublished data) demonstrating that it is not limited to responses initiated with BMDC. Instead, redistribution of activated CD8 T cells to Ag-free LN is a normal aspect of immune response whose significance has not been well understood.

Our work has also demonstrated that redistribution of activated CD8 T cells to most peripheral Ag-free LN is mediated by CD62L, in keeping with its already well-known involvement in the entry of naive T cells into LN. In separate work (39), we established that a small population of activated CD8 T cells expressing e4β7 redistributes from mesenteric to mediastinal LN in a process mediated by that integrin. However, in that instance, both LN were priming compartments. In the present study, we observed that activated e4β7<sup>+</sup> cells redistribute to Ag-free mesenteric LN in a CD62L-independent manner. Thus, redistribution of activated cells to Ag-free LN during the primary immune response is a general phenomenon that can be mediated by distinct mechanisms. Interestingly, regardless of the number of cells initially transferred, redistributed CD8 cells included subsets expressing high and low levels of CD62L, and the fraction of CD62L<sup>high</sup> cells was higher in cells that had redistributed than in priming LN. However, the representation of CD62L<sup>high</sup> and CD62L<sup>low</sup> cells in Ag-free LN was equally susceptible to anti-CD62L blockade. The relative frequencies of these populations also remained stable after redistribution, suggesting that they do not interconvert. However, it remains possible that a portion of redistributing cells down-regulate CD62L very rapidly after LN entry, and also that this process is subsequently balanced by up-regulation, leading to the appearance that CD62L<sup>high</sup> and CD62L<sup>low</sup> cells are stable and separate populations. Alternatively, CD62L<sup>low</sup> expression, which is measurably above that of CD62L<sup>neg</sup> cells, may be sufficient for LN entry. Regardless of the exact mechanism, we and others (24, 50) have also observed that previously activated CD8 cells with a reduced level of CD62L expression persist in LN long term.

Recent work has led to significant concerns about how accurately the processes of differentiation and memory are represented in animals transferred with large numbers of CD8 T cells. In particular, it was demonstrated that the fraction of activated CD8 cells that retain CD62L<sup>high</sup> expression after infection with an Ag expressing pathogen is higher in animals transferred with relatively high numbers of cells (24, 47). It was also shown that long-term CD62L<sup>low</sup> memory cells rescued from such animals reacquired a CD62L<sup>high</sup> phenotype after adoptive transfer, leading to their designation as “transitional memory cells” (24). The development of transitional memory cells was diminished by increasing the number of endogenous APC, suggesting that an elevated ratio of Ag-specific T cells to APC results in compromised or altered activation. Whether activated early stage CD62L<sup>low</sup> cells show a similar reacquisition of CD62L is unknown. However, asymmetrical division of a single T cell has been shown to produce memory and effector lineage cells that are CD62L<sup>high</sup> and CD62L<sup>low</sup>, respectively, as early as the first cell division (51). Thus, while adoptive transfer may alter the fraction of CD62L<sup>high</sup> and CD62L<sup>low</sup> expressing cells, both are physiologically relevant offspring to the CD8 T cell activation process. Larger adoptive transfer numbers were also associated with a higher fraction of activated early stage
cells that retained CD127 (47), although a second study using a distinct immunogen observed different kinetics of CD127 loss and re-expression, and a more complex relationship to adoptive transfer number (52).

We have demonstrated a direct correlation between adoptive transfer number and CD62L^high expression on activated early stage cells similar to that described previously (24, 47). However, we found that the percentage of activated cells expressing CD127 on day 7 was elevated, not diminished, when lower numbers of cells were transferred. This difference with previous work is likely attributable to our use of BMDC as an immunogen. The administration of exogenous DC may reduce the elevated T cell:APC ratio thought to be responsible for altered T cell differentiation in other models using pathogens presented by endogenous DC. Finally, larger adoptive transfer numbers were associated with a smaller fraction of cells expressing granzyme B, but a larger fraction expressing IL-2 and TNF (47). In our hands, we found that the fraction of cells expressing IFN-γ was enhanced at high adoptive transfer numbers. Nonetheless, in both studies, these differences were quantitative rather than qualitative. Thus, although adoptive transfer number can clearly affect the proportions of cells expressing effector or memory markers, it does not obviate the fundamental observation that activated CD8 T cells that have redistributed to an Ag-free LN contain both effectors and memory precursors.

Another important observation in the present study is that the CD8 cells that redistribute to Ag-free LN, regardless of their level of CD62L expression, have characteristics of well-differentiated effectors. Their production of IFN-γ was equivalent to that of “classical” splenic effectors, and they had acquired high level expression of either αββ1 or αβββ integrin (39), enabling them to home to peripheral nonlymphoid tissue. The biological significance of having CD8 T cells with effector activity in LN where there is no Ag or active infection is not clear. In contrast, we have also demonstrated that redistributed CD8 cells are not simply terminal effectors that die as the primary response contracts, but contain the precursors of memory cells. Regardless of adoptive transfer number, a significant fraction of redistributed cells express CD127 by day 7, which has been associated with a memory or prememory phenotype (25, 53). More directly, redistributed cells rescued from LN 3 days after immunization persisted as memory cells after adoptive transfer into naive recipients. This time frame for the establishment of memory precursors is consistent with that of other work in which activated cells were recovered from priming LN (18). From this perspective, the observation of effector activity in the cells that redistribute to Ag-free LN is also consistent with other work suggesting that memory cells go through a stage in which they express effector activity (18, 25, 29).

Interestingly, the size of the recall response was ~5-fold higher in animals that had received redistributed OT-I cells with a CD62L^high phenotype than in those receiving and equivalent number of CD62L^low cells. One possibility is that this reflects enhanced survival or homeostatic proliferation of CD62L^high cells after adoptive transfer (24). However, the ratio of these two populations did not change from day 3 to 9 in the LN of animals treated with FTY720, nor in the LN of untreated animals up to 40 days after immunization. Alternatively, it is possible that the CD62L^high cells distribute more efficiently into lymphoid tissue after adoptive transfer, while the CD62L^low cells are more likely to enter peripheral tissue, and that this alters the extent to which they engage with DC after vaccinia infection. This is in keeping with other work on the distribution of adoptively transferred CD62L^high and CD62L^low cells (6, 54, 55), although the cells used were usually propagated in vitro or isolated from spleen rather than LN. Finally, it is possible that the CD62L^high cells have greater capacity to proliferate in response to the vaccinia challenge. This possibility is also consistent with other work (10, 11, 29, 31). Regardless of the exact mechanism, our results suggest that early stage redistributed CD62L^high cells are enriched for central memory precursors. CD62L^low cells also harbor memory precursors, although these may represent central, transitional, or effector memory lineages.

High and low level expression of CD62L is often used, irrespective of location, to define TCM and effector T memory subsets, respectively (6, 31). However, the stable representation of both CD62L^high and CD62L^low Ag experienced OT-I cells in LN for at least 40 days, and the fact that both populations contain memory precursors that give rise to recall responses in LN after adoptive transfer, indicates that residence in an Ag-free LN is a more inclusive definition of TCM. In relation to this, we also suggest that the rapid redistribution of CD8 T cells to Ag-free LN represents an additional marker of TCM Precursors, and is a process that leads to the establishment of systemic memory.

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Disclosures

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References


