Early Intrahepatic Accumulation of CD8$^+$ T Cells Provides a Source of Effectors for Nonhepatic Immune Responses[1]

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Interactions between the liver and CD8$^+$ T cells can lead to tolerance, due in part to CD8$^+$ T cell death. To test whether this was the case in an extrahepatic infection, we investigated the fate and effector capacity of intrahepatic CD8$^+$ T cells during lung-restricted influenza infection in mice. Virus-specific T cells accumulated in livers without detectable intrahepatic presentation of viral Ags, and this accumulation was not restricted to the contraction phase, but was apparent as early as day 5. Intrahepatic influenza-specific cells were functionally similar to those recovered from the bronchioalveolar lavage, based on ex vivo cytokine production and specific target lysis. Both adoptive transfer of liver lymphocytes and orthotopic liver transplant of organs containing accumulated effector T cells revealed that activated CD8s from the liver were viable, expanded during reinfection, and generated a memory population that trafficked to lymphoid organs. Thus, intrahepatic CD8$^+$ T cells re-enter circulation and generate functional memory, indicating that the liver does not uniformly incapacitate activated CD8$^+$ T cells. Instead, it constitutes a substantial reservoir of usable Ag-specific effector CD8$^+$ T cells involved in both acute and recall immune responses. *The Journal of Immunology,* **2007, 179: 201–210.**

During acute phases of immune responses, including those to influenza infections, the liver accumulates activated CD8$^+$ T cells (1–5). However, tolerance phenomena associated with the liver and an abundance of apoptotic cells have given rise to the idea that the liver is a "graveyard" for these cells during resolution of acute infections (6). Dysfunction and apoptosis have been reported for CD8$^+$ T cells during infections with hepatotropic viruses and in the development of tolerance to oral and transplantation Ags (7–9). There is also evidence that the liver alters CD4$^+$ T cell function and a higher proportion stain with annexin V than in other organs (10). During influenza infection, intrahepatic virus-specific CD8$^+$ T cells have been reported to have a defect in IFN-γ secretion (5).

However, tolerance induction by the liver is not universal. The outcome of CD8$^+$ T cell interactions with the liver may depend on where T cells first see Ag. Thus, when Ag was expressed within both the liver and lymph nodes (LNs),[3] T cells primed for the first time in the liver were dysfunctional and did not cause hepatocellular injury, whereas those primed in LNs were functional and caused hepatitis (11). In contrast, in an experimental model using orthotopic transplantation to limit Ag expression to the liver, CD8$^+$ T cells primed in the liver were functionally competent in terms of IFN-γ synthesis and CTL activity (12). In the influenza model, we found a focal transient hepatitis that was the consequence of intrahepatic accumulation of influenza-specific CD8$^+$ T cells (13). CD8$^+$ T cell-dependent liver injury was detectable within 5 days of infection and influenza-specific T cells were identified in the foci (13), indicating hepatic accumulation of influenza-specific T cells occurred very early in the response (3, 4, 14).

In light of these findings, it is important to understand repercussions of intrahepatic localization on T cell function following classical LN priming, in the absence of Ag expression in the liver. Because influenza in mice causes only localized respiratory infection, this virus provides a convenient model for investigating the liver's involvement in distal immune responses. Our results show that Ag-specific CD8$^+$ T cells remained functionally competent after localization to the liver and retained the capacity for acute function and memory formation after transfer or transplant.

Keating et al. (15) have concurrently found high viability among Ag-specific T cells in the liver at peak and resolution phases. Thus, mounting evidence indicates that during resolution, the liver does not merely harbor apoptosing T cells. The present study advances these findings by showing significant influenza-specific T cell accumulation at very early phases even before the systemic response is fully established, as well as the ability of these accumulating cells to exit the liver and to generate viable memory populations. This indicates an active role for the liver in contributing to mounting immune responses, which has been suggested (15) but not demonstrated.

Our findings reveal a previously unrecognized potential for the liver to act as a significant repository for fully competent effector cells. As such, it is a prototype for how extralymphoid tissues can contribute to systemic immunity and memory formation.

Materials and Methods

Viral infection and tissue collection

C57BL/6 (B6) and BALB/c mice were obtained from The Jackson Laboratory, whereas B6.SJL (CD45.1$^+$), B6.PL (Thy1.1$^+$), and OT-I $\times$ B6.PL

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[3] Abbreviations used in this paper: LN, lymph node; BAL, bronchioalveolar lavage; β-gal, β-galactosidase; MLN, mediastinal LN; EID_{50}, 50% egg infectious dose; NLT, nonlymphoid tissue; NP, nucleoprotein; PA, acid polymerase; PR8, influenza A/PR/8/34; x31, influenza A/HK/x31; WSN-OVA_I, influenza A/WSN/OVA_I.

mice were bred in-house. Mice were maintained pathogen-free in accordance with animal committee guidelines. Intranasal infection was with 10^5 50% egg infective dose (EID_{50}) of influenza A/HKx31 (H3N2) or A/PR/8/34 (H1N1) (recall) (16, 17), or 10^3 PFU of H1N1 recombinant influenza A/WSN-OVA_I (WSN-OVA_I), expressing SIINFEKL ($OVA_{257–264}$) (18). Virus titers in the liver were measured by hemagglutination assay as described (16), after expansion in embryonated eggs; EID_{50} was calculated using the Reed-Muensch equation.

Flow cytometry

For flow cytometry, spleen, LN, macrophage-depleted bronchioalveolar lavage (BAL), and lung cells were prepared (16). Livers were perfused, excised, and digested in collagenase/DNase as reported (13), hepatocytes were pelleted by low-speed spins (2×30 *g*, 3 min), and lymphocytes were isolated by density gradient centrifugation (4°C, $1500 \times g$, 20 min) using 21.5% Optiprep (Axis-Shield). Cells were FcR-blocked and stained with mAbs against surface markers as stated. Influenza-specific cells were detected with PE-conjugated H-$2D^b$MHC class I/$NP_{366–374}$ tetramers (made (19) in collaboration with Trudeau Institute). Annexin V PE-Cy5 (Abcam) labeling was done in buffer plus 2.5 mM $CaCl_2$, 15 min, room temp; Cytox blue was added for 10 min to discriminate dead cells, diluted 2-fold, and immediately acquired on an LSRII. Intracellular cytokine staining was done after 6-h ex vivo culture with 1 μl/ml Golgi Plug (BD Biosciences) and 50 U/ml rIL-2 $\pm$ 1 μM nucleoprotein (NP) peptide. Flow samples were acquired on FACSCalibur or LSR II (BD Biosciences) and analyzed using FlowJo (Tree Star). The Student *t* test $p < 0.05$ was considered significant.

In vitro proliferation/hepatocyte isolation

Organs from infected mice (day 5.5 after WSN-OVA_I infection) were mechanically disrupted and plated in 6-well plates. Livers were perfused 10 min with oxygenated 37°C HBSS 0.05% collagenase IV, then excised and disrupted with tweezers to liberate liver cells into hepatocyte medium (DMEM-F12, 10% FBS, 1/100 insulin-transferrin-selenium, 100 nM dexamethasone). Following agitated incubation 30 min at 37°C, hepatocytes were pelleted $47 \times g$, 2×3 min. Supernatants were combined as non-hepatocytes. Hepatocytes were cultured 5×10^5 per well (precoated with 50 μg/ml collagen in 0.02 M acetic acid). A total of 1.5×10^6 CD8-enriched CFSE-labeled OT-I cells were added, and after 5-day culture, CFSE dilution indicated the presence of viral Ags. Some cultures were pulsed with 1 μM SIINFEKL peptide before addition of OT-I cells.

Competitive accumulation assay

Single-cell suspensions from OT-I spleen/LN (CD90.1 homozygous) were CD8-enriched, transferred into B6 mice, and activated in vivo with WSN-OVA_I infection for 8 days. Enriched $CD8^+$ T cells (>90% pure) from these BAL provided the activated $CD8^+$ fraction. Spleen/LN of $CD45.1^+$/$CD45.2^+$ heterozygous B6 mice were negatively enriched for $CD8^+$ T cells (>70% pure) for the naive $CD8^+$ fraction. Equal numbers of activated and naive cells (3×10^6 each) were injected i.v. into B6.SJL mice. Recipient livers were analyzed on day 4 for $CD8^+$ accumulation from the fractions.

Lysispot (20)/ELISPOT assays

Lymphocytes were isolated from infected BALB mice. Millipore MAIP ELISPOT plates were coated with either anti-IFN-γ (2 μg/ml, AN18; American Type Culture Collection) or anti-β-galactosidase (β-gal; 4 μg/ml, D19-2F3-2; Roche) Ab in PBS. β-gal-transduced P815.LacZ target cells were pulsed 1 h with 2 μg/ml $NP_{147–156}$ peptide (Invitrogen Life Technologies) or left untreated, then plated 30,000 cells/well in 50 μl of α20 medium. Lymphocyte dilutions from organs were added in 50 μl of IMDM10 medium (HyClone), 10% FCS. Lysispot wells contained 0.4 ng/ml IL-2. After 4 h at 37°C, biotinylated anti-β-gal Ab GAL13 (1/1000; Sigma-Aldrich) in 50 μl of IMDM10 was added to Lysispot cultures without disturbing the cell layer. After 20 min, plates were washed with PBS 0.01% Tween 20 (PBST); 2 μg/ml biotinylated XMG1.2 (BD Biosciences) was used to detect IFN-γ spots. Both spot assays were incubated with 1 μg/ml alkaline phosphatase-SA (Jackson Immunobiology) in PBST plus 2% BSA. For spot development, substrate mix (Vector Laboratories) was used. Plates were analyzed with CTL ImmunoSpot Series 1 Analyzer scanner and software (Cellular Technology).

In vivo cytotoxicity assay

RBC-lysed splenocytes from naive B6 mice were divided in half; one pool was labeled with 2 μM CFSE and pulsed with 1 μM influenza $NP_{366–374}$ or $PA_{224–233}$ (Biosynthesis) for 1 h at 37°C. The other pool, stained with 0.2 μM CFSE, was pulsed with 1 μM HEL peptide. Pools were mixed 1:1, then coinjected i.v. into influenza-infected mice (8×10^6 total) on specified day of infection. Recipient organs were analyzed by flow after 18 h for changes in ratio of high-to-low CFSE populations (Ag-specific target cells to control cells). Specific lysis was calculated as described (21) using the formula: percent-specific lysis = (1 − ratio in uninfected mice/ratio infected mice) × 100, where ratio = (% $CFSE^{low}$/% $CFSE^{high}$).

In vivo activation and adoptive transfer of OT-I cells

Spleen/LN cells from Thy 1.1^+ OT-I mice were depleted using rat mAbs to CD4 (GK1.5), MHC class II (TIB120), and goat anti-mouse Ig- and goat anti-rat Ig-coated beads (Dynal). A total of 5×10^6 $CD8^+$ T cells (>92% pure) in 500 μl of PBS were i.v. transferred into congenic B6 mice. After 24 h, mice were infected with WSN-OVA_I to activate OT-I T cells, and sacrificed on day 8. Pooled livers or spleens were processed using to the liver isolation protocol, enriched for $CD8^+$ T cells, and analyzed by flow cytometry for OT-I $CD8^+$ T cell proportion (in some experiments, OT-I $CD8^+$ cells were instead sorted by flow cytometry from livers or spleens). Spleen and liver fractions were normalized for OT-I $CD8^+$ T cell content and between 1×10^4 and 2×10^5 (different experiments) were transferred i.v. into two B6.SJL cohorts which had been infected the previous day with WSN-OVA_I. Alternatively, cells were transferred into naive recipients for memory experiments and infected with influenza 45 days after transfer.

Mouse liver transplantation

Orthotopic mouse liver transplants were performed as previously described (12, 22). Briefly, under isoflurane inhalation anesthesia, donor livers were dissected out, and the right adrenal vein, pyloric vein, and hepatic artery were cut. The gallbladder was removed and a polyethylene stent tube was inserted into the common bile duct for later biliary drainage reconstruction. The infrahepatic inferior vena cava and portal vein were clamped and the organ was extensively perfused for over 5 min with 4°C normal saline via portal vein, then excised, and kept in 4°C 0.9% saline solution until the recipient's liver was completely removed. The donor liver was placed orthotopically into the abdominal cavity. The supra- and infrahepatic inferior vena cava were reconstructed by microsurgical hand suture anastomoses and the portal vein was anastomosed using a cuff technique. Bile flow was restored by a stent tube into the recipient common bile duct. Long-term survival (>30 days) was >90%.

Results

Differential kinetics of $CD8^+$ T cell accumulation in lung and liver during primary and memory influenza responses

The observation that histologically visible focal lesions dependent on virus-specific $CD8^+$ cells developed as early as 5 days after intranasal influenza infection (13) suggested that these cells should be detectable intrahepatically at such early time points. In B6 mice infected with influenza A/Hong Kong/x31 (x31), D^b/$NP_{366–374}$ tetramer staining showed the presence of virus NP-specific T cells in mediastinal lymph nodes (MLN) draining the infected lung by day 5 (Fig. 1*A*). On day 6, the numbers in the MLN had increased, but relatively few NP^+CD8^+ T cells were detectable in lung tissue or airways (BAL). However, relatively large numbers of NP-specific $CD8^+$ cells were detectable in livers (~20,000) and spleens (~55,000) of infected animals (Fig. 1*C*). Numbers in the MLN and liver were similar at this time point, with splenic numbers significantly ($p < 0.001$) greater than in any other tested organs. Liver ($p < 0.006$), MLN ($p < 0.016$), and spleen ($p < 0.001$) were all significantly higher than either the infected airways or lung tissue. The large magnitude of the liver population continued such that, by the peak influenza response at day 8, numbers of NP-specific $CD8^+$ T cells in the liver approached numbers in the spleen and were not statistically different (Fig. 1*A*).

To emphasize relative rates of $CD8^+$ T cell accumulation normalized to organ size, numbers of accumulating cells are depicted in Fig. 1*B* as a percent of each organ's day-8 accumulation: the maximum accumulation for all but the MLN. By day 5, the MLN contained over 17% of its total influenza-specific $CD8^+$ cell number of day 8. By day 6, intrahepatic accumulation had already reached 17.8% of its day 8 total, while accumulation was just beginning in airways. These data reinforce that lung accumulation

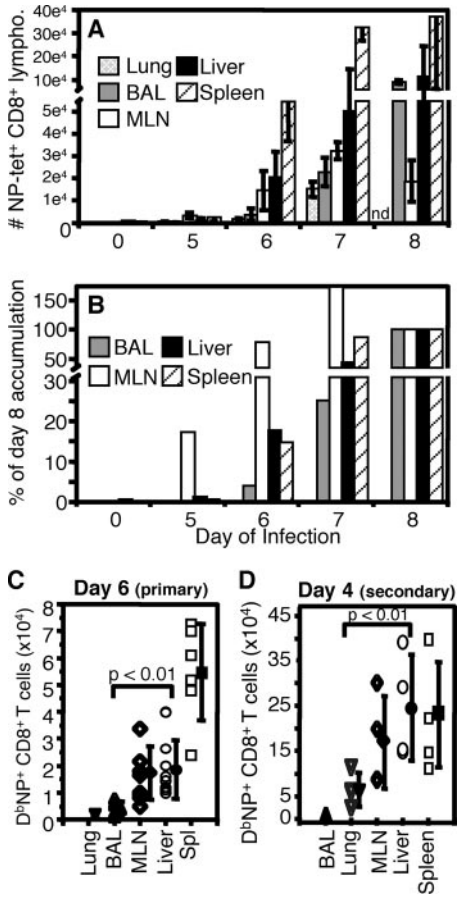

FIGURE 1. Early accumulation of influenza-specific T cells is greater in the liver than in the infected lung. *A*, C57BL/6 mice infected intranasally with influenza/x31 virus; Ag-specific numbers determined by tetramer staining of airway (BAL), lung, spleen, liver, and MLN lymphocytes on days 5–8. Mean ± SD from up to 10 independent experiments of minimum $n = 3$ each. *B*, Accumulation in each organ is expressed as a proportion of day 8 total accumulation, calculated as the ratio (values from (*A*))/(organ day 8 cell number). *C*, B6 mice were infected as in *A*, and day 6 tetramer$^+$ lymphocytes were assessed. Open symbols are individual mice; closed symbols and bars are mean ± SD. *D*, x31-immune mice from *A* were challenged with heterosubtypic PR/8 influenza after 40 days. NP$^+$CD8$^+$ lymphocytes were quantified on day 4. Significance by Wilcoxon rank sum test, $p < 0.05$.

lagged behind liver accumulation not only in number but also in proportion of its own maximum capacity.

Secondary pulmonary infection of x31-primed mice with serologically distinct influenza A/Puerto Rico/8 (PR8) stimulates a robust memory NP-specific CD8$^+$ T cell response, but non-cross-reactive Abs (23). This secondary NP$^+$CD8$^+$ response is both more rapid and of greater magnitude than in primary infection, resulting from the contribution of both tissue-resident memory T cells in the lung at the beginning of infection (24, 25) and more rapid secondary expansion of lymphoid memory T cells in the nodes. Comparative analysis of NP$^+$CD8$^+$ T cells in organs during PR8 challenge revealed that, as in primary infection, the largest numbers of specific cells could be detected in the MLN, liver, and spleen. By day 4 (Fig. 1*D*) after secondary infection, NP-specific cells were apparent in the draining LN, liver, and spleen at similar magnitudes. All three of these organs had significantly more cells than either the lung tissue or infected airways ($p < 0.01$), in which accumulation was just beginning.

One interpretation is that as virus-specific CD8$^+$ T cells are initially generated in the MLN (26) and enter the circulation, they accumulate in the liver and spleen instead of selectively accumulating at the site of infection where presumably most needed. However, the results did not distinguish whether this is due to specialized affinity for activated T cells, the size and unique vascular organization of the liver, or the presence of viral Ags, which could also affect the fate of the cells.

Absence of viral Ag in the liver

The liver has been reported to act as a site of primary CD8$^+$ activation when the specific Ag is expressed by hepatocytes (12). However, influenza productively infects only the epithelial lining of the respiratory tract, due to restricted expression of a host enzyme (27, 28). In addition, viral RNA could not be detected in livers using real-time PCR analysis for viral genome (13). Homogenized livers from animals undergoing primary and secondary influenza infection were used to infect highly permissive eggs (29) and viral titers were determined after 3 days of incubation. Though a weak virus titer was detected in 3 of 32 livers (Fig. 2*A*), the estimated number of infectious virions was fewer than five per positive liver (1 EID_{50}), an amount that does not explain the magnitude and consistency of virus-specific T cell infiltrate (which occurred in all individuals). In sharp contrast, infected lungs had $>10^5$ EID_{50} (Fig. 2*A*, *inset*), and the few weakly positive liver values were possibly a result of inadvertent contamination from concurrently harvested lungs. These results showed the uniform hepatic CD8$^+$ accumulation is unlikely to be a response to the nonuniform virus detected intrahepatically.

Despite the lack of evidence for intrahepatic influenza infection, the possibility of Ag presentation by APCs traveling to the liver during pulmonary infection remained. As an additional control to test whether viral Ags were present intrahepatically in a form recognizable by CD8$^+$ T cells, the immunodominant WSN-OVA_I epitope SIINFEKL was used as a surrogate viral Ag. The influenza virus WSN-OVA_I infects mice in a manner parallel to x31, and transgenic CD8$^+$ OT-I T cells (which recognize SIINFEKL in the context of MHC class I) respond with similar kinetics as NP-specific T cells (18). Cell populations were isolated from WSN-OVA_I influenza-infected mice on day 5.5 (when influenza-specific T cells first became detectable intrahepatically), and were tested in vitro for their ability to stimulate division of CFSE-labeled CD8$^+$ OT-I T cells.

The liver isolation protocol was modified to collagenase digestion only, which does not interfere with MHC class I presentation (12), so that all potential APCs, including hepatocytes, dendritic cells, and Kupffer cells, remained in the homogenate. This homogenate was then divided by low-speed centrifugation into a hepatocyte fraction and a nonparenchymal fraction including bone marrow-derived APC. Digested liver cells failed to induce CFSE dilution in CD8$^+$ OT-I cells when cocultured for either 4 (data not shown) or 6 days (Fig. 2*B*), although exogenous addition of SIINFEKL peptide to cultures did promote OT-I T cell division. Tissue homogenates from lung or draining LN (Fig. 2*B*) were all capable of inducing division of OT-I cells, indicating Ag was present in an appropriate form. Livers from mice infected with vaccinia virus expressing SIINFEKL, which establishes a systemic viral infection, were also capable of stimulating several divisions of OT-I cells (data not shown). We conclude that although liver cells can present Ag when it is added exogenously and when the liver is infected, influenza Ag is not endogenously presented in livers of influenza-infected mice to an extent detectable by T cells. Activated CD8$^+$ T cell accumulation in the liver is therefore unlikely to be a response to local infection or intrahepatic Ag presentation.

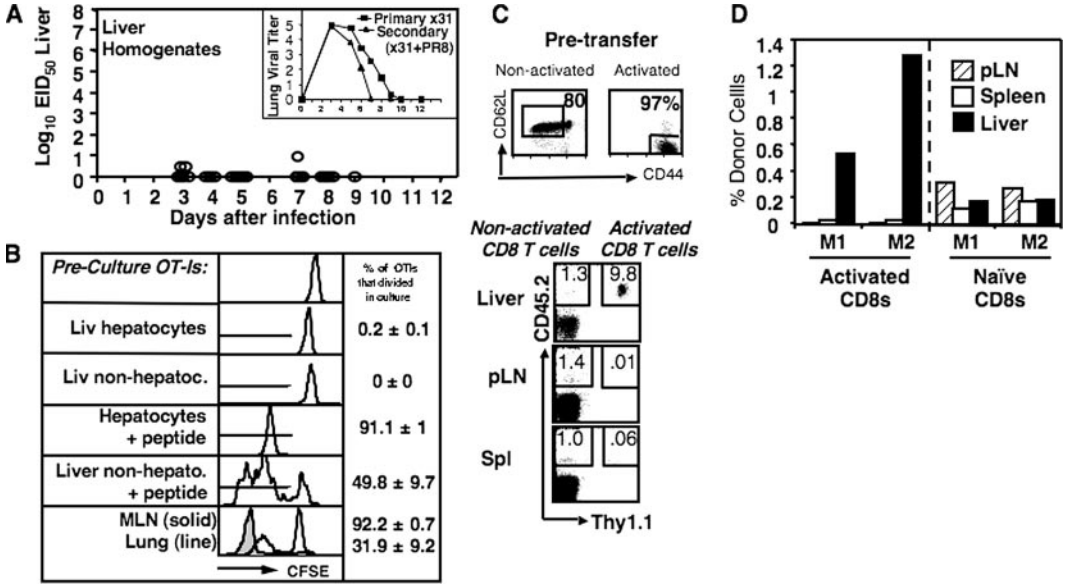

FIGURE 2. Accumulation of influenza-specific T cells in the liver is not a response to intrahepatic Ag presentation. *A*, Viral hemagglutination assay after egg inoculation with 27 liver homogenates from infected mice; expressed as 50% endpoint of $\log_{10}$ egg ID. *Inset*, Concurrent viral titers in lungs during primary and secondary infections. *B*, Ex vivo assay indicates no Ag presentation by liver cells. Cells from WSN-OVA_I-infected mice cultured with $CFSE^+CD8^+$OT-I cells. Gated on $Thy1.1^+CD8^+$ lymphocytes; divided cell percentage ± SEM of three or more organs. *C*, Intrahepatic accumulation of activated $CD8^+$ T cells in the absence of pulmonary infection. Activated $Thy1.1^+CD45.2^+CD8^+$ T cells from airways of day 8-infected mice, enriched, and transferred with equal number (1.5×10^6) of naive $Thy1.2^+CD45.1^+CD45.2^+CD8^+$ T cells into naive $Thy1.2^+CD45.1^+$ mice. Fractions stained for CD44/CD62L and gated on $CD8^+$ lymphocytes. Four days after injection, recipient organs stained for donor cells, gated on $CD8^+$ lymphocytes. *D*, Competitive assay from *C* shows activated cells preferentially trapped in the liver. Graphed are the percentages of lymphocytes in each organ that are the transferred cells; M1 and M2 indicate individual mice.

Accumulation of $CD8^+$ T cells in the absence of infection in the lung

Although there was no evidence of virus or viral Ag presentation in the liver, these studies did not absolutely rule out the possibility that infection has systemic effects that modify the accumulation of activated T cells in the liver. To determine whether activated, virus-specific $CD8^+$ T cells could become trapped in the liver in the absence of infection, we used an internally controlled competitive assay in which we transferred a mixture of naive cells plus $CD8^+$ T cells activated in infected mice, into uninfected congenic recipients.

Donor $Thy1.1^+CD45.2^+CD8^+$OT-I T cells were isolated from airways of day 8-infected B6 mice, the peak of the $CD8^+$ response and a point when there is relatively little infectious virus in the lung. This CD8-enriched fraction was 97% $CD44^{high}CD62L^{low}$ (Fig. 2*C*). At minimum, 30% of the $CD8^+$ population in the airways on this day is directed against influenza epitopes, not including several minor epitopes (30). The cells were washed extensively and mixed one to one with naive $Thy1.2^+CD45.2^{+/-}$ $CD8^+$ splenocytes as an internal control. The mixture of activated and naive cells was transferred i.v. into naive $CD45.1^+$ B6.SJL mice so that the activated, nonactivated, and host cells were distinguishable based on Thy1 and CD45 markers (Fig. 2*C*). An aliquot of the transferred cells, inoculated into eggs, was negative for influenza virus.

Four days after cell transfer, donor cells were detectable in lymphoid tissue and livers, but in significantly differing proportions. Naive $CD8^+$ T cells distributed throughout all organs evenly (representing 1–2% of the $CD8^+$ population in each organ, Fig. 2*C*), with a slight predisposition for peripheral LN (Fig. 2*D*). In sharp contrast, activated $CD8^+$ T cells accumulated almost exclusively in livers of recipient animals (Fig. 2*D*), making up to 10% of the hepatic $CD8^+$ population (Fig. 2*C*). Furthermore, by immunohistochemistry (data not shown), activated cells induced the formation of inflammatory foci characteristic of influenza-induced hepatitis (13), even in the absence of infection. This showed that in a system lacking virus, activated virus-specific $CD8^+$ T cells localized to the liver in the absence of infection, further supporting that this phenomenon is Ag independent.

Effector capacity of intrahepatic T cells

Intrahepatic $CD8^+$ T cells were reported to have altered cytotoxic or cytokine effector functions in several infections, including hepatitis B, hepatitis C, and influenza (5, 31). Given the early and substantial accumulation of cells in the liver, any functional deviation could be detrimental to the pulmonary antiviral response. We therefore sought to understand the full functional status of the influenza-specific cells accumulating in the liver. Lymphocytes from liver, spleen, and BAL were restimulated ex vivo with the $NP_{366-374}$ peptide and then stained for IFN-γ. At day 9 after infection, Ag-specific $CD8^+$ lymphocytes from the liver demonstrated robust IFN-γ production (Fig. 3*A*). Of liver lymphocytes, 6.0% were IFN-γ^+CD8^+ T cells and 2.2% were TNF-α^+ in response to the peptide, comparing favorably with tetramer staining which was 4.8% of liver lymphocytes. In airways, 10.3% made IFN-γ, while 5.6% of the BAL were NP-specific $CD8^+$ T cells, in range with the literature (10–11% for IFN-γ and 6.3–11% for tetramer staining at day 10 of infection) (5, 23). The data do not support the contention that cytokine production is reduced among intrahepatic influenza-specific cells as previously proposed (5).

To examine the cytotoxic potential of intrahepatic $CD8^+$ T cells in relation to their ability to secrete IFN-γ, a single-cell assay of target killing was performed in parallel with an IFN-γ ELISPOT. The Lysispot assay measures immediate ex vivo cytotoxic function such that the number of spots generated is a precise estimate of the number of CTL (20). $CD8^+$ T cells from influenza-infected BALB

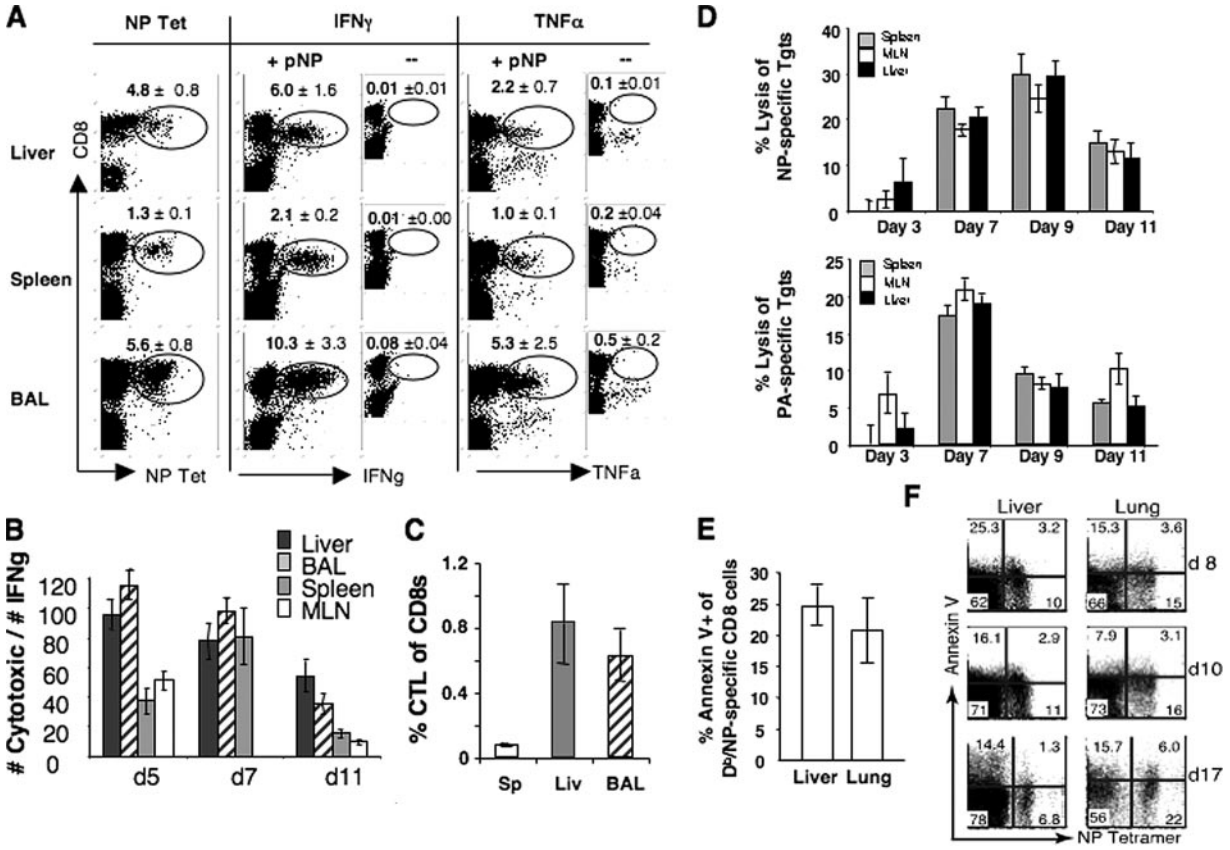

FIGURE 3. Intrahepatic influenza-specific CD8$^+$ T cells are functional. *A*, Normal cytokine production: cells from day 9 influenza-infected B6 mice stained with D^b/NP-tetramer and anti-CD8, or cultured 6 h with brefeldin A and IL-2 ± 1 μM NP peptide and stained for cytoplasmic IFN-γ or TNF-α. Average ± SD (CD8$^+$tetramer$^+$ or CD8$^+$cytokine$^+$) in lymphocyte gate, $n = 5$. *B*, Ex vivo-specific target lysis: depicted is ratio of Lysispot spots (killing of peptide-presenting targets) to ELISPOT spots (Ag-specific IFN-γ production) from x31-infected BALB/c mice, average of $n = 4$ ± SEM. *C*, Cytotoxicity as a proportion of total CD8$^+$ T cells in organ. Determined by day 7 Lysispots/(number of input lymphocytes × flow percentage CD8). *D*, Equivalent target cell recovery in each organ by in vivo cytotoxicity assay: x31-infected mice received 1:1 peptide-pulsed (NP or PA) CFSE^{high} plus irrelevantly pulsed CFSE^{low} splenocytes. Organs evaluated for relative ratio of CFSE^{high}-to-CFSE^{low} (18 h); average of $n = 3$ or more per time point ± SEM. *E*, Intrahepatic lymphocytes are not proportionally more annexin V$^+$ than in the lung. Large lymphocyte gate on day 8-infected B6 mice included dying cells, then gated on CD8$^+$, then D^{b}NP-tetramer$^+$, then annexin V; mean ±SD of 3. *F*, Lymphocytes from days 8, 10, and 17 gated on CD8$^+$; percent of CD8s indicated.

mice were assayed for cytotoxicity ex vivo at the beginning, peak, and resolution of intrahepatic accumulation. The CD8$^+$ T cells were combined in vitro with excess targets pulsed with the MHC class I epitope NP. The ratio of specific target lysis to frequency of IFN-γ production in ELISPOT indicated that on day 7, the proportion of cytotoxicity among Ag-specific cells was equivalent in all sampled organs, with >75% of IFN-γ^+CD8$^+$ cells capable of target lysis (Fig. 3*B*). Significantly, over 50% of liver-resident Ag-specific cells were cytolytic throughout the time course, even at early and late time points (up to day 15, data not shown), supporting that many cells were not disarmed during contraction. Of note, a significantly greater proportion of total CD8$^+$ T cells in the liver were cytotoxic in comparison to the spleen (Fig. 3*C*), suggesting the intrahepatic CD8$^+$ population may be more activated than splenic cells. In fact, the liver rivaled the airways, where cytotoxic effectors are most needed. The data also show that the Lysispot assay can detect low-frequency CTL not observed previously using bulk assays.

The killing capacity of CTL in vivo in the liver vs other sites was tested using an in vivo cytotoxic assay. On days 0, 7, 9, and 11 after influenza infection, mice were injected with a mixture of two splenocyte pools, one CFSE^{high}-labeled and pulsed with the immunodominant influenza NP or acid polymerase (PA) peptides, and the other CFSE^{low}-labeled and pulsed with a noninfluenza peptide. Organs were then sampled for the presence of CFSE-labeled targets. Reductions in influenza peptide$^+$ target cells in relation to irrelevant peptide-expressing targets were similar in all three organs, whether pulsed with NP or PA (Fig. 3*D*). Thus, there was no difference between organs in proportional recovery of NP-pulsed targets vs irrelevantly pulsed targets, with 25–30% lysis of Ag-specific targets across the board. Together, the Lysispot and in vivo assays provide evidence that there is no cytotoxic defect among influenza-specific CD8$^+$ T cells in the liver.

Apoptotic phenotype of intrahepatic CD8$^+$ T cells

Under some circumstances, higher apoptosis among liver T cells contributes to local tolerance (5). Higher TUNEL staining was reported among bulk CD8s in the liver as compared with the spleen during influenza infection (5), but the proportion of NP-tetramer$^+$CD8$^+$ lymphocytes was over twice that of TUNEL$^+$ lymphocytes. This indicated that either TUNEL staining underestimated dying cells or the majority of Ag-specific T cells were not apoptotic. As an alternative assay for early signs of apoptosis, we measured the appearance of phosphatidylserine on the outer cell leaflet using annexin V, which is associated with induction of programmed cell death (32, 33) and the detection and engulfment by macrophages (34).

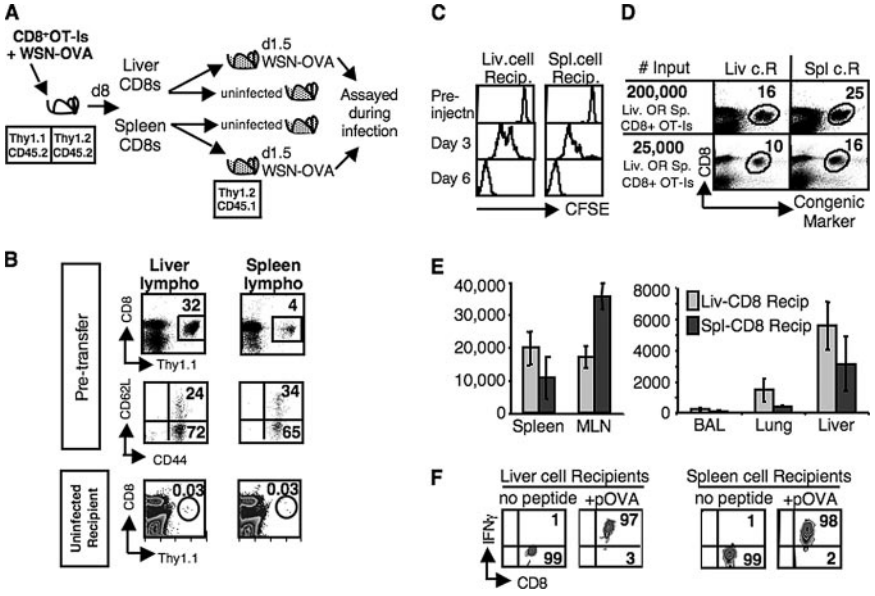

FIGURE 4. Intrahepatically trapped effector CD8$^+$ T cells are responsive in recipients. *A*, CD8-enriched (>90% pure) B6.PL TCR-transgenic OT-I T cells were activated in B6 hosts by influenza WSN-OVA$_I$ infection, then enriched day 8 from either liver or spleen. These activated B6.PL OT-I T cells were transferred i.v. into naive B6.SJL recipients. *B*, Pretransfer CD8$^+$ pools from B6 livers or spleens (liver 70% pure, spleen 90% pure), percentages of lymphocytes indicated (*top*); activation as percent of Thy1.1$^+$CD8$^+$ Ag-specific cells (*middle*); reisolation from uninfected-recipient B6.SJL spleens on day 8 as a percentage of CD8s (*bottom). C*, CFSE dilution of donor liver or spleen CD8s in recipient spleens. *D*, Activated OT-I CD8s sorted from liver or spleen (>99% pure) were transferred in equal amounts into infected mice and monitored on day 8 for expansion; percentage of CD8s indicated. *E*, Effector OT-I cells from liver or spleen were identically isolated and CD8 enriched; 10,000 cells transferred to infected recipient mice and reisolated day 6. Average of three spleen cell recipients and seven liver cell recipients. *F*, IFN-γ cytokine production in day 8 spleens of recipient mice, percentage of CD8$^+$ donor.

Influenza-specific CD8$^+$ T cells in the liver were not significantly more annexin$^+$ than in other nonlymphoid organs, including at the site of infection itself (Fig. 3*E*). Less than 25% of Ag-specific CD8$^+$ T cells were annexin V$^+$ in livers and lung tissue at the peak of infection (Fig. 3*E*). Values were also similar among total CD8$^+$ T cells, with 26.5 $\pm$ 2.3 in the liver and 22.7 $\pm$ 7.8 in the lung. These results were in agreement with findings in lymphocytic choriomeningitis virus model, where the majority of intrahepatic lymphocytic choriomeningitis virus-specific T cells were annexin negative throughout acute infection, including at the end of the response (35). In influenza, the proportion of annexin V$^+$ cells in the liver actually decreased among the Ag-specific subset during contraction (Fig. 3*F*), and reached basal levels among total CD8s (<20%) at day 17.

Therefore, there is no evidence among intrahepatic CD8$^+$ T cells of decreased functional capacity at the peak of the response and no evidence of increased death at the resolution. The finding that not all activated intrahepatic CD8$^+$ cells are slated to die led us to consider what purpose and possible outcome such a sizeable accumulation could have for the immune response.

Function in response to infection

Though Ag-specific CD8$^+$ T cells from the liver functioned normally ex vivo, there may be a defect in ability to properly proliferate and traffic in response to in vivo challenge. Thus, a more stringent test was to ask whether these cells were capable of effector function upon transfer to infected recipients. In vivo-activated OT-I-transgenic CD8$^+$ T cells (Fig. 4*A*) were isolated from either livers or spleens. The enriched liver-derived CD8$^+$ T cell pool contained 32% CD8$^+$OT-I cells that were 96% CD44^{high}, and the splenocyte pool was 4% OT-I$^+$ that were 99% CD44^{high} (Fig. 4*B*). Both pools contained mixed CD62L populations. The splenic and intrahepatic CD8$^+$ pools were normalized for OT-I T cells and transferred into separate cohorts of B6.SJL mice.

In uninfected controls (Fig. 4*B*, *bottom*), donor OT-I cell numbers approached the level of detection (<500 cells were recovered in any organ). However, when transferred into infected mice, CFSE diluted fully among both liver-derived and spleen-derived daughter CD8$^+$ T cells (Fig. 4*C*). After 8 days, donor OT-I progeny were readily detectable in spleens of infected host mice (Fig. 4*D*), demonstrating the capacity of effector cells in nonlymphoid tissue (NLT) to seed lymphoid organs. Both populations had increased from an input of <1% (in uninfected controls) to 10–25% of the splenic CD8$^+$ population. However, expansion of liver CD8$^+$ effectors was up to 50% less than spleen effectors in several experiments over a range of input numbers (Fig. 4*D* and data not shown). The difference in recovery suggested liver lymphocytes were unequal to their splenic counterparts in proliferation or survival. However, we hypothesized the more rigorous processing conditions for liver lymphocytes could have compromised the transfer.

To control for this, the experiment was repeated, but splenic and liver lymphocytes were processed identically using collagenase/DNase digestion. Upon transfer to infected recipients, intrahepatic lymphocytes performed at least as well as transferred splenocytes in expansion capacity (Fig. 4*E*). It is noteworthy that again, effectors localized in more significant numbers to the liver than to the lung and airways, regardless of original organ residency. Furthermore, both lymphocyte pools produced OT-I daughter cells fully capable of IFN-γ production (Fig. 4*F*). Ultimately, Ag-specific CD8$^+$ T cells isolated from the liver during the acute phase did persist and expand comparably to splenocytes. We next asked whether they persisted equally well long-term if parked in the absence of Ag.

Generation of memory from intrahepatic lymphocytes

Adoptive transfer into infected hosts could have induced expansion of a subset of viable effector T cells. To determine whether the

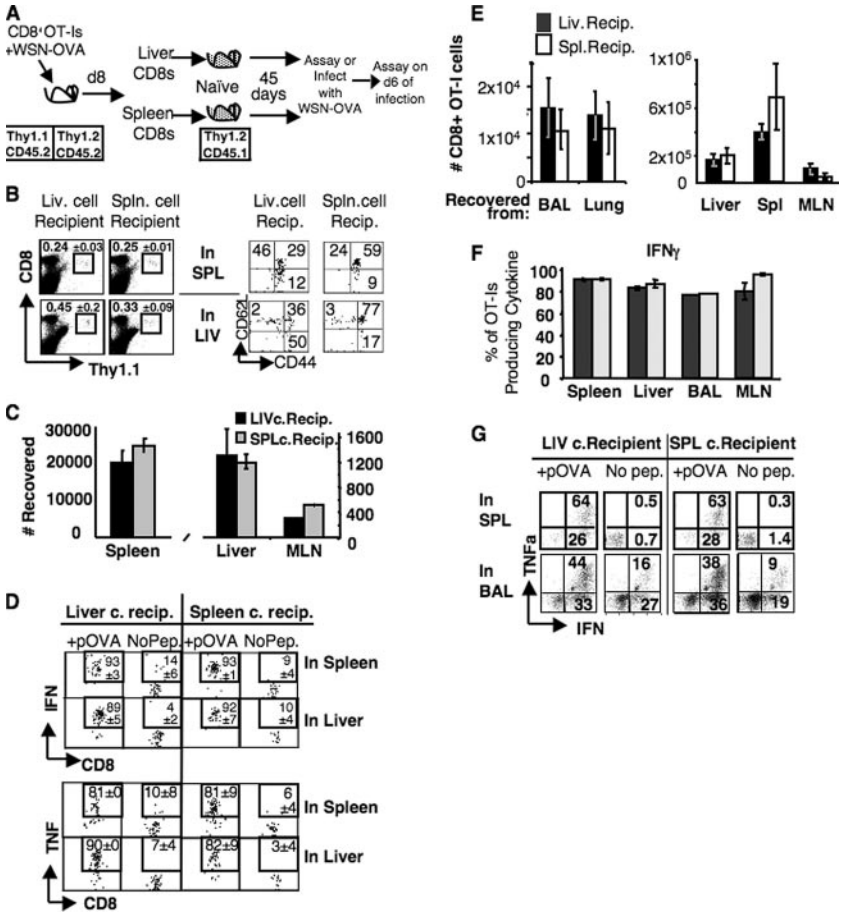

FIGURE 5. Effector CD8$^+$ T cells from the liver can become memory. *A*, CD8-enriched B6.PL TCR-transgenic OT-I T cells were activated in B6 hosts by WSN-OVA$_I$ infection, reisolated, and enriched on day 8 from either liver or spleen. Activated cells were transferred (1.3×10^5 CD8$^+$ effector splenocytes or liver lymphocytes per recipient) i.v. into naive B6.SJL recipients. *B*, Donor cell recovery in uninfected recipient mice on day 50, shown is average percent of CD8s ± SEM (*left*); activation markers (*rt*), average percent of donor CD8$^+$ cells. *C*, Numbers recovered in uninfected recipients $n = 3$, day 50; black, liver cell recipients, gray, spleen cell recipients. *D*, Cytokine production among OT-I cells recovered from uninfected recipients ± SD. *E*, Recipients ($n = 4$) were infected on day 45 and sacrificed 6 days later, cells stained for congenic markers. *F*, Intracellular cytokine staining of recalled donor CD8$^+$ cells on day 6 after WSN-OVA$_I$ influenza infection, $n = 4$. *G*, Cytokine double producers, gated on donor CD8$^+$ lymphocytes, average of four.

intrahepatic pool also contained T cells that could survive without infection, uninfected recipients of activated OT-I cells were not challenged with WSN-OVA$_I$ until 45 days after transfer (Fig. 5*A*).

Liver-derived donor CD8$^+$ T cells survived in recipient mice in the absence of infection and could be isolated from host lymphoid and NLT (Fig. 5, *B–D*). In spleens of naive hosts, similar percentages were recovered regardless of original donor organ (Fig. 5*B*) with 0.24–0.25% of the CD8$^+$ T cells in recipient spleens of both groups being donor-derived, representing 0.05% (±0.005) of the host splenocytes. Percentages recovered in livers (Fig. 5*B*) and MLN (data not shown) were also comparable between groups, again between 0.04 and 0.05% of lymphocytes. Expression profiles of CD44 and CD62L on recovered cells were heterogeneous depending on the sampled organ (Fig. 5*B*, *right*). In recipient spleens, percentages translated to 20,315 (±2,685) OT-Is originating from donor livers and 24,530 (±1,970) spleen-derived OT-Is (Fig. 5*C*). Equivalent amounts (~1,200 donor cells) were also isolated from livers in both cohorts, though too few cells were recovered from lungs to accurately detect and compare.

Regardless of the organ sampled, over 80% of OT-Is were capable of IFN-γ production following 6-h restimulation and this was not dependent on the transferred organ (Fig. 5*D*). Over 70% of memory CD8$^+$ OT-I T cells from either donor group could also make TNF-α, suggesting even fully differentiated double producers survived contraction and did not revert in phenotype. Furthermore, acute-phase intrahepatic T cells could gain entry into both lymphoid and nonlymphoid organs in the absence of inflammation.

Upon infection, donor lymphocytes expanded, and on day 6 after challenge (Fig. 5*E*) they generated an equivalent response in all organs whether descended from spleen or liver lymphocytes. These recalled cells showed no strong organ-specific bias, trafficking similarly regardless of pretransfer origin, with $4–6 \times 10^5$ localizing to spleen, $1–1.2 \times 10^4$ localizing to BAL, and ~2×10^5 in livers. Approximately 3% of airway lymphocytes were of donor origin after expansion from both donor organs. These cells were uniformly CD44^{high} and CD127 expression was comparable (data not shown).

Recalled cells also resembled one another in capacity for cytokine production (TNF-α, IL-2, and IFN-γ) regardless of donor organ. In direct comparison to spleen cell progeny, liver cell progeny had comparable IFN-γ production in lymphoid tissue (over 90% in spleens), at the site of infection (BAL), and in the liver itself following ex vivo restimulation with their cognate OVA peptide (Fig. 5*F*). When cultured without peptide, <5% of OT-I cells stained positive for IFN-γ in spleen, liver, and MLN, while up to 50% were positive in the unrestimulated BAL (Fig. 5*G*), which may be a result of sustained Ag presentation by airway APCs in the culture. Donor cells in both cohorts performed equivalently in terms of TNF-α and comparable proportions were positive for both cytokines regardless of ancestry (Fig. 5*G*).

Therefore, memory CD8$^+$ T cell cytokine-producing capabilities in response to Ag were uncompromised irrespective of whether the cells had localized to liver or spleen during primary infection. Clearly, intrahepatic effector CD8$^+$ T cells developed into long-lived memory cells when isolated from the liver during acute infection and could generate a recall response of similar quality to transferred splenocyte effectors.

In vivo release of influenza-specific CD8$^+$ T cells from the liver

Given the functional competence and lymphoid-extralymphoid trafficking potential of the hepatic T cell pool, we asked whether a

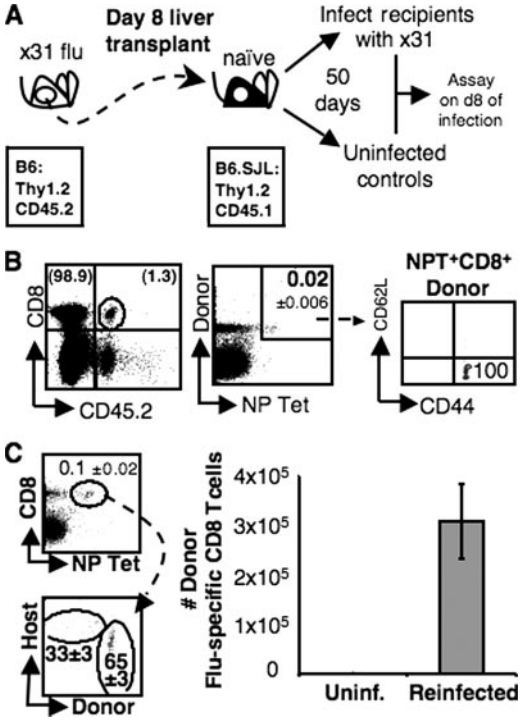

FIGURE 6. The liver releases trapped Ag-specific $CD8^+$ T cells that function in a recall response. *A*, Livers were orthotopically transplanted from day 8 influenza/x31-infected mice; recipients were naive. After 50 days, recipients were infected with influenza/x31. *B*, Donor cells were recovered from spleens of uninfected recipient mice on day 50. *Left*, Gated on lymphocytes, numbers indicate the percent of $CD8^+$; *middle*, gated on CD8, average percent, $n = 4$ is shown. *C*, Liver transplant recipients were infected on day 50 with influenza-x31; organs recovered on day 6 of infection (*top*). Gated on lymphocytes, all plots indicate average percent. Graphed is the number of Ag-specific donor CD8s recovered, as determined by staining $\times$ total splenocytes ($n = 4$ uninfected, 6 reinfected).

proportion of these cells were capable of migrating out in the absence of mechanical organ disruption. If capable of independently exiting, the accumulation of fully armed $CD8^+$ T cells before pulmonary viral clearance could act as a stockpile of viable cells to draw from.

At the peak of infection, livers were orthotopically transplanted into naive animals (Fig. 6*A*). Transplanted livers were first perfused thoroughly, leaving no evidence of blood-borne leukocytes (12); this was also verified by histological sections of perfused livers (data not shown). Thus, only intrahepatically trapped lymphocytes were transplanted with the liver. This created transplant recipients otherwise naive to influenza but containing livers from acutely infected mice, along with their accumulated influenza-specific effector $CD8^+$ T cells. Recipients were rested for 50 days before monitoring surviving and released intrahepatic T cells.

Sufficient numbers of intrahepatic effectors survived organ transplantation and acute-phase contraction to generate a systemic memory population. Donor $CD8^+$ T cells could be recovered in recipient spleens on day 50 following transplant, such that 1.3% of the $CD8^+$ T cell lymphocytes were of donor origin (Fig. 6*B*), and 2.1 ± 0.4 of the $CD8^+$ donor cells were NP $tetramer^+$. An average of 2406 ± 186 Ag-specific T cells of donor origin were recovered in spleens of uninfected recipients, significant ($p < 0.01$) over background staining of $tetramer^+$ host CD8s. Donor influenza-specific $CD8^+$ T cells were 100% $CD44^{high}$ (Fig. 6*B*). Given that seeding of lymphoid organs by donor lymphocytes occurred before introduction of Ag, and because viral Ags are not presented intrahepatically during influenza (Fig. 2), subsequent activation of these donor cells likely occurs primarily in the draining LN.

When liver transplant recipients were infected with influenza (Fig. 6*C*), donor Ag-specific T cells on day 6 made up almost 65% of the total D^b/NP-$tetramer^+$ response in the spleen. Their numbers approached $300{,}000 \pm 74{,}380$, significantly more than in uninfected controls (Fig. 6*C*, *right*). Thus, a notable proportion of intrahepatic T cells emerged from the liver and responded to infection. The cumulative data suggest that not all intrahepatic CD8 cells are culled intrahepatically and, in fact, the liver contains all the necessary and functional subsets to reconstitute a complete immune $CD8^+$ population.

Discussion

Ag-specific $CD8^+$ T cells begin accumulating in the liver very early during the influenza response. At a point where intrahepatic accumulation is maximal, these cells are fully functional and we find no evidence of increased cell death compared with other compartments. Both after adoptive transfer as a cell suspension, and orthotopic transplantation from acutely infected mice, these intrahepatic influenza-specific cells can participate in recall antiviral responses. The magnitude of hepatic involvement in immune responses is reinforced by the finding that the liver is the next largest site besides the spleen of specific $CD8^+$ T cell accumulation among sampled organs, including the infected lung.

Activated $CD8^+$ T cell accumulation in the liver has been described in numerous models, both in the presence (3, 5) and absence (4, 36) of Ag. Binding between liver parenchyma and activated T cells is promoted by ICAM-1/LFA-1 (36) and VCAM-1/VLA-4 interactions; in the absence of Ag on hepatocytes, VLA-4 plays the more significant role (37). In the lung, retention is dependent on LFA-1 but not VLA-4 (38, 39). Thus, in combination with the large intrahepatic surface area, differential expression of adhesion molecules on endothelia in lung and liver could explain preferential early accumulation in the liver.

In contrast to models in which locally presented Ag in the liver induces activation (12, 40), and apoptosis or tolerance (9, 11, 41, 42), the influenza model describes the fate of extrahepatically activated T cells that accumulate in the liver in the absence of intrahepatic Ag. The fate of T cells may differ when accumulation is not engendered by intrahepatic Ag, which could explain the disparity in T cell mortality and function in comparison to studies with systemic (including intrahepatic) Ag (3, 43). Thus, findings in models of intrahepatic Ag presentation do not predict the same consequences for extrahepatically activated T cells residing in the liver.

Upon cotransfer of activated and naive $CD8^+$ T cells into naive hosts, the activated T cells preferentially accumulated in livers, while naive cells dispersed throughout organs. It is interesting that these activated cells, isolated for transfer from airways of day 8-infected mice, selectively favored the liver over the lungs. This suggests that neither a differential activation of the cells nor homing influence their trafficking patterns, though we cannot rule out changes in surface molecule expression during their 4 days in recipient mice. Thus, while inflammation in the lung is not enough to trump the liver during infection, in a system lacking inflammation, the cells still go to the liver first.

The significance of early intrahepatic trapping for the pulmonary immune response is unclear, but in restricted infections such as influenza, regulating the number of circulating virus-specific CTL may limit indiscriminate immunopathogenesis at the infection site. In situations where a high apoptosis rate was observed (3, 43), accumulation was interpreted to mean the liver can have a

negative impact on immune responses, particularly during the contraction phase (6), and this is reinforced by observations that allogeneic liver transplants can induce T cell tolerance (44). In contrast, the large magnitude of the intrahepatic pool early in the response suggests that during expansion, the liver is instead a substantial reservoir of fully functional T cells. The capacity of these cells to exit from a transplanted liver and repopulate other compartments argues that the liver (and possibly other large extralymphoid tissues) could have a significant positive role by temporarily storing $CD8^+$ effectors during acute extrahepatic responses, in a manner akin to the splenic reservoir.

An adequate understanding of the liver during $CD8^+$ T cell immune responses activated at remote sites, including the lungs, needs to reconcile its function as a site of T cell sequestration and elimination with its function as a reservoir of activated, fully functional T cells. The liver as an immune reservoir during the acute antiviral response stands in contrast to its function in limiting the magnitude of $CD8^+$ T cell memory responses (37). These experiments used transplantation of TLR4-deficient livers to reveal that a defect in intrahepatic accumulation of activated $CD8^+$ T cells led to their increase in blood and memory compartments and to enhanced memory responses. One possibility is that the liver shifts modes from the early to late phase of the immune response. During the expansion phase of an extrahepatic immune response, for example, many activated $CD8^+$ T cells accumulate in the liver, retain function, and can move out to repopulate other tissues and the memory compartment. As Ag-specific T cells become abundant in the blood, the liver's role may shift to elimination, preventing the memory compartment from becoming overloaded. Potential shifts in liver-T cell interaction and the consequences need to be formally investigated. The current study highlights our limited understanding of the dynamics of recirculation (lymphoid-nonlymphoid exchange) and the length of stay in NLT.

Memory cells isolated from different organs can perform similarly in recall responses (1), but it was not clear where the memory localized within nonlymphoid tissues originates. The current report indicates both lymphoid and extralymphoid effectors can contribute to systemic immunity and memory, suggesting that generation of memory may be decentralized. This raises new questions concerning the nature of central and effector memory T cells and the potential for exchange between lymphoid and nonlymphoid tissues. The collective contribution of all extralymphoid tissues to memory formation and maintenance remains to be investigated, but it is possible that $CD8^+$ T cells can mobilize from other large organs such as the skin or gut as well. This can have consequences in cases where extralymphoid memory formation may be impeded, such as in cirrhosis of the liver. In contrast, $CD8^+$ T cell mobilization from the liver may have significant benefit in cases of compromised lymphoid tissue, such as following splenectomy in humans. At the very least, the liver can function as an immune organ that regulates peripheral immune homeostasis during viral infections in which large numbers of $CD8^+$ CTL are generated.

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Disclosures

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References

1. Masopust, D., V. Vezys, E. J. Usherwood, L. S. Cauley, S. Olson, A. L. Marzo, R. L. Ward, D. L. Woodland, and L. Lefrancois. 2004. Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. *J. Immunol.* 172: 4875–4882.
2. John, B., and I. N. Crispe. 2004. Passive and active mechanisms trap activated $CD8^+$ T cells in the liver. *J. Immunol.* 172: 5222–5229.
3. Mehal, W. Z., F. Azzaroli, and I. N. Crispe. 2001. Antigen presentation by liver cells controls intrahepatic T cell trapping, whereas bone marrow-derived cells preferentially promote intrahepatic T cell apoptosis. *J. Immunol.* 167: 667–673.
4. Bowen, D. G., A. Warren, T. Davis, M. W. Hoffmann, G. W. McCaughan, B. Fazekas de St. Groth, and P. Bertolino. 2002. Cytokine-dependent bystander hepatitis due to intrahepatic murine CD8 T-cell activation by bone marrow-derived cells. *Gastroenterology* 123: 1252–1264.
5. Belz, G. T., J. D. Altman, and P. C. Doherty. 1998. Characteristics of virus-specific $CD8^+$ T cells in the liver during the control and resolution phases of influenza pneumonia. *Proc. Natl. Acad. Sci. USA* 95: 13812–13817.
6. Crispe, I. N., T. Dao, K. Klugewitz, W. Z. Mehal, and D. P. Metz. 2000. The liver as a site of T-cell apoptosis: graveyard, or killing field. *Immunol. Rev.* 174: 47–62.
7. Gruener, N. H., F. Lechner, M. C. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G. R. Pape, and P. Klenerman. 2001. Sustained dysfunction of antiviral $CD8^+$ T lymphocytes after infection with hepatitis C virus. *J. Virol.* 75: 5550–5558.
8. Limmer, A., J. Ohl, G. Wingender, M. Berg, F. Jungerkes, B. Schumak, D. Djandji, K. Scholz, A. Klevenz, S. Hegenbarth, et al. 2005. Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur. J. Immunol.* 35: 2970–2981.
9. Qian, S., L. Lu, F. Fu, Y. Li, W. Li, T. E. Starzl, J. J. Fung, and A. W. Thomson. 1997. Apoptosis within spontaneously accepted mouse liver allografts: evidence for deletion of cytotoxic T cells and implications for tolerance induction. *J. Immunol.* 158: 4654–4661.
10. Klugewitz, K., F. Blumenthal-Barby, A. Schrage, P. A. Knolle, A. Hamann, and I. N. Crispe. 2002. Immunomodulatory effects of the liver: deletion of activated $CD4^+$ effector cells and suppression of IFN-γ-producing cells after intravenous protein immunization. *J. Immunol.* 169: 2407–2413.
11. Bowen, D. G., M. Zen, L. Holz, T. Davis, G. W. McCaughan, and P. Bertolino. 2004. The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J. Clin. Invest.* 114: 701–712.
12. Klein, I., and I. N. Crispe. 2006. Complete differentiation of $CD8^+$ T cells activated locally within the transplanted liver. *J. Exp. Med.* 203: 437–447.
13. Polakos, N. K., J. C. Cornejo, D. A. Murray, K. O. Wright, J. J. Treanor, I. N. Crispe, D. J. Topham, and R. H. Pierce. 2006. Kupffer cell-dependent hepatitis occurs during influenza infection. *Am. J. Pathol.* 168: 1169–1178; quiz 1404-1165.
14. Ando, K., K. Hiroishi, T. Kaneko, T. Moriyama, Y. Muto, N. Kayagaki, H. Yagita, K. Okumura, and M. Imawari. 1997. Perforin, Fas/Fas ligand, and TNF-α pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J. Immunol.* 158: 5283–5291.
15. Keating, R., W. Yue, J. A. Rutigliano, J. So, E. Olivas, P. G. Thomas, and P. C. Doherty. 2007. Virus-specific $CD8^+$ T cells in the liver: armed and ready to kill. *J. Immunol.* 178: 2737–2745.
16. Allan, W., Z. Tabi, A. Cleary, and P. C. Doherty. 1990. Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting $CD4^+$ T cells. *J. Immunol.* 144: 3980–3986.
17. Liang, S., K. Mozdzanowska, G. Palladino, and W. Gerhard. 1994. Heterosubtypic immunity to influenza type A virus in mice: effector mechanisms and their longevity. *J. Immunol.* 152: 1653–1661.
18. Topham, D. J., M. R. Castrucci, F. S. Wingo, G. T. Belz, and P. C. Doherty. 2001. The role of antigen in the localization of naive, acutely activated, and memory $CD8^+$ T cells to the lung during influenza pneumonia. *J. Immunol.* 167: 6983–6990.
19. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.
20. Snyder, J. E., W. J. Bowers, A. M. Livingstone, F. E. Lee, H. J. Federoff, and T. R. Mosmann. 2003. Measuring the frequency of mouse and human cytotoxic T cells by the Lysispot assay: independent regulation of cytokine secretion and short-term killing. *Nat. Med.* 9: 231–235.
21. Coles, R. M., S. N. Mueller, W. R. Heath, F. R. Carbone, and A. G. Brooks. 2002. Progression of armed CTL from draining lymph node to spleen shortly after localized infection with herpes simplex virus 1. *J. Immunol.* 168: 834–838.
22. Steger, U., B. Sawitzki, A. M. Gassel, H. J. Gassel, and K. J. Wood. 2003. Impact of hepatic rearterialization on reperfusion injury and outcome after mouse liver transplantation. *Transplantation* 76: 327–332.
23. Flynn, K. J., G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, and P. C. Doherty. 1998. Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia. *Immunity* 8: 683–691.
24. Hogan, R. J., E. J. Usherwood, W. Zhong, A. A. Roberts, R. W. Dutton, A. G. Harmsen, and D. L. Woodland. 2001. Activated antigen-specific $CD8^+$ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol.* 166: 1813–1822.
25. Hikono, H., J. E. Kohlmeier, K. H. Ely, I. Scott, A. D. Roberts, M. A. Blackman, and D. L. Woodland. 2006. T-cell memory and recall responses to respiratory virus infections. *Immunol. Rev.* 211: 119–132.
26. Lawrence, C. W., and T. J. Braciale. 2004. Activation, differentiation, and migration of naive virus-specific $CD8^+$ T cells during pulmonary influenza virus infection. *J. Immunol.* 173: 1209–1218.
27. Eichelberger, M. C., M. L. Wang, W. Allan, R. G. Webster, and P. C. Doherty. 1991. Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. *J. Gen. Virol.* 72(Pt. 7): 1695–1698.

28. Hamilton-Easton, A., and M. Eichelberger. 1995. Virus-specific antigen presentation by different subsets of cells from lung and mediastinal lymph node tissues of influenza virus-infected mice. *J. Virol.* 69: 6359–6366.
29. Tripp, R. A., D. J. Topham, S. R. Watson, and P. C. Doherty. 1997. Bone marrow can function as a lymphoid organ during a primary immune response under conditions of disrupted lymphocyte trafficking. *J. Immunol.* 158: 3716–3720.
30. Belz, G. T., W. Xie, J. D. Altman, and P. C. Doherty. 2000. A previously unrecognized H-2D^b-restricted peptide prominent in the primary influenza A virus-specific CD8$^+$ T-cell response is much less apparent following secondary challenge. *J. Virol.* 74: 3486–3493.
31. Rehermann, B., and M. Nascimbeni. 2005. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat. Rev. Immunol.* 5: 215–229.
32. Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* 182: 1545–1556.
33. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. *J. Immunol. Methods* 184: 39–51.
34. Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals, and M. H. van Oers. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 84: 1415–1420.
35. Wang, X. Z., S. E. Stepp, M. A. Brehm, H. D. Chen, L. K. Selin, and R. M. Welsh. 2003. Virus-specific CD8 T cells in peripheral tissues are more resistant to apoptosis than those in lymphoid organs. *Immunity* 18: 631–642.
36. Mehal, W. Z., A. E. Juedes, and I. N. Crispe. 1999. Selective retention of activated CD8$^+$ T cells by the normal liver. *J. Immunol.* 163: 3202–3210.
37. John, B., I. Klein, and I. N. Crispe. 2007. Immune role of hepatic TLR-4 revealed by orthotopic mouse liver transplantation. *Hepatology* 45: 178–186.
38. Galkina, E., J. Thatte, V. Dabak, M. B. Williams, K. Ley, and T. J. Braciale. 2005. Preferential migration of effector CD8$^+$ T cells into the interstitium of the normal lung. *J. Clin. Invest.* 115: 3473–3483.
39. Thatte, J., V. Dabak, M. B. Williams, T. J. Braciale, and K. Ley. 2003. LFA-1 is required for retention of effector CD8 T cells in mouse lungs. *Blood* 101: 4916–4922.
40. Bertolino, P., D. G. Bowen, G. W. McCaughan, and B. Fazekas de St Groth. 2001. Antigen-specific primary activation of CD8$^+$ T cells within the liver. *J. Immunol.* 166: 5430–5438.
41. Liu, Z. X., S. Govindarajan, S. Okamoto, and G. Dennert. 2001. Fas-mediated apoptosis causes elimination of virus-specific cytotoxic T cells in the virus-infected liver. *J. Immunol.* 166: 3035–3041.
42. Limmer, A., J. Ohl, C. Kurts, H. G. Ljunggren, Y. Reiss, M. Groettrup, F. Momburg, B. Arnold, and P. A. Knolle. 2000. Efficient presentation of exogenous antigen by liver endothelial cells to CD8$^+$ T cells results in antigen-specific T-cell tolerance. *Nat. Med.* 6: 1348–1354.
43. Huang, L., G. Soldevila, M. Leeker, R. Flavell, and I. N. Crispe. 1994. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. *Immunity* 1: 741–749.
44. Qian, S., A. J. Demetris, N. Murase, A. S. Rao, J. J. Fung, and T. E. Starzl. 1994. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology* 19: 916–924.