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

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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 Vβ8+ and Vβ5+ CD8+ T cells into congenic hosts reveals TLM clusters that are composed of all Vβ5+ or Vβ8+, not mixed Vβ5+/Vβ8+ 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 TEM/TCM 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 CD62LlowCCR7− 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, bifurcated 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+ 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 Thy1.1/CD8a 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 CD8a T cells

CD8+ T cells were purified by panning (19). Purified 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 B220 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 (HBS 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 x 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 paraaffin-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). Fluorochrome-conjugated mAbs used include: AlexaFluor488 (A488)-IgG anti-2C TCR idotype (22), A488- and PE-conjugated anti-Thy-1.2 (23), A488–anti-Vβ8 (24), PE-anti-CD8 (23), A647–anti-Vβ8 (25), A647–anti-GFAP (BD Pharmingen, San Diego, CA), A546-anti-pan cytokeratin (Sigma-Aldrich). All images were collected by LSM510 (Zeiss). For CD8 IHC staining, liver sections were incubated with 53-6-7 rat anti-mouse CD8 (4°C, 16 h), followed by HRP-goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 4°C, 6 h), ImmPACT DAB (Vector Laboratories, Burlingame, CA), counterstained with Mayer’s hematoxylin (Sigma-Aldrich). For IL-15, Thy-1.2, and IL-15Rα IHC staining, three serial liver sections were individually stained with rat anti-Thy-1.2 (clone: 30H12), goat anti-miIl5 (R&D), and goat anti-miIl5Rα (Santa Cruz Biotechnology, Santa Cruz, CA) primary Abs, followed by staining with appropriate HRP-anti-rat or HRP-anti-goat secondary Abs. DAB precipitation and hematoxylin counterstain were then performed. All images were collected by Axiovision software (Zeiss).

For electron microscopy, the liver was perfused with 4% paraformaldehyde, fixed for 4 h, made into cubes [~1 mm3], 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 (Tecnai G2 Spirit TWIN, FEI Company, Hillsboro, OR).

In vitro proliferation assay

Sorted memory CD8+ T cell subsets were stimulated (2 x 10^5 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 [3H]thymidine pulse.

CTL assay

Ag-specific cytolytic activity by 2C memory CD8+ T cells was determined by the JAM assay (26). L512-bearing 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 x 10^6 CFU per mouse).

Statistical analysis

The association of TLM clusters with hepatic stellate cells (HSCs), neutrophils, and IL-15Rα and IL-15+ 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

Naïve 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+ T cell clusters were found most frequently in the liver, followed by bone marriow (BM), spleen, and lastly the lymph node (Fig. 1A). Absolute counts of donor CD8+ T cells were the highest for the spleen, followed by the liver, followed by BM, and lastly the lymph node (LN) (Fig. 1B).

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

Memory CD8+ T cells are divided into CD62LhighCCR7+ TSM+ and CD62LlowCCR7− TSM− subsets that reside in lymphoid and nonlymphoid organs or tissues, respectively (1). The current thinking is that TSM+ but not TSM− 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+ TSM+ cells. Consistent with this expectation, the vast majority of TLM cells expressed the CD62Lhigh...
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 x 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-1.2+CD8+ TLM cells, spleen Thy-1.2+CD62LhiCD8+ TLM cells, liver Thy-1.2+CD62LloCD8+ TLM cells, and BM Thy-1.2+CD62LloCD8+ 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 L4-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 TEM, 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 x 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 x 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 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 VB5+ and VB8+ 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 VB5+ and VB8+ 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 VB5+ and no VB8+ cells, and six (46%) that contained exclusively VB8+ and no VB5+ cells (Fig. 4). Similarly, of the 20 TLM clusters identified in day 60 PAT host liver sections, 10 (50%) contained exclusively VB5+ and no VB8+ cells, and the other 10 (50%) contained exclusively VB8+ and no VB5+ 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% VB8+ cells, which is much higher than the ~25% VB8+ cells within T cells of nonimmunized mice (Fig. 5A). This finding of highly enriched VB8+ cells is consistent with biased expansion of VB8+ T cells in some individual mice responding to *L. monocytogenes* infection (27). If liver CD8+ T cells were generated elsewhere and homed to micrometastases that support the survival of memory CD8+ T cells, then each CD8+ T cell cluster would be expected to contain ~60% VB8+ 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 VB8+ cells (a representative cluster is shown in Fig. 5B, lower row). For the other six CD8+ T cell clusters, VB8+ cells were readily detectable and constituted ~80–100% of Thy-1.2+ CD8+ cells in the clusters (a representative cluster is shown in Fig. 5B, upper row). The nonrandom nature of nearly all or no VB8+ T cell presence in all 12 clusters examined indicates that these clusters were clonal descendents of single cells and that specialized niches exist in the liver that provide the necessary microenvironment for single CD8+ T cells to undergo clonal expansion. By considering the thickness of liver sections and the total area scanned, the 12 CD8+ T cell clusters found in 40 sections translates to ~5.8 × 10^3 CD8+ T cell clusters per entire liver of an *L. monocytogenes*-immune mouse. Liver sections from age-matched naive mice that had not been exposed to

**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 × 10^6) 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, endothelial cells; H, hepatocytes; L, mononuclear lymphoid cells; N, neutrophils; R, RBCs.

**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 VB5+ and VB8+ 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–VB5, PE-anti–Thy-1.2 and A647-anti–VB8, 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 VB8+ or all VB5+ cells, with no mixed VB5+VB8+ cells (scale bar, 5 μm; original magnification ×630). C, Of the total of 13 donor clusters found in day 7 PAT host liver sections, six contained VB8+ but no VB5+ cells, and six contained VB5+ but no VB8+ cells, whereas one contained mixed VB8+ and VB5+ cells. Of the 20 donor clusters found in day 60 PAT host liver sections, 10 contained VB8+ but no VB5+ cells, and 10 contained VB5+ but no VB8+ cells.

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**FIGURE 5.** Clonal CD8+ T cell expansion in the livers of *L. monocytogenes*-infected mice. B6 mice were given a sublethal dose of *L. monocytogenes* i.v. A. Spleen cells and intrahepatic lymphocytes were obtained from B6 mice that had been infected with *L. monocytogenes* 60 days previously and stained with PE-anti-CD8 and Cy5–anti-Vβ8. Histograms of Vβ8 expression, with the percentages of Vβ8+ cells indicated, are shown. B. A total of 40 randomly chosen liver sections from three day 60 post *L. monocytogenes*-infected mice were stained with A488-anti-Thy-1.2, PE-anti-CD8, and A647-anti-Vβ8, and subjected to confocal microscopy examination. A total of 12 CD8+ clusters were identified. Of these 12 CD8+ clusters, six were Vβ8+ and six were Vβ8−. One representative each of Vβ8+ (top row) and Vβ8− (lower row) clusters are shown (scale bar, 20 μm; original magnification ×630). A total of 45 liver sections from three age-matched naive mice that had not been exposed to *L. monocytogenes* were stained under conditions to L. monocytogenes-immune liver sections. From these 45 naive liver sections, only one Vβ8+ cluster was identified. The sum of scanned areas of the 40 L. monocytogenes-immune liver sections is similar to the sum of the scanned area of the 45 naive liver sections.

*L. monocytogenes* were stained under conditions identical to the *L. monocytogenes*-immune liver sections. Examination of naive liver sections comprising an area similar to that of *L. monocytogenes*-immune liver sections revealed only one Vβ8+ cluster as opposed to the 12 clusters found for *L. monocytogenes*-immune livers. Because the frequency of CD8+ T cell clusters we observed in the livers 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 ± 5.2 TLM clusters/mm3, a total of 4.4 ± 103 and <8.1 × 107 TLM clusters per entire liver, 5 × 105 and <9.2 × 107 total donor CD8+ T cells per entire liver. Flow cytometric analysis of liver cells of WT and IL-15Rα 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-15Rα 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 detected in all the 11 and 14 TLM 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 TLM 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 of TLM clusters. In day 7 and day 69 liver sections, 6 of 11 and 10 of 14 TLM clusters contained neutrophils. No neutrophils were found in random fields away from TLM clusters, and statistical analysis revealed highly significant differences for neutrophils for areas associated and away from TLM clusters (day 7, $p = 0.006$; day 69, $p < 0.001$).

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 TLM 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.

**TLM cells can convert to TCM in lymphoid tissues**

Ag$^+$IL-4 activated 2C CD8$^+$ T cells were adoptively transferred into B6 hosts to allow their development into memory T cells. On day 60 PAT, host TCM and TLM cells from the spleen and TLM cells were sorted out and retransferred into a second set of hosts. When TLM cells were adoptively transferred, 0.05, 0.01, and 1.44% of donor CD8$^+$ T cells among total CD8$^+$ T cells were found in the spleen, lymph node, and liver, respectively (Fig. 8). Clearly, TLM cells homed to the liver preferentially as the relative liver/spleen and liver/LN ratios were 29 (1.44 $\pm$ 0.05) and 144 (1.44 $\pm$ 0.01), respectively. When TLM cells were adoptively transferred, 0.16, 0.02, and 1.09% of donor CD8$^+$ T cells among total CD8$^+$ T cells were found in the spleen, lymph node, and liver, respectively. Relative tissue abundance for transferred TLM cells was 6.8 (1.09 $\pm$ 0.16) and 55 (1.09 $\pm$ 0.02) for liver-to-spleen and liver-to-LN, respectively. For transferred TCM cells, 0.73, 0.33, and 1.29% of donor CD8$^+$ T cells among total CD8$^+$ T cells were found in the spleen, lymph node, and liver, respectively. The relative tissue abundances for transferred TCM cells were 1.8 (1.29 $\pm$ 0.73) and 3.9 (1.29 $\pm$ 0.33) for liver-to-spleen and liver-to-LN, respectively. Based on these results, TCM cells home to the spleen and LN with the highest efficiency, followed by TLM cells, with TLM cells being the poorest. All TLM, TCM, and TCM cells, in contrast, homed to the liver with similar efficiency. Adoptively transferred CD62L$^{low}$ TLM and TLM cells that had been sorted to $>$99% purity showed highly significant conversion to CD62L$^{hi}$ phenotype in host spleen and LN, but remained mostly CD62L$^{low}$ in the liver. Adoptively transferred CD62L$^{hi}$ TCM cells that had been sorted to $>$99% purity remained unchanged in the LN, with a minor subset changing to CD62L$^{low}$ in the spleen, and significant loss of CD62L expression for the entire population (with 41% falling within the CD62L$^{low}$ region) in the liver. These results indicate that the CD62L$^{hi}$ states of all TCM, TCM, and TLM cells can either change or remain the same depending on the anatomic locations in which they are found.

**Discussion**

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 TLM clusters presumably plays critical roles by providing general and unique requirements for TLM clonal expansion. The formation of TLM clusters in the liver provides a unique and advantageous opportunity for gaining detailed mechanistic understanding at cellular and molecular levels. Following this reasoning, we looked for non-T cells that are intimately associated with TLM clusters. We found...
The mostly CD62L<sup>low</sup> phenotype of T<sub>LM</sub> cells found in the liver is expected, because memory CD8<sup>+</sup> T cells in tissues are classified as CD62L<sup>low</sup> T<sub>LM</sub> cells (1). If T<sub>LM</sub> cells belong to T<sub>TEM</sub> cells that do not undergo significant homeostatic expansion (2), how can our finding that T<sub>LM</sub> cells are undergoing clonal expansion be reconciled? We show that CD62L<sup>low</sup> T<sub>LM</sub> cells obtained from the spleen possess different functional attributes (e.g., ability to mount TCR-stimulated proliferation, IFN-γ production, and cytotoxicity) from T<sub>TEM</sub> cells obtained from the liver. A simple explanation is that T<sub>LM</sub> 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 T<sub>LM</sub> cells. Alternatively, when peripheral T<sub>TEM</sub> 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 T<sub>LM</sub>-similar and T<sub>TEM</sub>-dissimilar properties of T<sub>LM</sub> cells. The current T<sub>CM</sub> and T<sub>EM</sub> 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 T<sub>LM</sub> 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<sup>+</sup> memory T cell subsets.

Although T<sub>LM</sub> cells are similar to T<sub>TEM</sub> cells in regard to CD62L<sup>low</sup> expression and the ability to gain access to tissues, they are nevertheless much poorer than T<sub>TEM</sub> cells at expressing IFN-γ inducibility and cytolytic activities. The deficient functional attributes of T<sub>LM</sub> 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 T<sub>LM</sub> 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<sup>+</sup> 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 CD62L<sup>hi</sup> T<sub>CM</sub> cells are engaged in cell cycle progression, then two issues arise. First, the small fraction of T<sub>LM</sub> cells engaged in cell cycle progression may be mistakenly classified as CD62L<sup>low</sup> T<sub>LM</sub> cells. Second, when there is a cell division-associated loss of CD62L expression, it is not clear how long it will take to regain CD62L<sup>hi</sup> expression after cell cycle progression ceases.

Adoptively transferred CD62L<sup>low</sup> T<sub>LM</sub> cells become CD62L<sup>hi</sup> cells in host spleen and LN; this is consistent with the T<sub>CM</sub> → T<sub>LM</sub> linear development model (2, 34). However, the transferred CD62L<sup>low</sup> T<sub>LM</sub> cells remained mostly CD62L<sup>low</sup> in the liver, which supports the idea of the liver as a continuing source of memory T cells. When inside the liver, CD62L<sup>low</sup> T<sub>LM</sub> cells remain CD62L<sup>low</sup> and become CD62L<sup>hi</sup> T<sub>TEM</sub> cells only after their departure from the liver. If so, the liver is a site in which T<sub>TEM</sub>-like T<sub>LM</sub> cells undergo clonal expansion, which is consistent with the self-renewing model of memory T cell development (2, 35).

Adoptively transferred T<sub>LM</sub> cells were found in the liver. If T<sub>LM</sub> cells can convert to CD62L<sup>low</sup> T<sub>TEM</sub> cells, then they would be expected to gain access to nonlymphoid tissues. Adoptively transferred T<sub>LM</sub> 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 T\textsubscript{CM} donor cells recovered from host liver, such that more than 40% were scored as CD62L\textsuperscript{lo}. One possible interpretation of this result is that T\textsubscript{CM} cells could turn into T\textsubscript{EM} (T\textsubscript{CM}) 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 T\textsubscript{EM} \rightarrow T\textsubscript{CM} conversion (36). Alternatively, whereas it may be true that all T\textsubscript{CM} cells are CD62L\textsuperscript{hi}, the converse that all CD62L\textsuperscript{hi} cells are T\textsubscript{CM} cells is not true. If so, a small subset of T\textsubscript{EM} cells exists within CD62L\textsuperscript{hi} T\textsubscript{CM} cells, and it is these CD62L\textsuperscript{hi} T\textsubscript{CM} cells that have homed to the liver. There is a third possibility that the liver environment is incompatible with CD62L\textsuperscript{hi} expression, and that when CD62L\textsuperscript{hi} 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\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} T cell clusters in L. monocytogenes-infected mice nevertheless provides an example that the formation of CD8\textsuperscript{+} clusters can be seen in real infections. In this connection, it has been reported that the number of dividing Ag-specific CD8\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} T cells (9). We show in this study that the liver, the largest organ of the body, is also a site of CD8\textsuperscript{+} 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\textsuperscript{+} memory T cells do more than gain entry and survive in the liver, our results show that they actually grow in the liver. Why and how the liver promotes memory CD8\textsuperscript{+} 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
The authors have no financial conflicts of interest.

References
Figure S1. Intrahepatic lymphocyte isolation procedure does not damage CD8+ T cells. B6 spleen CD8+ T cells were isolated either by anti-CD8 panning alone or by the intrapatic lymphocyte isolation procedure (collagenase digestion and Histodenz centrifugation) followed by anti-CD8 panning procedure. CD8+ T cells isolated by these two procedures were stimulated in wells coated with 10 μg/ml anti-CD3+anti-CD28 for 24h and proliferation assessed by 3H-thymidicine incorporation as described in Materials and Methods.
Figure S2. CD62L expression of memory CD8$^+$ T cell in different organs induced by Ag- and IL-4-activation. The Ag- and IL-4-activated 2C memory CD8$^+$ T cells were adoptively transferred into B10.7L host mice (8×10$^6$ cells/mouse). Single cell suspensions from the host spleen, bone marrow, LN, and liver were obtained on d90 after adoptive transfer and stained with FITC-anti-CD62L, PE-anti-CD8, and Cy5-anti-2C TCR. Histograms of CD62L expression of 2C CD8$^+$ T cells are shown.
Figure S3. The distribution and the size of the memory CD8+ T cell clusters. Ag- and IL-4-activated 2C CD8+ T cells (8x10^6) were adoptively transferred into B6 hosts. Liver sections were obtained from recipient mice on day 60 after adoptive transfer hosts for CD8 immunohistochemical staining. To facilitate comparison, the numbers of clusters counted were converted to a volume of 1 mm^3. The size (means±SEM) of the cell cluster was determined by averaging of long axis & short axis. The frequency (A) and size (B) of CD8+ T cell clusters located in portal triad zone, central vein zone, and midzonal were counted in bar plot (means±SEM). *Significant differences between the centrilobular and portal triad groups (p=0.013). **Significant differences between the central vein and midozonal groups (p=0.008).
Figure S4

**Figure S4.** Ag- and IL-4-activated 2C donor CD8⁺ T cells did not damage or undergo apoptosis in the liver.  A. Sera were collected from groups of 5 host mice on day 7 pat (open symbols) and control mice (no adoptive transfer, solid symbols) and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) determined.  B. Cryo-sections prepared from host liver on day 14 pat were stained by PE-anti-Thy-1.2 (Red) and subjected to fluorescent (FITC-dUTP)-based Tunel assay (Roche) performed according to manufacturer’s protocol. Scale bar, 10 μm.
Figure S5

A. day 7

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B. day 60

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Figure S5. Non-selective clonal expansion of TCR Vβ5 and Vβ8 usage memory CD8+ T cells activated by TCR+IL-4-activation in the 7d and 60d post-adoptive transfer host livers. TCR Vβ5+ and Vβ8+ B10 CD8+ T cells were activated in vitro by anti-CD3 and anti-CD28 mAbs presented by LPS-activated B blasts and exogenous IL-4. Equal numbers (4x10^6 each) of the TCR- and IL-4-activated Vβ5+ and Vβ8+ CD8+ T cells were mixed and adoptively transferred into B10.TL host mice. The hosts were sacrificed on day 7 (A) and day 60 (B) after adoptive transfer. Single cell suspensions from the host spleen and liver were stained with A488-anti-TCR Vβ5, PE-anti-Thy-1.2, A647-anti-TCR Vβ8, and A680-anti-CD8 mAbs. Contour plots of correlated Vβ5+ and Vβ8+ donor cells are shown with their respective percentages indicated. Typical results from one of the three experiments are shown.
Figure S6

Figure S6. Few donor memory CD8$^+$ T cells are found in the liver of IL-15R$\alpha$-ko host. Ag+IL-4-activated 2C CD8$^+$ T cells (8x10$^6$) were adoptively transferred into each of three B6 or IL-15R$\alpha$-ko hosts. On d28 pat, single cell suspensions from the liver of host mice were stained with A680-anti-CD8 and Cy5-anti-2C TCR mAbs. Total numbers of donor 2C CD8$^+$ T cells (mean±SEM) were determined by multiplying the percentage of 2C donor cells within the total recovered live liver cells and the total recovered live liver cells. *, significant difference between the Wt and IL-15R$\alpha$-ko liver (p=0.006).
Figure S7. **IL-15 and IL-15Rα gene expression by hepatic stellate cells.** FSC\textsuperscript{lo}SSC\textsuperscript{hi} hepatic stellate cells were sorted from single cell suspensions of naïve B6 mice. RNA samples from total liver and sorted HSC were prepared. The levels of IL-15 and IL-15Rα gene expression were determined by real-time RT-PCR and normalized against GAPDH gene expression. Triplicates were performed for each sample. *, significant difference between the HSC and total liver (p=0.003). **, very significant difference between the HSC and total liver (p=0.00003).