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Effector Function-Deficient Memory CD8+ T Cells Clonally Expand in the Liver and Give Rise to Peripheral Memory CD8+ T Cells

Yu-Chia Su,* Chen-Cheng Lee,* and John T. Kung*,†

Upon adoptive transfer into histocompatible mice, naïve CD8+ T cells stimulated ex vivo by TCR+IL-4 turn into long-lived functional memory cells. The liver contains a large number of so formed memory CD8+ T cells, referred to as liver memory T cells (TLM) in the form of cell clusters. The CD62Llow expression and nonlymphoid tissue distribution of TLM cells are similar to effector memory (TEM) cells, yet their deficient cytotoxicity and IFN-γ inducibility are unlike TEM cells. Adoptive transfer of admixtures of TCR+IL-4–activated VB5+ and VB8+ CD8+ T cells into congenic hosts reveals TLM clusters that are composed of all VB5+ or VB8+, not mixed VB5+/VB8+ cells, indicating that TLM clusters are formed by clonal expansion. Clonally expanded CD8+ T cell clusters are also seen in the liver of Listeria monocytogenes-immune mice. TLM clusters closely associate with hepatic stellate cells and their formation is IL-15/IL-15R-dependent. CD62Llow TLM cells can home to the liver and secondary lymphoid tissues, remain CD62Llow, or acquire central memory (TCM)-characteristic CD62Lhi expression. Our findings show the liver as a major site of CD8+ memory T cell growth and that TLM cells contribute to the pool of peripheral memory cells. These previously unappreciated TLM characteristics indicate the inadequacy of the current TCM/TEM classification scheme and help ongoing efforts aimed at establishing a unifying memory T cell development pathway. Lastly, our finding of TLM clusters suggests caution against interpreting focal lymphocyte infiltration in clinical settings as pathology and not normal physiology. The Journal of Immunology, 2010, 185: 7498–7506.

Antigen-specific memory T cells that develop in response to Ag stimulation have been classified as CD62LhiCCR7+ central memory T (TCM) cells or CD62LloCCR7− effector memory T (TEM) cells, with TCM and TEM cells homing to lymphoid tissues and nonlymphoid tissues, respectively (1). Although significant advances have been made in the functional characterization of memory T cell subsets, a unifying development pathway has not emerged. As such, linear differentiation, bifurcative differentiation, and self-renewing effector models have been proposed (2). The process of memory T cell development and maintenance is now known to be influenced by cytokines, Ag-specific T cell frequency, and anatomic locations (3–6) and is thus characterized by considerable complexity.

Because memory CD8+ T cells can be maintained in the absence of secondary lymphoid organs (7, 8), nonlymphoid tissues and organs are expected to play critical roles in their maintenance. Bone marrow has been reported to be the preferred site for homeostatic proliferation of memory CD8+ T cells (9, 10), a process that is dependent on IL-15 and IL-15Rα (11, 12). Whether the expression of IL-15 and IL-15Rα by the many nonlymphoid tissues and organs, such as the liver (13, 14), also contribute to the homeostasis of memory CD8+ T cells is largely unknown.

We have reported previously that adoptive transfer of Ag+IL-4–activated naïve CD8+ T cells into histocompatible hosts resulted in potent development of long-lived functional memory cells (5, 15). Using this highly efficient CD8+ memory T cell generation system, we show in this study that the liver, the largest organ of the body, is a site of CD8+ memory T cell growth through the process of clonal expansion. We also describe cells that may participate in this clonal expansion process. Our results are also discussed in the context of memory development pathways.

Materials and Methods

Mice

H2-Ld–restricted 2C TCR transgenic mice (16) were bred onto C57BL/10ScN (B10) and C57BL/6J (B6) backgrounds (5, 17). B6.TL and B10.TL are B6- and B10-histocompatible Thy1+CD8+ congenic strains, respectively. IL-15Rα knockout breeders (18) were purchased (The Jackson Laboratory, Bar Harbor, ME). All mice were bred in the animal facility of the Institute of Molecular Biology under specific pathogen-free conditions and used between 6 and 12 wk of age unless otherwise indicated. All experimental procedures were performed in accordance to active protocols approved by Institutional Animal Care and Utilization Committee of Academia Sinica.

Generating and monitoring memory CD8+ T cells

CD8+ T cells were purified by panning (19). Purified 2C CD8+ T cells were activated by antigenic peptide in the presence of IL-4 for 3 d, cultured for 2 d in IL-2–supplemented medium, and adoptively transferred into indicated hosts as described previously (5). Vβ5+ and Vβ8+ CD8+ T cells were obtained by cell sorting of wild type (WT) CD8+ T cells stained with F–anti-Vβ5, Cy5–anti-TCR Vβ8, and PE–anti-CD8. WT CD8+ T cells were activated in vitro by anti-CD3/CD28 presented by LPS-activated B blasts (20). Indicated numbers of donor CD8+ T cells were transferred i.v. into indicated hosts. The presence of donor CD8+ T cells in host mice was
monitored by flow cytometry and expressed as the ratio of donor to total CD8+ T cells (5).

Isolation of liver lymphocytes

Intrahepatic lymphocytes (IHLs) were isolated by a modification described previously (21). The liver was perfused, excised, homogenized by Cell Strainer (BD Falcon, Franklin Lakes, NJ), and digested (HBSS containing 0.02% collagenase IV [Sigma-Aldrich, St. Louis, MO], 0.002% DNase I [Roche, Basel, Switzerland], and 5% FCS, at 37°C for 45 min). IHLs were enriched by gradient centrifugation (24% HistoDenz [Sigma-Aldrich], 20 min, 1580 × g, 4°C). Spleen CD8+ T cells obtained using the IHL isolation procedure proliferated similarly to anti-CD3/CD28 stimulation as those isolated by our routine CD8+ panning procedure (Supplemental Fig. 1), indicating that the IHL isolation procedure did not cause any damage to CD8+ T cells.

Examination of liver memory CD8+ T cells

H&E staining of paraffin-embedded liver sections was performed according to standard procedures. OCT-embedded liver sections (5 μm thick) were fixed in acetone, stained with indicated Abs (4°C, 8 h), and observed by confocal microscopy (Zeiss LSM 510, Oberkochen, Germany). Fluoro-chrome-conjugated mAbs used include: AlexaFluor488 (A488)-1B2 anti-2C TCR idotype (22), A488- and PE-conjugated anti–Thy-1.2 (23), A488–anti-Vβ5 (24), PE–anti-CD8 (23), A647–anti-Vβ8 (25), A647–anti-γδT (26). Liver was postfixed with 2.5% OsO4 (2 h, 4°C), washed, dehydrated, embedded in Spurr resin, sectioned with a diamond knife (Ultracut, Reichert-Jung, Vienna, Austria) and examined by electron microscopy (Zeiss EM109).

For electron microscopy, the liver was perfused with 4% paraformaldehyde, fixed for 4 h, made into cubes (24), postfixed with 2.5% glutaraldehyde plus 4% paraformaldehyde (overnight, 4°C) and then 1% Oso4 (2 h, 4°C), washed, dehydrated, embedded in Spurr resin, sectioned with a diamond knife (Ultracut, Reichert-Jung, Vienna, Austria) and examined by electron microscopy (Zeiss EM109).

In vitro proliferation assay

Sorted memory CD8+ T cell subsets were stimulated (2 × 10^6 cells/well per 0.2 ml) in wells that had been coated previously with 10 μg/ml each of anti-CD3+ anti-CD28 for 24 h. Proliferation by memory 2C CD8+ T cells was accessed by a 6-h, 0.5-μCi/ml [3H]thymidine pulse.

FIGURE 1. High numbers of CD8+ memory T cells in the liver. A, Ag + IL-4–activated naive CD8+Thy-1.2+ T cells (8 × 10^6) from 2C TCR transgenic mice were adoptively transferred into a group of 10 congenic B10.TL (Thy-1.2+) hosts. On the indicated days PAT, single-cell suspensions from the spleen, liver, LN, and BM from two hosts were stained with PE–anti-CD8 and Cy5-anti–Thy-1.2 mAbs, and ratios (means ± SEM) of donor to total CD8+ T cells are shown. B, On day 28 PAT, single-cell suspensions from the indicated organs of two host mice were stained as in A, and the total numbers of donor cells were also determined. Because BM and LN cells were recovered from two femurs and mesenteric LN, respectively, they constituted part of all BM and LN cells and are therefore under estimates of total BM and LN cells in the entire host.

CTL assay

Ag-specific cytolytic activity by 2C memory CD8+ T cells was determined by the JAM assay (26). L. monocytogenes P815 target cells were [3H]thymidine labeled (6-h pulse, 10 μCi/ml) and used at indicated effector/target ratios.

Scoring of CD8+ T cell clusters found in the liver

A cluster is defined as six or more closely situated CD8+ T cells. Vβ8+ clusters are defined as CD8+ clusters containing >80% Vβ8+ cells and no Vβ5+ cells, or CD8+ clusters containing >80% Vβ5+ cells and no Vβ8+ cells.

Listeria monocytogenes immunization

Listeria monocytogenes 5334, a clinical isolate from the National Taiwan University Hospital, was injected i.v. at a sublethal dose (6 × 10^6 CFU per mouse).

Statistical analysis

The association of TLM clusters with hepatic stellate cells (HSCs), neutrophils, and IL-15+ and IL-15Rα cells was analyzed by Fisher’s exact probability test, in which the frequencies of test cells found within the confines of TLM clusters were compared against the frequencies of test cells found in areas away from TLM clusters; p < 0.05 is considered statistically significant.

Results

Memory CD8+ T cells are found in large numbers in the liver

Naive 2C CD8+ T cells activated ex vivo by Ag in the presence of added IL-4 become long-lived functional memory cells after adoptive transfer into congenic histocompatible hosts (5, 15). Using this high-efficiency CD8+ T cell memory generation system, we examined for the presence of donor-derived memory T cells in different host tissues and organs (Fig. 1A). Donor CD8+ memory T cells were found most frequently in the liver, followed by bone marrow (BM), spleen, and lastly the lymph node (Fig. 1A). Absolute counts of donor CD8+ T cells were the highest for the spleen, then the liver, followed by BM, and lastly the lymph node (LN) (Fig. 1B).

CD8+ TLM cells express TCM-like and TCM-unlike properties

Memory CD8+ T cells are divided into CD62L+CCR7+ TCM and CD62LlowCCR7− TLM subsets that reside in lymphoid and nonlymphoid organs or tissues, respectively (1). The current thinking is that TLM but not TCM CD8+ memory cells possess the genetic programming required for entry into nonlymphoid organs or tissues. Because TLM cells are found in the liver, a nonlymphoid organ, they are expected to be similar to CD8+ TCM cells. Consistent with this expectation, the vast majority of TLM cells expressed the CD62Llow
phenotype (Supplemental Fig. 2). To more comprehensively characterize TLM cells, effector functions were studied (Fig. 2A). Whereas spleen TCM cells mounted a strong TCR-stimulated proliferative response, TLM cells were replication incompetent. Spleen TEM cells showed much stronger proliferation than TLM cells, although the magnitude was significantly less than that of TCM cells. TLM cells expressed Ag-specific cytolytic activity at a level that was one third and half those of spleen TEM and TCM cells, respectively (Fig. 2B). Similar to TLM cells, CD8+ memory T cells from the BM were also replication incompetent and expressed poor cytotoxicity in comparison with spleen TEM. IFN-γ production by TLM cells was much weaker than those of TEM and TCM cells upon anti-CD3/CD28 stimulation (Fig. 2C). Memory CD8+ T cells from the spleen were CFSE-labeled and transferred into a histocompatible host. On day 28 postadoptive transfer (PAT), CFSE fluorescence patterns of donor cells in various host organs were examined. The majority of memory CD8+ T cell from the liver and BM divided four to six times, and the majority population of memory CD8+ T cell from the spleen and LN divided for three or four times (Fig. 2D). In sum, TLM cells display TEM-like and TEM-unlike properties.

FIGURE 2. Characterization of liver memory CD8+ T cells. A, Ag+IL-4-activated 2C CD8+ T cells (16 × 10^6) were adoptively transferred into each of six B6.TL hosts to allow their development into memory T cells. On d70 PAT, single cell suspensions from indicated organs of two host mice were stained with FITC-anti-CD62L, Cy5-anti-Thy-1.2, and TR-anti-CD8. Indicated memory 2C CD8+ T cell subsets, spleen CD62LThy-1.2CD8+ TCM cells, spleen Thy-1.2CD62LlowCD8+ TLM cells, liver Thy-1.2CD62LlowCD8+ TLM cells, and BM Thy-1.2CD62LlowCD8+ TBM cells were isolated by cell sorting. Sorted memory CD8+ T cell subsets were activated in wells coated with anti-CD3+anti-CD28 for 24 h. Typical results from one of two experiments are shown. B, Subsets of 2C memory CD8+ T cells as indicated were obtained from two host mice by cell sorting as in A and assayed for Ag-specific cytolytic activity at indicated effector/target cell ratios against L3-bearing P815 target cells. Typical results from one of two experiments are shown. C, On day 60 PAT, subsets of CD8+ memory T cell subsets were obtained from two host mice as in A, naive CD8+ T cells (CD8+CD44low) were isolated from the spleen of IFN-γ knockout mice by cell sorting. Indicated memory subsets and naive CD8+ T cells were activated in anti-CD3+anti-CD28–coated wells for 9 h, followed by intracellular staining with Cy5-anti–IFN-γ. Intracellular IFN-γ staining histograms are: naive IFN-γ knockout CD8+ T cells, gray-filled; liver TLM, thick line; spleen TCM, thin line; spleen TLM, dashed line. Typical results from one of three experiments are shown. D, This experiment involved two adoptive transfers performed sequentially. For the first adoptive transfer, each of two B10.TL hosts received (8 × 10^6) Ag+IL-4-activated 2C CD8+ T cells. On day 7 PAT, total spleen CD8+ T cells from these B10.TL hosts were obtained and CFSE-labeled (2 μM, 37˚C, 15 min). CFSE-labeled CD8+ T cells that contained the 5 × 10^5 cell equivalent of 2C CD8+ T cells were adoptively transferred into a second B10.TL host. On day 28 PAT, single-cell suspensions from the indicated organs were stained with PE-anti-Thy-1.2, Cy5-anti-2C TCR (1B2), and A680-anti-CD8 mAbs; CFSE distribution was analyzed for cells of donor origin (1B2+Thy-1.2+CD8+). The number of cell divisions is shown on the top of the histogram. Typical results from one of three experiments are shown.
mice were analyzed by CD8 immunohistochemical staining. Distribution of the Ag+IL-4-activated CD8+ T cell clusters in the host liver was estimated (Supplemental Fig. 3A). TLM clusters in periportal and midzonal regions were 3.5- and 6-fold more frequently found, respectively, than those in centrilobular regions, with the size of clusters being similar for all regions (Supplemental Fig. 3B). Normal levels of serum AST and ALT and the lack of apoptotic cells found, respectively, than those in centrilobular regions, with the size of clusters being similar for all regions (Supplemental Fig. 3B). Normal levels of serum AST and ALT and the lack of apoptotic cells in the immediate vicinity of TLM clusters indicate that no pathology is associated with the presence of TLM clusters in the liver (Supplemental Fig. 4).

TLM clusters form through the process of clonal expansion

Purified Vβ5+ and Vβ8+ CD8+ T cells were activated in separate cultures by anti-CD3+IL-4, mixed in equal proportions, and adoptively transferred into histocompatible congenic hosts. At the population level, similar proportions of Vβ5+ and Vβ8+ memory CD8+ T cells were found, indicating no biased maintenance (Supplemental Fig. 5). Confocal analysis of 13 TLM clusters of day 7 PAT host liver sections revealed six (46%) that contained exclusively Vβ5+ and no Vβ8+ cells, and six (46%) that contained exclusively Vβ8+ and no Vβ5+ cells (Fig. 4). Similarly, of the 20 TLM clusters identified in day 60 PAT host liver sections, 10 (50%) contained exclusively Vβ5+ and no Vβ8+ cells, and the other 10 (50%) contained exclusively Vβ8+ and no Vβ5+ cells. These results indicate that liver CD8+ T cells arise through in situ clonal expansion of single cells.

Clonal nature of CD8+ T cell clusters in the liver of L. monocytogenes-immune mice

To examine whether mice subjected to live infectious agents also develop CD8+ T cell clusters in the liver, WT mice were given a sublethal dose of L. monocytogenes. Single-cell suspension recovered from the liver of L. monocytogenes-immune hosts contained CD8+ T cells that were 64% Vβ8+ cells, which is much higher than the ~25% Vβ8+ cells within T cells of nonimmunized mice (Fig. 5A). This finding of highly enriched Vβ8+ cells is consistent with biased expansion of Vβ8+ T cells in some individual mice responding to L. monocytogenes infection (27). If liver CD8+ T cells were generated elsewhere and homed to microniches that support the survival of memory CD8+ T cells, then each CD8+ T cell cluster would be expected to contain ~60% Vβ8+ cells. A total of 40 randomly chosen liver sections from three L. monocytogenes-immune mice were examined for the presence of CD8+ T cell clusters. Scanning of all the 40 liver sections yielded a total of 12 CD8+ clusters, six of which contained either no or very few Vβ8+ cells, with no mixed Vβ5+/Vβ8+ cells (scale bar, 5 μm; original magnification ×630). C. The total of 13 donor clusters found in day 7 PAT host liver sections, six contained Vβ8+ but no Vβ5+ cells, and six contained Vβ5+ but no Vβ8+ cells, whereas one contained mixed Vβ8+ and Vβ5+ cells. Of the 20 donor clusters found in day 60 PAT host liver sections, 10 contained Vβ8+ but no Vβ5+ cells, and 10 contained Vβ5+ but no Vβ8+ cells.

![FIGURE 3. Clusters of Ag+IL-4-activated CD8+ T cells are found in the liver. Ag+IL-4-activated 2C CD8+ T cells (8 × 10^5) were adoptively transferred into each of three B10.TL hosts. A. On day 7 PAT, all three hosts were sacrificed and H&E staining of a liver paraffin section was performed. A representative cell cluster is shown (scale bar, 10 μm; original magnification ×1000). Mononuclear lymphoid cells, neutrophils, hepatocytes, RBCs, and sinusoids outlined by endothelial cells are marked. B. On day 7 PAT, three hosts were sacrificed and liver frozen section was stained with A488-anti–Thy-1.2 and PE–anti-CD8. Fluorescence image of one representative donor cell cluster, showing colocalization of CD8+ and Thy-1.2+ cells, is displayed. Scale bar, 10 μm. Original magnification ×630. E. Representative cell cluster, showing colocalization of CD8+ and Thy-1.2+ cells, is displayed. Scale bar, 10 μm. Original magnification ×630.](http://www.jimmunol.org/)

![FIGURE 4. Clonal expansion of in vitro TCR+IL-4-activated CD8+ T cells in the liver. Equal numbers (4 × 10^6 each) of the TCR+IL-4-activated Vβ5+ and Vβ8+ CD8+ T cells were mixed and adoptively transferred into each of two B10.TL host mice. On days 7 and 60 PAT, one of the two hosts were sacrificed, and liver frozen sections were made, stained with A488-anti–Vβ5, PE-anti–Thy-1.2 and A647-anti–Vβ8, and examined by confocal microscopy. Confocal images of two representative clones (labeled #1 and #2) from day 7 PAT (A) and day 60 PAT (B) liver sections are shown. These clones contained cells that were either all Vβ8+ or all Vβ5+ cells, with no mixed Vβ5+/Vβ8+ cells (scale bar, 5 μm; original magnification ×630). C. The total of 13 donor clusters found in day 7 PAT host liver sections, six contained Vβ8+ but no Vβ5+ cells, and six contained Vβ5+ but no Vβ8+ cells, whereas one contained mixed Vβ8+ and Vβ5+ cells. Of the 20 donor clusters found in day 60 PAT host liver sections, 10 contained Vβ8+ but no Vβ5+ cells, and 10 contained Vβ5+ but no Vβ8+ cells.)
IL-15R requirement for CD8+ TLM cluster formation

Therefore, these naive liver sections revealed only one Vβ8+ cluster as opposed to the 12 clusters found for L. monocytogenes-immune mice. Because the frequency of CD8+ T cell clusters we observed in the liver of L. monocytogenes-immune mice was >10-fold higher than that of naive livers, the vast majority of CD8+ T cell clusters seen in the liver of L. monocytogenes-immune mice must therefore have been formed as a result of L. monocytogenes infection.

IL-15R requirement for CD8+ TLM cluster formation

Because IL-15 plays a critical role in the homeostasis of memory CD8+ T cells, we examined whether IL-15 and IL-15R immunoreactive signals can be detected in the vicinity of memory CD8+ TLM clusters (Fig. 6). Using serial liver sections, IL-15 and IL-15R immunoreactive signals in the form of dendrite-like processes were found (Fig. 6B, 6C). Of the 13 total TLM clusters analyzed, all contained IL-15+ and IL-15R+ dendrite-like processes. Statistical analysis of TLM-associated IL-15+ and IL-15R+ signals against those in areas away from TLM clusters showed differences that were highly significant (for IL-15, \( p = 0.02 \); for IL-15R, \( p = 0.0002 \)). To further study the IL-15R role in TLM cluster formation in the liver, we adoptively transferred Ag+IL-4–activated CD8+ T cells into IL-15R knockout hosts. Whereas 8 TLM clusters were found in nine entire liver sections of WT hosts, none were found in 54 entire liver sections of IL-15R knockout hosts (Fig. 6D).

Considering the area and thickness of the liver sections examined, these numbers can be converted, respectively, for WT and IL-15R knockout hosts to 28.4 and <0.52 TLM clusters/mm², a total of 4.4 \( \times 10^4 \) and <8.1 \( \times 10^2 \) TLM clusters per entire liver, 5 \( \times 10^5 \) and <9.2 \( \times 10^3 \) total donor CD8+ T cells per entire liver. Flow cytometric analysis of liver cells of WT and IL-15R knockout mice that had previously received adoptively transferred Ag+IL-4–activated CD8+ T cells also showed a highly reduced number of donor 2C CD8+ T cells in IL-15R knockout hosts when compared with WT hosts (Supplemental Fig. 6).

CD8+ TLM clusters are closely associated with HSCs

The clonal nature of liver memory CD8+ T cells is most consistent with the existence of specialized niches in the liver that provide the microenvironment required for clonal expansion of single memory CD8+ T cells. Because HSCs possess dendrite-like processes that are similar in structure to IL-15+ and IL-15R+ signals associated with TLM clusters, we examined for possible HSC presence near TLM clusters, using 1B2 mAb, GFAP, and pancytokeratin to mark donor 2C CD8+ T cells, HSCs, and hepatocytes, respectively. GFAP+ HSCs were present near TLM clusters, with some clusters surrounded by multiple HSCs. These findings suggest that HSCs, together with other liver microenvironmental factors, provide the microenvironment required for clonal expansion of single memory CD8+ T cells in the liver.
HSCs were detected in all the 11 and 14 T_{LM} clusters found, respectively, for day 7 and day 69 PAT hosts (Fig. 7A, 7B). Statistical analysis of GFAP^+ signals that were associated with and away from T_{LM} clusters revealed significant differences (day 7 PAT, $p = 0.045$; day 69 PAT, $p = 0.02$).

Neutrophils with characteristic segmental nuclei and autofluorescence (28) were seen in a significant proportion of CD8^+ T cells. The identity of cells and organelles are marked as follows: *; lipid droplets; arrows, dendrite-like cytoplasmic processes; H, hepatocytes; T, T lymphocytes.

Our finding demonstrates that colocalization of the GFAP^+ HSCs and neutrophils are highly specific for CD8^+ T cell clusters. Because HSCs reside in the space of Disse, which in its normal state is too thin to be observed by conventional light microscopy, electron microscopy was used to reveal ultrastructural details of the spatial relationship among memory CD8^+ T cells of T_{LM} clusters, hepatocytes, and endothelial cells. Three T cells with characteristic scanty cytoplasm are shown on the lower half of the electron micrograph (Fig. 7C). An HSC, with two clearly visible and characteristic lipid droplets, situated over the leftmost T cell. The dendrite-like cytoplasmic processes of the HSC were seen to form close contact with each of the three T cells, indicating their intimate association.

The primary finding in this study is that CD8^+ memory T cells grow in the liver through the process of clonal expansion. Clonal growth in tissues can be visualized only when the rate of cell division exceeds the rate at which daughter cells die or migrate away. The microenvironment that surrounds T_{LM} clusters presumably plays critical roles by providing general and unique opportunities for gaining detailed mechanistic understanding at cellular and molecular levels. Following this reasoning, we looked for non-T cells that are intimately associated with T_{LM} clusters. We found

**Discussion**

The primary finding in this study is that CD8^+ memory T cells grow in the liver through the process of clonal expansion.
that all TLM clusters were closely associated with IL-15+ and IL-15R+ cells with dendrite-like processes. The failure to form TLM-like T cells from one day 60 PAT B6 host that had been adoptively transferred with $3 \times 10^5$ Ag+IL-4–activated 2C CD8+ T cells previously. Three B6.TL hosts were individually transferred with $3 \times 10^5$ each of TLM, TLM, and TCM, respectively. On day 14 PAT, cells from indicated organs were obtained by cell sorting from one day 60 PAT B6 host that had been adoptively transferred with $16 \times 10^5$ Ag+IL-4–activated 2C CD8+ T cells previously. Three B6.TL hosts were individually transferred with $3 \times 10^5$ each of TLM, TLM, and TCM, respectively. On day 14 PAT, cells from indicated organs were obtained and stained with FITC-anti-CD62L, A405-anti-CD8, and Cy5-anti-Thy-1.2, and ratios of donor CD8+ T cells to total CD8+ T cells for indicated tissues determined. B, CD8+ TCM cells, spleen TCM cells, and spleen TLM cells were obtained as in A. Three B6.TL hosts were individually transferred with $4.5 \times 10^5$ TLM, $5.2 \times 10^5$ TCM, and $6 \times 10^5$ TLM cells, respectively. To facilitate visualization of the relatively small numbers of donor cells, only cells of donor origin are shown. Types of CD8+ memory T cells and host tissues are as indicated. The purity of sorted TCM cells and TLM (TLM) cells was always $>99\%$ CD62Llow and CD62Lhi, respectively. CD62Lhi cells were boxed, and the percentages of donor cells that are CD62Lhi given. Typical results from one of two experiments are shown.

The mostly CD62Llow phenotype of TLM cells found in the liver is expected, because memory CD8+ T cells in tissues are classified as CD62Llow TLM cells (1). If TLM cells belong to TCM cells that do not undergo significant homeostatic expansion (2), how can our finding that TLM cells are undergoing clonal expansion be reconciled? We show that CD62Llow TLM cells obtained from the spleen possess different functional attributes (e.g., ability to mount TCR-stimulated proliferation, IFN-γ production, and cytotoxicity) from TLM cells obtained from the liver. A simple explanation is that TLM cells are capable of location- or organ-specific homeostatic expansion. Thus, the microenvironment in the liver but not the spleen meets all the requirements for homeostatic expansion of TLM cells. Alternatively, when peripheral TLM cells gain access to the liver, they undergo specific changes, possibly through their close association with HSCs and neutrophils (e.g., to enable their homeostatic expansion). An additional noteworthy point is concerned with our finding of TLM-similar and TLM-dissimilar properties of TLM cells. The current TCM and TLM classification scheme for memory T cell subsets (1) may work well for commonly studied memory T cells, but it appears to be inadequate for memory cells that have not been studied extensively, such as the TLM cells reported in this study. Development of new markers, along with CD62L and CCR7, will be required for a comprehensive classification scheme of all CD8+ memory T cell subsets.

Although TLM cells are similar to TLM cells in regard to CD62Llow expression and the ability to gain access to tissues, they are nevertheless much poorer than TLM cells at expressing IFN-γ inducibility and cytolytic activities. The deficient functional attributes of TLM cells may be caused by their engagement in cell cycle progression, a state that may be incompatible with high-function capabilities. Deficient functional activities may also be the consequence of active homeostatic expansion. To express potent functional properties, memory cells must not have been engaged recently in cell cycle progression. The findings that TLM cells undergo clonal growth in the liver and yet fail to proliferate in response to TCR stimulation appear paradoxical, but could be explained by the mutually exclusive genetic programs for cytokine-mediated homeostatic expansion and TCR-stimulated expansion.

CD8+ memory cells undergo homeostatic expansion by dividing once every 2 wk or more (32). Thus, at any given time, a small fraction of the memory pool is engaged in cell cycle progression. T cell activation is known to be associated with loss of CD62L expression (33). If CD62L loss also occurs when CD62Lhi TLM cells are engaged in cell cycle progression, then two issues arise. First, the small fraction of TLM cells engaged in cell cycle progression may be mistakenly classified as CD62Llow TLM cells. Second, when there is a cell division-associated loss of CD62L expression, it is not clear how long it will take to regain CD62Lhi expression after cell cycle progression ceases.

Adoptively transferred CD62Llow TLM cells become CD62Lhi cells in host spleen and LN; this is consistent with the TLM → TLM linear development model (2, 34). However, the transferred CD62Llow TLM cells remained mostly CD62Llow in the liver, which supports the idea of the liver as a continuing source of memory T cells. When inside the liver, CD62Llow TLM cells remain CD62Llow and become CD62Lhi TLM cells only after their departure from the liver. If so, the liver is a site in which TLM-like TLM cells undergo clonal expansion, which is consistent with the self-renewing model of memory T cell development (2, 35).

Adoptively transferred TCM cells were found in the liver. If TLM cells can convert to CD62Llow TLM cells, then they would be expected to gain access to nonlymphoid tissues. Adoptively transferred TCM cells recovered from host spleen and LN showed no decrease in the level of CD62L expression. However, there was...
a significant decrease in CD62L expression at the population level for TCM donor cells recovered from host liver, such that more than 40% were scored as CD62L−. One possible interpretation of this result is that TCM cells could turn into TEM (TCM) cells either before or after gaining entry into the liver. This possibility contradicts the linear development model (2, 34), but is consistent with the reported TCM → TEM conversion (36). Alternatively, whereas it may be true that all TCM cells are CD62L+, the converse that all CD62L+ cells are TCM cells is not true. If so, a small subset of TCM cells exists within CD62Lhi TCM cells, and it is these CD62Lhi TCM cells that have homed to the liver. There is a third possibility that the liver environment is incompatible with CD62Lhi expression, and that when CD62Lhi cells enter the liver, they lose CD62L expression either by shedding or by downregulation of CD62L gene expression. Although our system of generating memory CD8+ T cells is highly efficient, the generated memory T cells might not be entirely identical to those generated as a result of natural infection. Given that significant heterogeneity exists within the known memory subsets, activation of highly purified naive CD8+ T cells in vitro under controlled Ag+IL-4 stimulation will likely yield memory T cells characterized by a more uniform differentiation state and functionality. Natural infection, although highly relevant to human disease, is rather complex because tissue tropism and many types of APCs may be involved, and it is likely to generate memory T cells characterized by considerable heterogeneity. Our finding of clonally expanded CD8+ T cell clusters in L. monocytogenes-infected mice nevertheless provides an example that the formation of CD8+ clusters can be seen in real infection. In this connection, it has been reported that the number of dividing Ag-specific CD8+ T cells in mice immune to lymphocytic choriomeningitis virus is most numerous in the bone marrow, and few are found in the liver (9), thus raising the possibility that Ag-specific CD8+ T cell clonal growth in the liver may be different for different infectious agents and the conditions under which the immune response is initiated. Clearly, more studies are needed to address this issue. All these possibilities point to the importance of development of new markers that can comprehensively and unambiguously define memory T cells in the context of subsets, tissue location, their activation and homeostatic expansion history.

Bone marrow has been shown to be an important homeostatic proliferation site for memory CD8+ T cells (9). We show in this study that the liver, the largest organ of the body, is also a site of CD8+ memory T cell growth. Having two anatomical locations that allow homeostatic expansion is consistent with its critically important nature, and when normal physiology of the liver or bone marrow is temporarily interrupted, homeostatic expansion can still take place. Alternatively, the liver and the bone marrow are responsible for homeostatic expansion of nonoverlapping memory T cell subsets, or that renewed memory T cells from the liver and bone marrow may be functionally distinct. Reports contrasting the long-held view that the liver is a graveyard for activated T cells have been published (37–39). CD8+ memory T cells do more than simply reside in the liver, our results show that they actually grow in the liver. Why and how the liver promotes memory CD8+ T cell renewal and the cause death of other activated T cells is a question of fundamental importance that is worthy of further research. Caution should be exercised in interpreting clinical findings of focal lymphocyte infiltration in patient liver biopsy specimens because focal lymphocyte growth may represent normal physiology and not necessarily pathology.

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Disclosures

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References