Cell Reports
Article

Liver-Primed Memory T Cells Generated under Noninflammatory Conditions Provide Anti-infectious Immunity

Jan P. Böttcher,[1] Oliver Schanz,[1] Dirk Wohlleber,[1] Zeinab Abdullah,[1] Svenja Debey-Pascher,[2] Andrea Staratschek-Jox,[2] Bastian Höchst,[1] Silke Hegenbarth,[1] Jessica Grell,[3] Andreas Limmer,[1] Imke Atreya,[6] Markus F. Neurath,[6] Dirk H. Busch,[4] Edgar Schmitt,[7] Peter van Endert,[8] Waldemar Kolanus,[3] Christian Kurts,[1] Joachim L. Schultze,[2] Linda Diehl,[1] and Percy A. Knolle[1,5,*]

[1]Institutes of Molecular Medicine and Experimental Immunology
[2]Genomics and Immunoregulation, LIMES Institute
[3]Molecular Immunology, LIMES Institute
Rheinische Friedrich-Wilhelms-Universität, 53105 Bonn, Germany
[4]Institute of Microbiology and Hygiene
[5]Institute of Molecular Immunology
Technische Universität München, 81675 München, Germany
[6]Department of Medicine 1, Universitätsklinikum Erlangen, 91054 Erlangen, Germany
[7]Institute for Immunology, Johannes Gutenberg-Universität, 55122 Mainz, Germany
[8]INSERM U1013 and Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France
*Correspondence: pknolle@uni-bonn.de
http://dx.doi.org/10.1016/j.celrep.2013.02.008

SUMMARY

Development of CD8$^+$ T cell (CTL) immunity or tolerance is linked to the conditions during T cell priming. Dendritic cells (DCs) matured during inflammation generate effector/memory T cells, whereas immature DCs cause T cell deletion/anergy. We identify a third outcome of T cell priming in absence of inflammation enabled by cross-presenting liver sinusoidal endothelial cells. Such priming generated memory T cells that were spared from deletion by immature DCs. Similar to central memory T cells, liver-primed T cells differentiated into effector CTLs upon antigen re-encounter on matured DCs even after prolonged absence of antigen. Their reactivation required combinatorial signaling through the TCR, CD28, and IL-12R and controlled bacterial and viral infections. Gene expression profiling identified liver-primed T cells as a distinct Neuropilin-1$^+$ memory population. Generation of liver-primed memory T cells may prevent pathogens that avoid DC maturation by innate immune escape from also escaping adaptive immunity through attrition of the T cell repertoire.

INTRODUCTION

Generation of CD8$^+$ T cell (cytotoxic T lymphocyte [CTL]) immunity occurs through priming of naive CD8$^+$ T cells by professional antigen-presenting cells (APCs) such as dendritic cells (DCs) in secondary lymphatic tissues (Takada and Jameson, 2009; Zhang and Bevan, 2011). Appropriate innate immune stimulation causes DC maturation into immunogenic APCs that can cross-prime naive CD8$^+$ T cells, causing differentiation into effector and memory T cells (Harty and Badovinac, 2008; Kaech et al., 2002b; Kurts et al., 2010; Parish et al., 2009). In the absence of innate immune stimulation, immature APCs cross-presenting exogenous antigens induce peripheral tolerance characterized by anergy, suppression, or clonal deletion of T cells (Redmond and Sherman, 2005; Steinman et al., 2003). Alternatively, presentation of self-antigens by stromal cells in lymphatic tissue also contributes to peripheral T cell deletion (Gardner et al., 2008). Surviving tolerant self-antigen specific T cells generated under noninflammatory conditions retain their functional nonresponsiveness even after challenge with pathogens (Schietinger et al., 2012). While innate immune activation and inflammation are a precondition for induction of adaptive immunity, it has become clear that some infectious microorganisms such as hepatitis viruses employ stealth mechanisms to escape or prevent induction of innate immunity while disseminating viral antigens systemically (Protzer et al., 2012). Cross-presentation under noninflammatory conditions by immature DCs threatens to elicit peripheral tolerance in antigen-specific CTLs and thereby facilitate viral persistence.

Here, we report on the existence of a third outcome of naive CD8$^+$ T cell priming in addition to immunogenic priming by matured DCs and tolerogenic priming by immature DCs. This third way of T cell priming is enabled by liver sinusoidal endothelial cells (LSECs) cross-presenting circulating exogenous antigen during noninflammatory conditions. Once primed by LSECs, T cells were not deleted any more by antigen-presenting immature DCs. Although priming by LSECs induces T cell nonresponsiveness toward isolated T cell receptor (TCR) signaling (Diehl et al., 2008; Limmer et al., 2000; Schurich et al., 2010), liver-primed T cells were not terminally committed to their

Cell PRESS
Open ACCESS

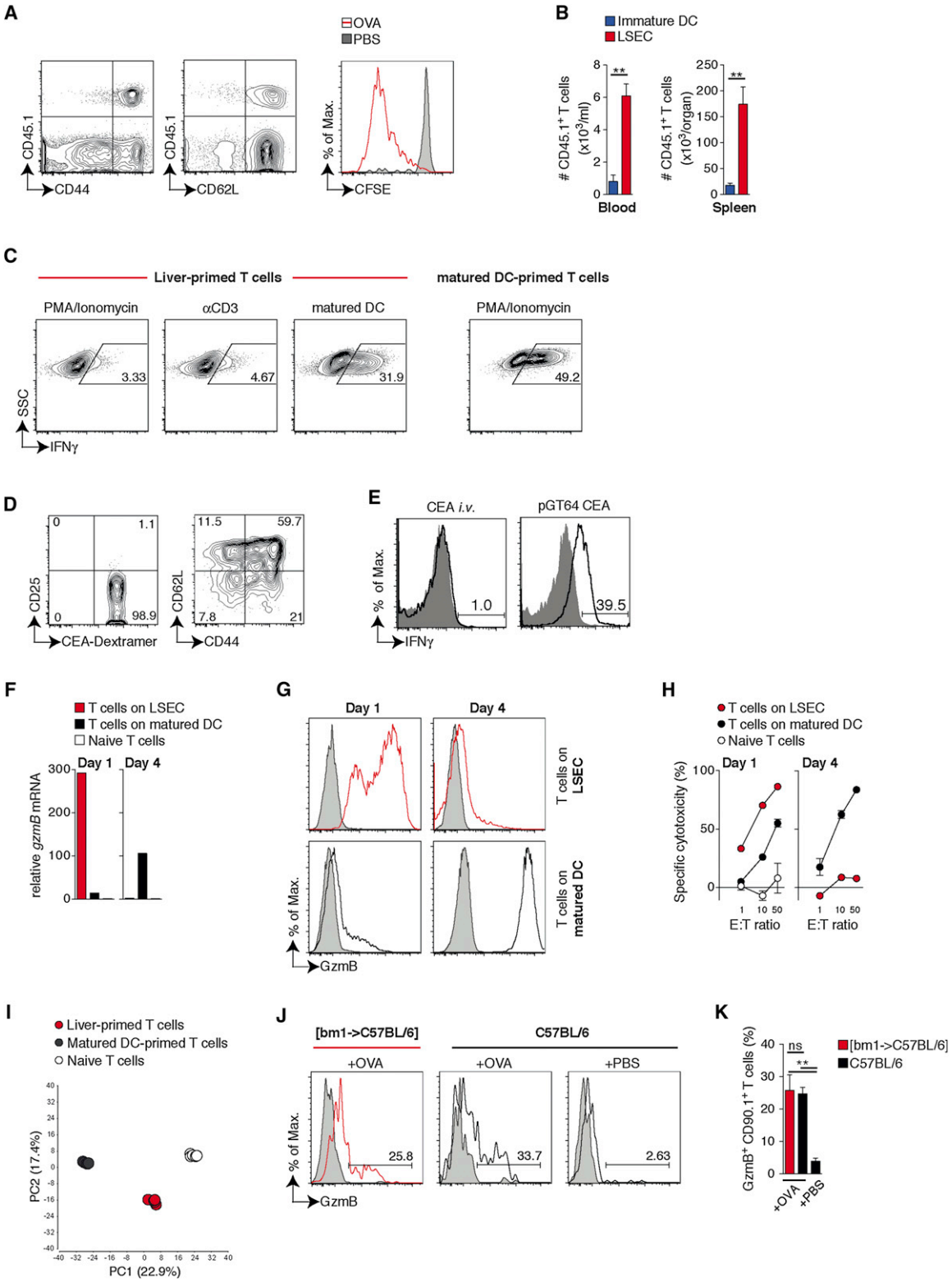

Figure 1. Naive CD8⁺ T Cells Primed by Cross-Presenting LSECs in the Absence of Inflammation Survive and Are Reactivated by Matured DCs

(A) Surface phenotype and proliferation of adoptively transferred CD45.1⁺ CD8⁺ OT-I T cells (1 × 10^6 cells/mouse) 4 days after antigen-specific priming by LSECs in CD45.2⁺ (bm1 → C57BL/6) chimeric mice in vivo (see Figure S1).

(legend continued on next page)

nonresponsive state but rather acquired a differentiation state that enabled them to generate effector T cells under inflammatory conditions. Similar to central memory T cells, reactivation of liver-primed T cells for generation of protective effector CTLs required combinatorial stimulation through CD28 and interleukin (IL)-12 in addition to TCR signaling. Our results define a liver-primed memory T cell population that is generated by nonprofessional APCs, which rescues T cells from tolerization by immature DCs and enables the subsequent induction of pathogen-specific CTL immunity.

RESULTS

Naive CD8$^+$ T Cells Primed by Cross-Presenting LSECs in the Absence of Inflammation Survive and Can Be Reactivated by Matured DCs

We investigated the fate of T cells stimulated by LSEC (liver-primed T cells) in vivo using an established chimeric mouse model, in which cross-presentation occurs by liver-resident LSECs but not bone-marrow-derived DCs (von Oppen et al., 2009). In this model (Figure S1A), radiation-resistant LSECs efficiently cross-present nonself antigen to circulating naive CD8$^+$ T cells, whereas no cross-presentation is observed by professional APCs in spleen or liver (von Oppen et al., 2009), which correlates with H-2K^b expression in the chimeras that is detected on LSECs but not bone-marrow-derived cells (Figures S1B and S1C). To study the consequences of cross-presentation of soluble proteins by LSECs to naive CD8$^+$ T cells under noninflammatory conditions, we adoptively transferred naive carboxyfluorescein-diacetate-succinimidyl-ester (CFSE)-labeled CD45.1$^+$ OVA-specific TCR transgenic T cells (OT-I) into these chimeras. Four days after injection of soluble LPS-free OVA, naive OT-I T cells had proliferated and upregulated the activation marker CD44 while expressing CD62L at high levels (Figure 1A). T cell proliferation was not observed in the absence of antigen excluding homeostatic proliferation as a cause. As CD8$^+$ T cell priming under noninflammatory conditions can result in clonal deletion (Mueller, 2010; Steinman et al., 2003), we investigated the fate of liver-primed T cells and compared it to that of naive T cells primed by immature DCs, i.e., following injection of OVA coupled to DEC205 into C57BL/6 mice (Bonifaz et al., 2002). As expected, T cell numbers were reduced 7 days after priming by immature DCs (Figure 1B), which is compatible with DEC205-OVA-dependent deletion through DC-mediated cross-tolerance. Of note, OVA-DEC205 was not cross-presented by LSECs (Figure S1D), which may be related to the absence of DEC205 expression from LSECs (data not shown) and the very low antigen-concentration used. Seven days after priming by LSECs, however, we found high numbers of viable T cells in blood and spleen (Figure 1B), indicating that LSEC priming did not induce T cell elimination. These results demonstrate that T cell priming by LSECs or by immature DCs leads to different outcomes. Consistent with our previous reports (Diehl et al., 2008; Limmer et al., 2000), we found that T cells at d4 after priming by LSECs failed to respond to stimulation via TCR triggering or phosphomyristolic acid (PMA)/ionomycin treatment (Figure 1C). However, when liver-primed T cells recognized their antigen on matured DCs they started to produce interferon (IFN)-γ (Figure 1C). CD8$^+$ T cells with a different specificity (carcino-embryonic-antigen [CEA] specific) but similar phenotype, i.e., CD62L$^+$CD44$^+$ T cells that did not produce IFN-γ upon restimulation, were also found in HLA-A2 transgenic mice upon repeated i.v. injection of recombinant CEA (Figures 1D and 1E), indicating that they are derived from CEA cross-presentation by LSECs (Höchst et al., 2012). Collectively, these results indicated that the nonresponsiveness of liver-primed T cells toward TCR signaling was reversible and thereby distinct from the ultimately committed nonresponsiveness observed in tolerized T cells (Schietinger et al., 2012). This questioned our earlier reports that liver-primed T cells were tolerant (Diehl et al., 2008; Limmer et al., 2000; Schurich et al., 2010) and rather indicated that their nonresponsiveness is part of a as-yet-unknown T cell differentiation state.

The observed lack of deletion and the ability of matured DCs to evoke IFN-γ production in liver-primed T cells led us to characterize the early steps in their differentiation in vitro. Expression of

(B) Adoptive transfer of 5 × 10^5 OT-I T cells and determination of total numbers of OT-I T cells 7 days after antigen-presentation during noninflammatory conditions by LSECs (soluble OVA in [bm1 → C57BL/6] chimeric animals) or tolerogenic immature DCs (OVA coupled to anti-DEC205: DEC205-P3UOrv in C57BL/6 mice).

(C) In-vitro-generated liver-primed OT-I T cells (d4 after priming) were analyzed for IFN-γ production after stimulation by PMA/ionomycin, anti-CD3, or OVA-loaded matured DCs. IFN-γ expression within viable T cells was determined after 20 hr by intracellular staining. OT-I T cells primed by matured DCs and stimulated with PMA/ionomycin for 20 hr served as control.

(D and E) Recombinant CEA protein (2 mg/mouse) was injected i.v. into HLA-A*201 mice every second day for 14 days. Splenic CEA-specific CD8$^+$ T cells were identified by HLA-A*201 dextramer staining loaded with the CEA-specific peptide YLSGANLNL.

(D–G) These CEA-specific CD8$^+$ T cells were analyzed for (D) surface marker expression and (E) IFN-γ production determined by intracellular staining after 4 hr restimulation by PMA/ionomycin. CEA-specific CTLs isolated 2 weeks after DNA vaccination with pGT64 CEA plasmid served as positive control. Analysis of granzyme B mRNA (F) and protein (G) in OT-I T cells after coculture with OVA-loaded LSECs or splenic, matured DCs in vitro. One of three independent experiments is shown.

(H) Antigen-specific cytotoxicity; naive T cells cultured on LSECs in absence of OVA served as control. Mean ±SD is shown. Data are representative of three independent experiments.

(I) Principal component analysis based on most variably expressed genes (n = 616 genes, FDR 10%, $p < 0.000005$) in naive (n = 5), liver-primed T cells (n = 4), or matured DC-primed (n = 5) OT-I T cells (d4 after in vitro priming).

(J) Following transfer of naive CD90.1$^+$ OT-I T cells (10^6 cells/mouse), detection of granzyme B protein expression 18 hr after injection of LPS-free OVA (400 μg/mouse) or PBS in CD90.1$^+$ T cells in the livers of (bm1 → C57BL/6) mice or C57BL/6 mice. Representative flow cytometric graphs of granzyme B compared to isotype control and (I) percentage of granzyme B$^+$ among CD8$^+$CD90.1$^+$ OT-I T cells.

Data are representative of two to five independent experiments (A–H, and J and K).

See also Figure S1.

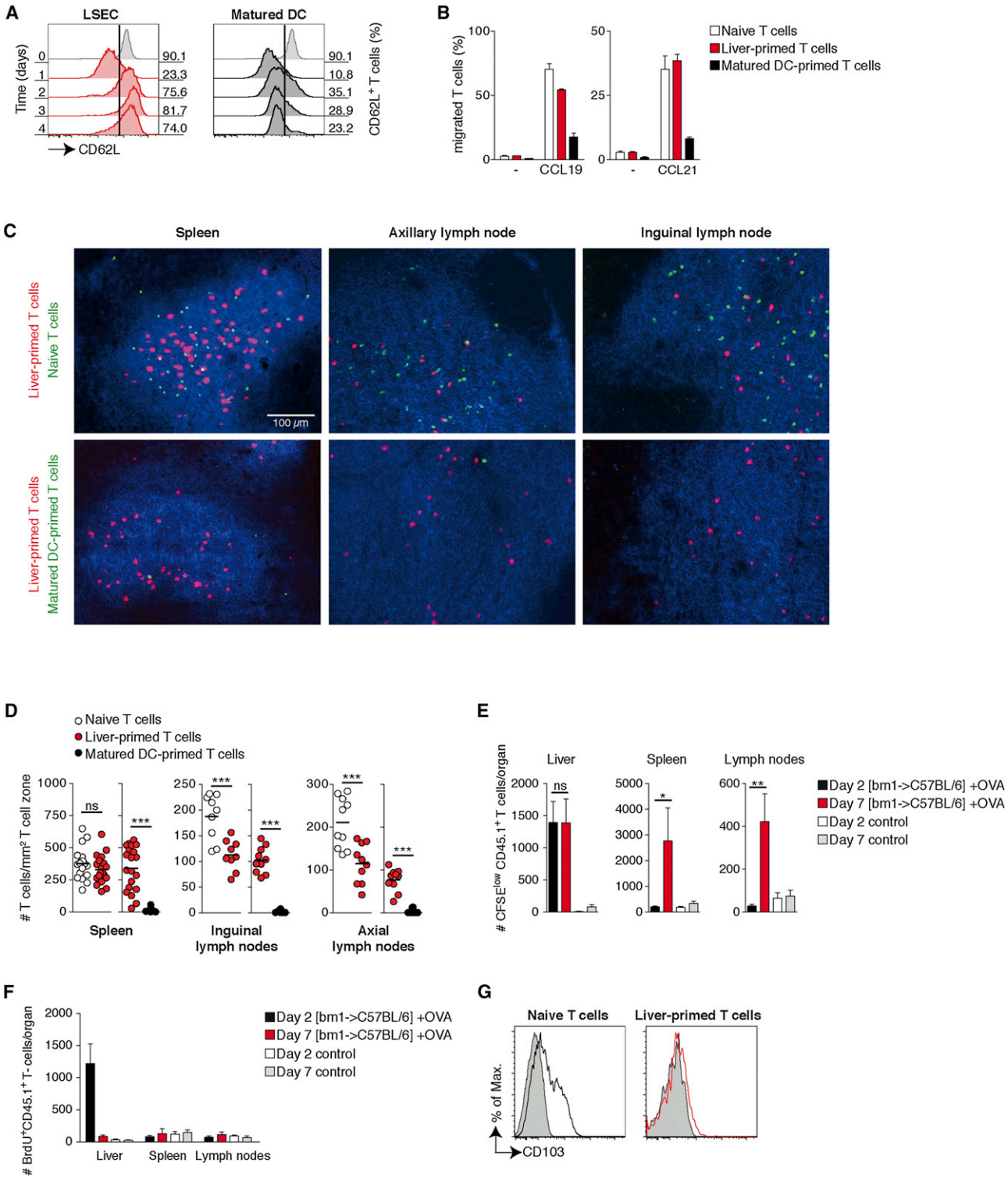

Figure 2. Liver-Primed CD8$^+$ T Cells Re-express CD62L and Migrate to Secondary Lymphoid Organs

(A) Time kinetics of CD62L expression by OT-I T cells primed in vitro by LSECs or matured DCs. Numbers on the right y axis show percentage of CD62L$^+$ T cells.

(B) Migration of OT-I T cells toward CCL19 or CCL21 in a transwell assay.

(C and D) After T cell priming in vitro, 1:1 ratios of differentially labeled liver-primed T cells (red) and naive OT-I T cells (green) or liver-primed (red) and OT-I T cells primed by mature DCs (green) (total of 5 × 10^6 cells/animal) were adoptively transferred into C57BL/6 mice. (C) T cell detection in the T cell zones of secondary lymphoid organs identified by anti-CD3 staining (blue). One representative image of at least ten T cell zones per organ is shown.

(legend continued on next page)

granzyme B is important for antigen-specific CTL cytotoxicity (Barry and Bleackley, 2002). We found that T cells primed by matured DCs that developed into effector CTLs expressed granzyme B and showed prominent cytotoxicity 4 days after priming (Figures 1F–1H). Liver-primed T cells neither expressed granzyme B nor showed cytotoxicity at this time point (Figures 1F–1H) consistent with our previous findings (Limmer et al., 2000). However, at day 1 (d1) after priming liver-primed T cells strongly expressed granzyme B and were highly cytotoxic (Figures 1F–1H). This indicates that liver-primed T cells transiently became effector cells before CTLs primed by matured DCs gained their full effector capacity, but subsequently lost their ability to elicit direct effector function upon TCR triggering (see Figure 1C). Global gene expression profiling followed by principle component analysis substantiated that liver-primed T cells were different from both naive T cells and CTLs primed by matured DCs (Figure 1I).

To characterize whether naive T cell stimulation by cross-presenting LSECs occurred also during direct competition with priming by immature DCs in vivo, we challenged wild-type C57BL/6 mice after naive CD90.1$^+$ OT-I T cell transfer with LPS-free OVA, where both LSECs and immature DCs had the chance to interact with naive OT-I T cells. Eighteen hours after OVA application, we detected a significant population of CD90.1$^+$ granzyme B$^+$ T cells in the liver (Figure 1J), suggesting that granzyme B expression was induced by LSECs. Induction of granzyme B in T cells through cross-presenting LSECs was confirmed in chimeric mice (Figure 1J). The percentage of granzyme B$^+$ cells among total CD90.1$^+$ OT-I-derived T cells was similar in OVA-challenged chimeric and C57BL/6 mice (Figure 1K), indicating that competition with cross-presenting DCs did not modify the ability of LSECs to interact with naive CD8$^+$ T cells and to induce granzyme B expression. Taken together, these results identify a third way of T cell priming in the absence of inflammation where T cells are not deleted but undergo a temporary activation phase without differentiating into effector CTLs. Because liver-primed T cells expressed the lymphoid homing receptor CD62L, which is employed by naive T cells and a subset of memory T cells (central memory T cells [TCMs]) to localize to lymphatic tissue, we next investigated whether liver-primed T cells migrated into spleen and lymph nodes.

Lymphoid Homing of Liver-Primed T Cells

Time kinetic analysis revealed a rapid re-expression of CD62L in liver-primed T cells but not in CTLs primed by immunogenic matured DCs in vitro (Figure 2A). Because T cell entry into lymph nodes requires CD62L and migration toward the chemokines CCL19/CCL21 (Förster et al., 1999), we next investigated their chemotactic behavior. Both liver-primed T cells and naive T cells migrated efficiently toward CCL19/21 in a transwell assay, whereas effector T cells primed by matured DCs did not (Figure 2B).

To address the relevance of these findings for in vivo migration, we directly compared liver-primed T cells to naive T cells or effector CTLs primed by matured DCs by cotransfer of differentially fluorochrome-labeled cells. Liver-primed T cells similar to naive CD8$^+$ T cells migrated to lymphatic tissue and localized to the T cell zones in lymph nodes and spleen, whereas effector CTLs did not locate to the same compartment (Figures 2C and 2D). To verify T cell migration to lymphatic organs after in vivo priming by LSECs, we used the chimeric mouse model, where cross-presentation is restricted to LSECs (see Figure S1; von Oppen et al., 2009). We compared the numbers of proliferating (CFSE^{low}) OT-I-derived CD45.1$^+$ T cells from d2 with those at d7 after in vivo priming by LSECs cross-presenting OVA. Whereas the numbers of CD45.1$^+$ CFSE^{low} T cells in the liver did not differ between d2 and d7, there was an increase of CD45.1$^+$ CFSE^{low} T cells in spleen and lymph nodes at d7 (Figure 2E). Bromodeoxyuridine (BrdU) incorporation was mainly observed in CD45.1$^+$ T cells in the liver at d2 after OVA challenge, whereas at later time points or in CD45.1$^+$ T cells in lymphatic tissue little if any BrdU incorporation was observed (Figure 2F). This indicates relocation of T cells to lymphatic tissues after local priming in the liver. Consistent with egress of liver-primed T cells from the liver in vivo, we further observed downregulation of CD103, which is involved in retention of T cells within peripheral organs (Figure 2G). Thus, priming by LSECs in the liver under noninflammatory conditions does not cause T cell deletion but generates an antigen-experienced T cell population that enters lymphatic tissues early after priming.

Memory-like Properties of Liver-Primed T Cells

Among the T cells primed initially by matured DCs during inflammation, some express CD62L and locate to lymph nodes such as early TCMs, which then increase in numbers over time (Harty and Badovinac, 2008; Wakim and Bevan, 2010). As liver-primed T cells rapidly re-expressed CD62L after priming and localized to lymphatic tissue, we reasoned that they might have a similar distribution to TCMs in vivo and directly compared lymphatic tissue localization of OT-I-derived T cells at d7 after priming by LSECs with that of OT-I-derived TCMs or effector memory T cells (TEMs) at >d45 postinfection with *Listeria monocytogenes* (*L.m.*)-OVA. Liver-primed T cells localized to lymph nodes and spleen similar to TCMs but not TEMs (Figure 3A). This led us to examine whether liver-primed T cells share further characteristics with TCMs.

Memory T cell generation and differentiation is regulated by complex transcriptional programs (Angelosanto and Wherry, 2010; Rutishauser and Kaech, 2010), in which the two T-box

(E and F) Quantification of T cell localization within T cell zones in lymph nodes and spleen (E). Adoptive transfer of 1 × 10^6 naive CFSE-labeled CD45.1$^+$ OT-I T cells into (bm1 → C57BL/6) chimeric animals or C57BL/6 mice and injection of soluble OVA into (bm1 → C57BL/6) or PBS into C57BL/6 mice (control). At d2 or d7 after injection of OVA (E), total numbers of liver-primed (CFSE^{low}) CD45.1$^+$ OT-I T cells and (F) total numbers of proliferating CD45.1$^+$ T cells assessed by BrdU incorporation were analyzed in liver, spleen, and inguinal lymph nodes. BrdU was injected i.p. 20 hr before analysis on d2 or d7, respectively.

(G) Naive CD45.1$^+$ OT-I T cells or CD45.1$^+$ OT-I T cells isolated from liver 3 days after priming by LSECs in (bm1 → C57BL/6) chimeric animals were analyzed for CD103 expression versus appropriate isotype controls (gray shaded area).

Data are representative of at least two separate experiments with three to four mice per group (C–F) are shown; ns, not significant.

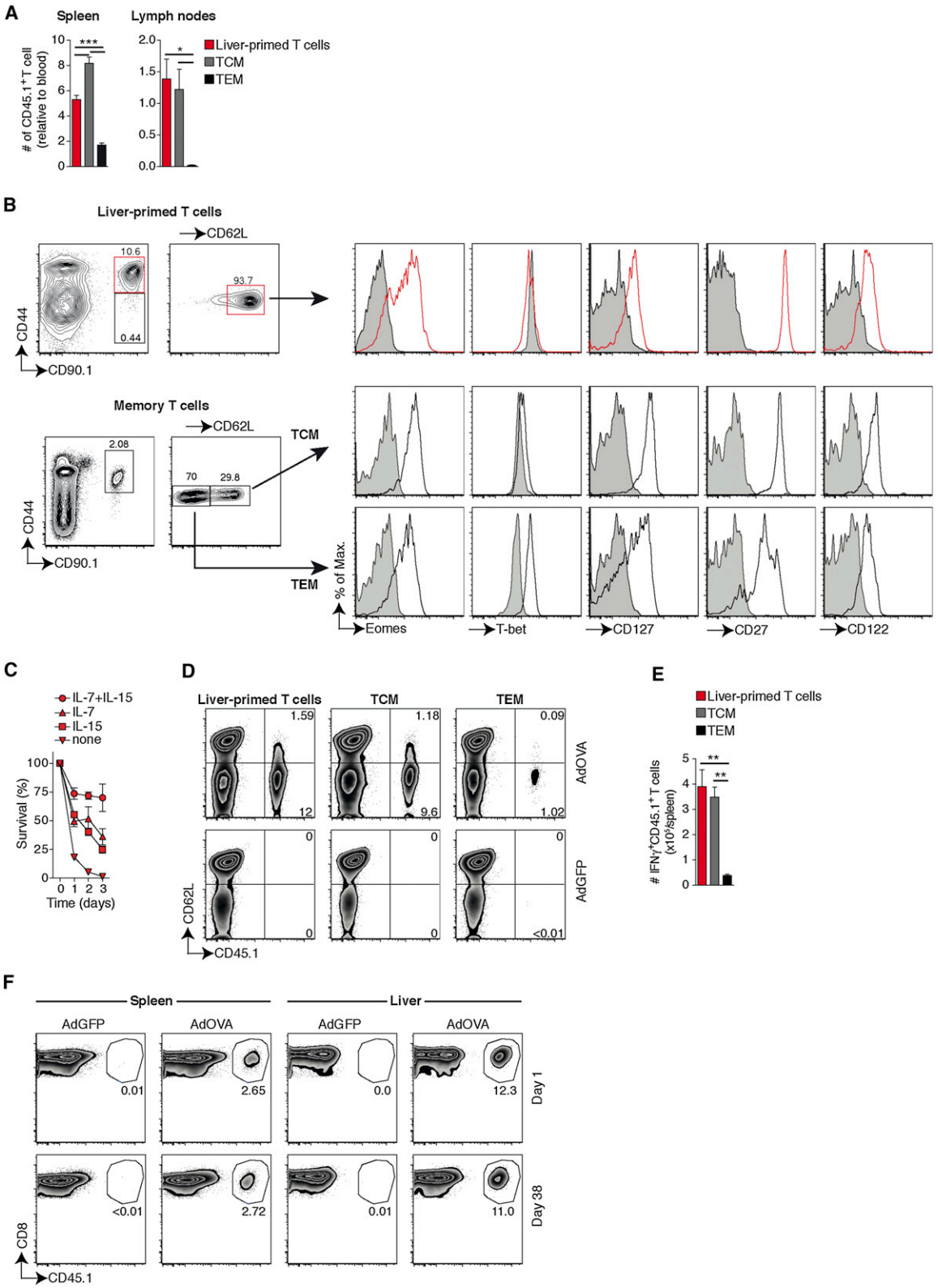

Figure 3. Liver-Primed T Cells Have Memory-like Functions and Differentiate into Effector T Cells upon Infection

(A) In vivo distribution of CD45.1$^+$ OT-I T cells 7 days after priming by LSECs in (bm1 → C57BL/6) chimeric animals or > d45 after *L.m.*-OVA infection in C57BL/6 mice.

(legend continued on next page)

transcription factors T-bet and Eomesodermin (Eomes) play key roles (Intlekofer et al., 2005; Rao et al., 2010). We found that liver-primed T cells similar to TCMs expressed high levels of Eomes (Figure 3B). However, whereas TCMs expressed T-bet at low levels, liver-primed T cells completely lacked T-bet expression when examined ex vivo (Figure 3B) or in vitro (Figure S2A). T cell factor 1 (TCF-1), a transcription factor required for memory T cell differentiation that controls Eomes expression (Zhou et al., 2010), was expressed at high levels in liver-primed T cells compared to effector T cells primed by matured DCs (Figure S2B). Furthermore, Bcl-6, a transcription factor that is important for memory T cell differentiation (Ichii et al., 2002), was upregulated in liver-primed T cells compared to naive T cells (Figure S2C). In contrast, the transcription factor Blimp-1 that is associated with effector cell differentiation (Kallies et al., 2009; Rutishauser et al., 2009) and T cell exhaustion (Shin et al., 2009) was not expressed in liver-primed T cells (Figure S2B). Finally, liver-primed T cells expressed the cytokine receptors CD127, CD122, and CD27 (Figure 3B) and responded with increased survival to signaling from the γc-cytokines IL-7/IL-15 in vitro (Figure 3C). There were no major differences in the phenotypic profile, frequency of granzyme B^+, or IFN-γ-producing liver-primed T cells isolated from the liver compared to those isolated from the spleen of chimeric mice at d7 after OVA application (Figure S3), suggesting that the surface and functional phenotype remained stable in liver-primed T cells irrespective of their tissue localization. Thus, there is remarkable similarity between liver-primed T cells and TCMs in the expression of transcription factors known to be associated with memory T cell differentiation and their ability to respond to survival signals.

Like Central Memory T Cells, Liver-Primed T Cells Can Be Reactivated and Generate Effector CTLs during Infection

The absence of T-bet expression on d4 in liver-primed T cells may explain their lack of direct effector function upon isolated TCR triggering (Cruz-Guilloty et al., 2009). The similarities to TCMs in expression of transcription factors determining memory T cell differentiation and IFN-γ production upon reactivation by matured DCs (see Figure 1C) indicated that liver-primed T cells might not be terminally committed to their nonfunctional state but possess the potential to give rise to effector T cells (Intlekofer et al., 2007; Joshi et al., 2007; Rutishauser and Kaech, 2010). To test for such plasticity, we adoptively transferred either $CD45.1^+$ liver-primed OT-I T cells sorted from the spleen of (bm1 → C57BL/6) chimeric mice or splenic OT-I-derived TEMs or TCMs sorted from mice at d45 postinfection with *L.m.*-OVA into $CD45.2^+$ recipients and infected them with an adenovirus expressing OVA (AdOVA). We excluded transfer of contaminating naive T cells within liver-primed T cells by FACSorting for $CD44^{hi}CD62L^{hi}$ T cells that had proliferated ($CFSE^{low}$) (Figure S3D). Five days after AdOVA infection, liver-primed T cells expanded extensively, downregulated CD62L, and produced IFN-γ upon restimulation (Figures 3D and 3E). This recall response was antigen specific and did not occur after AdGFP infection excluding bystander activation of transferred cells due to virus-induced inflammation (Figure 3D). The magnitude of the recall response generated by liver-primed T cells within 5 days after initial infection was similar to that elicited by the same number of transferred TCMs (Figure 3E). As expected, TEMs did not generate a prominent recall response (Bouneaud et al., 2005; Wherry et al., 2003). Even when liver-primed T cells remained without antigen contact for 38 days in vivo, we observed effector T cell generation following AdOVA infection (Figure 3F), pointing to another similarity with memory T cells, i.e., survival in the absence of antigen. These results demonstrate that liver-primed T cells were not terminally committed to their nonresponsive state that was initially reported (Diehl et al., 2008; Limmer et al., 2000; Schurich et al., 2010) in contrast to self-antigen specific tolerant T cells that retain their nonfunctional state even during infection (Schietinger et al., 2012). Priming by LSECs rather induced a differentiation state in T cells similar to TCMs where they gained the potential to generate effector T cell responses.

Molecular Requirements for Effector CTL Generation from Liver-Primed T Cells

Our results raised the question how reactivation of liver-primed T cells was achieved. The fact that liver-primed T cells produced IFN-γ after stimulation by antigen-presenting matured DCs (see Figure 1C) indicated that signals beyond TCR stimulation were necessary for their activation. We therefore introduced costimulatory signals for reactivation of liver-primed T cells and substituted matured DCs with αCD3/28-coated beads as artificial APCs combined with different proinflammatory cytokines. Liver-primed T cells produced IFN-γ upon reactivation with combinatorial stimulation with αCD3/28+IL-12 but not any other combination thereof (Figure 4A). Costimulation by IL-12 could be partially replaced by IFN-α but not other cytokines such as IL-2 (Figures S4A and S4B), demonstrating a fundamental difference of liver-primed T cells to IL-2-responsive anergic T cells (Schwartz, 2003). In order to determine whether IL-12 and potentially type I IFN mediated the reactivation of liver-primed T cells through matured DCs (see Figure 1C), we used antagonistic antibodies during coculture of matured DCs with liver-primed T cells.

(B) Analysis of surface marker expression and intracellular staining of T-box transcription factors of in-vivo-generated $CD90.1^+$ liver-primed T cells, TCMs, and TEMs. Representative flow cytometric analyses from at least four independent experiments with three to five mice per group are shown.
(C) In-vitro-generated liver-primed T cells were obtained at d4 and incubated with IL-7 (10 ng/ml) and/or IL-15 (10 ng/ml) and analyzed for T cell survival over time. Error bars show mean ±SD. Data are representative of three independent experiments.
(D and E) Wild-type C57BL/6 mice were challenged with AdOVA or AdGFP (5×10^6 pfu/mouse i.v.) after adoptive transfer of 1,000 in-vivo-generated and FACSorted $CD45.1^+$ OT-I-derived liver-primed OT-I cells, TCMs, or TEMs (see Figure S3D). Seven days after AdOVA or AdGFP infection, splenic $CD8^+$ T cells were analyzed for $CD45.1^+CD62L^-$ effector OT-I cells and total numbers of IFN-γ-producing $CD45.1^+$ OT-I cells after 4 hr of peptide restimulation.
(F) Transfer of 5×10^3 in-vivo-generated $CD45.1^+$ liver-primed OT-I cells and challenge with AdOVA or AdGFP (5×10^6 pfu/mouse) at d1 or d38 posttransfer. The frequency of $CD45.1^+CD8^+$ T cells was determined in spleen or liver at d5 after infection.
See also Figures S2 and S3.

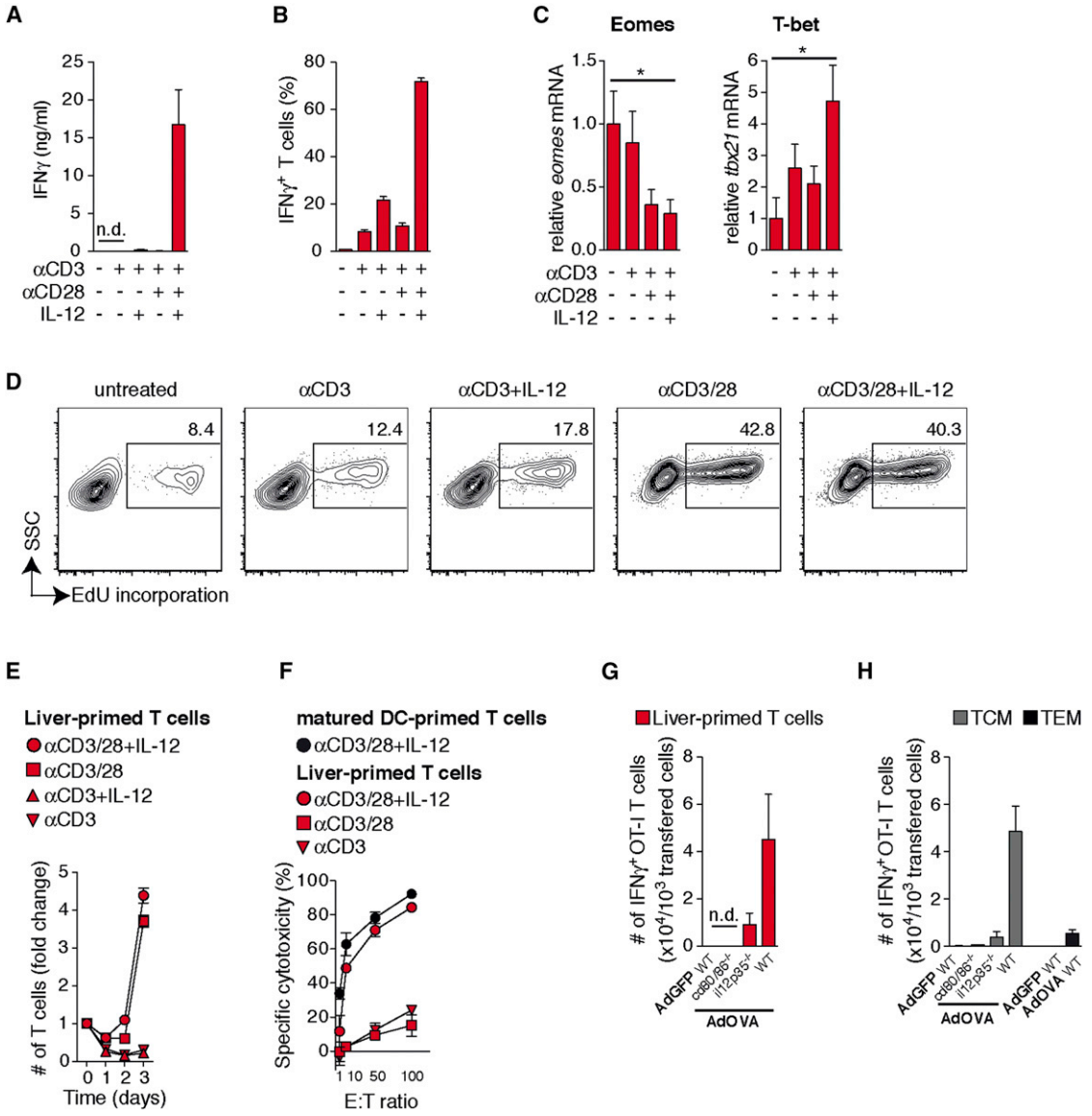

Figure 4. Combinatorial Stimulation of Liver-Primed T Cells Is Necessary for Effector T Cell Generation

(A–F) In-vitro-generated liver-primed T cells (d4 after priming) were analyzed for effector T cell functions after stimulation. Data are representative of three to five independent experiments. (A and B) Combinatorial stimulation for 20 hr and determination of IFN-γ expression in supernatant by ELISA (A) or frequency of IFN-γ-producing cells after additional 4 hr restimulation by PMA/ionomycin (B). (C) In-vitro-generated liver-primed T cells were stimulated with αCD3, αCD3/28, or αCD3/28+IL-12 for 10 hr, and total mRNA was isolated and analyzed by semiquantitative RT-PCR for expression levels of *tbx21* and *eomes*. Data show mean ±SEM of four independent experiments. (D) In-vitro-generated liver-primed T cells were stimulated as indicated and supplied with EdU-containing medium, and 20 hr later living T cells were analyzed for EdU incorporation. Representative data from one of three independent experiments are shown. (E) T cell expansion over time and specific cytotoxicity analyzed 72 hr after indicated in vitro stimulation (F).

(G and H) Transfer of identical numbers of in-vivo-generated OT-I-derived liver-primed T cells (G) or identical numbers of TCMs obtained >d45 after *L.m.*-OVA infection (H) into wild-type, *cd80/86*$^{-/-}$, or *il12p35*$^{-/-}$ mice followed by AdOVA challenge. TEMs from the same animals served as a control in (H). Five days postinfection, total numbers of OT-I T cells in spleen that produced IFN-γ after PMA/ionomycin stimulation relative to 1,000 initially transferred OT-I T cells. Data are representative of two independent experiments with three to four mice per group. n.d., not detected.

See also Figure S4.

Clearly, blockade of IL-12 or the type I IFN receptor (IFNAR1) reduced IFN-γ expression in liver-primed T cells cocultured with matured DCs, whereas only blockade of type I IFN impaired the generation of granzyme B^{+} CTLs (Figures S4C and S4D). In addition to the crucial role of IL-12 and type I IFN, further costimulatory molecules or cytokines known to induce IFN-γ expression and CTL effector function may also be involved.

To analyze how many liver-primed T cells gained the capacity to produce IFN-γ after combinatorial stimulation, we challenged these cells with PMA/ionomycin. Under these conditions, more

than 70% of liver-primed T cells reactivated initially by αCD3/28+IL-12-produced IFN-γ (Figure 4B). The development into cytokine producing cells did not depend on TCR affinity, because liver-primed T cells bearing a high (OT-I T cells) or intermediate (DES-TCR) affinity TCR produced IFN-γ equally well after combinatorial stimulation (data not shown). The combinatorial stimulation led to upregulation of *tbx21* (T-bet) and downregulation of *eomes* mRNA in liver-primed T cells (Figure 4C), suggesting the onset of effector CTL generation (Takemoto et al., 2006). Hence, we investigated the relevance of these signals for T cell expansion and gain of CTL effector function. Whereas αCD3/28 stimulation sufficed for proliferation and expansion of liver-primed T cells (Figures 4D and 4E), acquisition of CTL effector function required additional IL-12 signaling (Figure 4F). CD28 and IL-12 signaling were also essential for reactivation of liver-primed T cells in vivo during viral infection, because liver-primed T cells did not generate effector CTLs after transfer into cd80/86$^{-/-}$ or il12p35$^{-/-}$ mice (Figure 4G). A similar dependence on combinatorial stimulation for generation of CTLs was observed during bacterial infection with *Listeria* (Figure S4E). The emergence of some effector CTLs detected after transfer of liver-primed T cells into il12p35$^{-/-}$ mice may be explained by the compensatory function of type I IFN, which is likely elicited during viral infection and can partially replace IL-12.

We expected that TCMs unlike liver-primed T cells would not need costimulatory signals for effector T cell generation upon antigen re-encounter. However, TCMs adoptively transferred into cd80/86$^{-/-}$ or il12p35$^{-/-}$ mice failed to give rise to IFN-γ producing effector T cells after viral infection (Figure 4H). Adoptively transferred TEMs hardly expanded, as expected (Bouneaud et al., 2005). These results demonstrate that simultaneous stimulation through the TCR, CD28, and IL-12R is necessary for the development of effector CTLs from both liver-primed T cells and TCMs during microbial infection.

Liver-Primed T Cells Constitute a Distinct Antigen-Experienced CD8$^+$ T Cell Population

Our results demonstrate that TCMs and liver-primed T cells are similar with respect to their antigen-experienced state, localization to lymphatic tissues, and activation requirements for generation of effector CTLs. However, these cells were generated under fundamentally different situations: TCMs by matured DCs under inflammatory conditions in lymphatic tissue and liver-primed T cells under noninflammatory conditions in the liver. This raised the question how these T cell populations differed at the molecular level. We therefore compared these cell populations after FACSorting ex vivo at the global gene expression level. The global transcriptome was determined in naive CD8$^+$ OT-I T cells, liver-primed OT-I T cells, and OT-I-derived TCMs and TEMs obtained >d45 after *L.m.*-OVA infection and in exhausted virus-specific CD8$^+$ T cells isolated from mice suffering from chronic infection with the lymphocytic choriomeningitis virus (LCMV). Unbiased principal component analysis revealed that liver-primed T cells had a distinct gene expression profile that separated them from memory T cells (Figure 5A). They were also different from exhausted T cells (Figure 5A), and their genetic signature also showed no similarities to that of CD8$^+$ T cells undergoing deletion (Table S1) (Parish et al., 2009). There was a remarkable similarity in the regulation of genes between T cells primed by LSECs in vitro and those primed in the chimeric mice in vivo (Figure S5). This supports the notion that priming of naive T cells in vivo in the chimeric mouse was indeed performed by LSECs. Priming of naive CD8$^+$ T cells in vitro by Kupffer cells or liver dendritic cells leads to T cells with distinct functional and phenotypic properties (data not shown), further strengthening the point that proliferated CD44$^+$CD62L$^+$ CD8$^+$ T cells were initially stimulated by LSECs. In summary, these results establish liver-primed T cells as a distinct antigen-experienced CD8$^+$ T cell population.

The different global gene expression profile between liver-primed T cells and TCMs raised the question whether the observed functional similarities between liver-primed T cells and TCMs were reflected at the level of gene expression. We therefore directly compared the core gene expression signature related to memory T cell function (Wirth et al., 2010) with that of liver-primed T cells and TCMs. We found that TCMs isolated >d45 after *L.m.*-OVA infection showed similar regulation of approximately 33% of these core memory signature genes (Table S2). For those genes specifically regulated in TCMs, a similar regulation was detected in liver-primed T cells for 29 of 68 (43%) upregulated genes and 12 of 30 (40%) downregulated genes (Figure 5B; Table S2). Although these genes were significantly regulated in liver-primed T cells, their range of regulation was less pronounced compared to TCMs (Figure 5B; Table S2), indicating that beyond the similarities observed there are clear differences between these T cell populations.

KEGG pathway analyses using DAVID bioinformatics (Huang et al., 2009) revealed significant upregulation of the pathways for cytotoxicity, cytokine signaling, and TCR signaling in TCMs compared to liver-primed T cells (Table S3). In particular, TCMs showed upregulation of IFN-γ, FASL, and IL-18R1, which are all related to direct T cell effector function. This suggested that TCMs, in contrast to liver-primed T cells, directly exert effector function upon stimulation without the need to first develop into effector cells. Indeed, TCMs produced IFN-γ as efficiently as TEMs within 4 hr after stimulation with peptide or PMA/ionomycin (Figure 5C). Moreover, both TCMs and TEMs showed strong antigen-specific CTL effector function ex vivo (Figure 5D). As predicted from KEGG pathway analysis liver-primed T cells neither produced IFN-γ nor displayed antigen-specific CTL effector function upon such stimulation (Figures 5C and 5D), confirming the results obtained in vitro (see Figure 2). These results demonstrate that unlike memory T cells, liver-primed T cells are in a distinct differentiation state where they lack direct CTL effector function but possess TCM-like plasticity to give rise to effector T cells.

Neuropilin-1 Is a Potential Marker for Liver-Primed CD8$^+$ T Cells

As conventional markers for identification of antigen-experienced T cells did not suffice to distinguish between TCMs and liver-primed T cells (see Figure 3), we employed biolayout cluster analysis from the gene expression profiles of naive T cells, liver-primed T cells, TCMs, and TEMs to search for markers specific for liver-primed T cells. We identified clusters that contained 266 genes exclusively regulated in liver-primed T cells

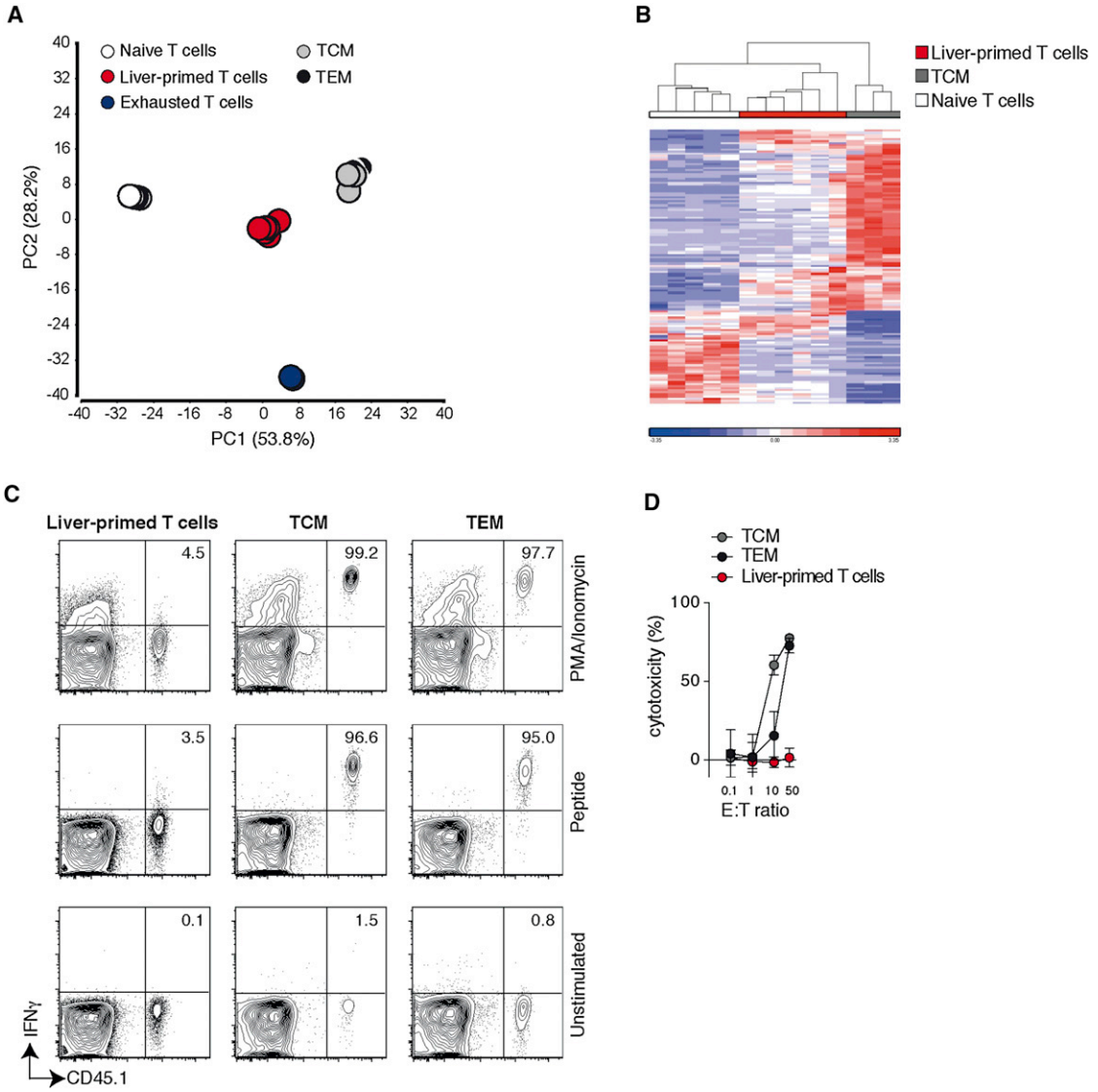

Figure 5. Liver-Primed T Cells Constitute a Distinct Antigen-Experienced CD8$^+$ T Cell Population

(A) Principal component analysis based on most variably expressed genes (n = 671 genes, FDR, $p < 0.000005$) in naive OT-I T cells (n = 5), OT-I-derived liver-primed T cells (d4–6 after in vivo priming, n = 6), exhausted LCMV-specific CD8$^+$ T cells (n = 2), and OT-I-derived TCMs (n = 3) or TEMs (n = 3) at d45–60 after *L.m.*-OVA infection.

(B) Regulation of TCM-specific genes (see Table S2) in liver-primed T cells. Genes were considered differentially expressed by the following criteria: FC ≥ 2, $p < 0.05$, difference of means >100 and passing 10% FDR.

(C and D) CD45.1$^+$ liver-primed T cells (d4 after in vivo priming), TCMs, and TEMs (>d45 postinfection) were FACSorted. (C) Restimulated with peptide or PMA/ionomycin on CD45.2$^+$ splenic feeder cells, IFN-γ production was measured by intracellular staining 4 hr later. (D) Specific cytotoxicity directly ex vivo. Representative data from at least two experiments with three to four mice per group are shown.

See also Figure S5 and Tables S1, S2, and S3.

(Table S4). The highest upregulated genes within these clusters (>4-fold change in liver-primed T cells compared to naive T cells) are involved in transcriptional regulation (e.g., Ear2, Zbtb32), signaling (e.g., axl), cytoskeletal organization (e.g., Anxa2, Synpo), are expressed as cell-surface molecules (e.g., Fc receptors, Sirpa, Nrp1), or function as transporter molecules (e.g., Slc40a1-ferroportin). Our analysis identified Neuropilin-1 (Nrp1) as the surface molecule with the highest differential expression on liver-primed T cells (Figure 6A; Table S4). Nrp1 was readily detectable at the protein level on liver-primed T cells with only very low-level expression on TCMs and TEMs (Figure 6B). This suggested that Nrp1 staining may be used to differentiate between liver-primed T cells and TCMs within the total population of antigen-experienced T cells. We therefore used this marker to investigate whether Nrp1$^+$ cells were present among splenic CD44$^+$CD62L$^+$CD8$^+$ T cells in C57BL/6 mice with a

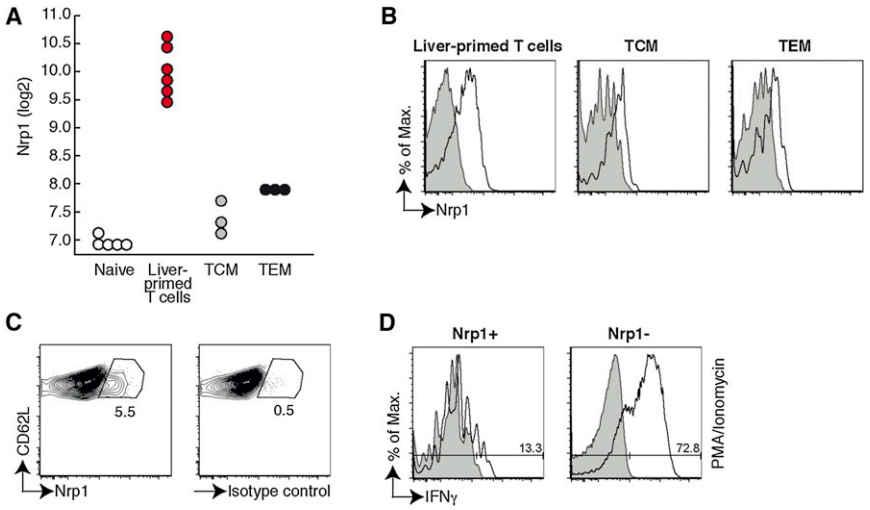

Figure 6. Nrp1 Is a Marker for Liver-Primed T Cells

(A) Analysis of Neuropilin-1 (Nrp1) mRNA expression based on global gene expression data.

(B) Surface staining for Nrp1 on OT-I-derived TCMs, TEMs, and liver-primed T cells compared to isotype control (shaded area).

(C) Frequency of Nrp1$^+$ T cells among CD44$^+$ CD62L$^+$CD8$^+$ T cells in the spleen of wild-type C57BL/6 mice.

(D) Functional characteristics of Nrp1$^+$ and Nrp1$^-$ T cells assessed by IFN-γ production in response to PMA/ionomycin stimulation within CD44$^+$CD62L$^+$CD8$^+$ T cells obtained from the spleen of normal mice. Data are representative for at least two independent experiments.

See also Table S4.

normal TCR repertoire. Approximately 3%–5% of these antigen-experienced CD62L$^+$ T cells expressed Nrp1 (Figure 6C). Similar to liver-primed T cells, Nrp1$^+$ T cells sorted from splenic CD44$^+$CD62L$^+$CD8$^+$ T cells in C57BL/6 mice were nonresponsiveness to PMA/ionomycin stimulation ex vivo and therefore lacked direct effector function, whereas Nrp1$^-$ T cells directly produced IFN-γ (Figure 6D). While Nrp1 has been identified as marker for CD4$^+$ T cells with regulatory function (Bruder et al., 2004; Sarris et al., 2008), expression of Nrp1 on a particular CD8$^+$ T cell populations has not been reported so far. Our marker analysis and the functional similarities with liver-primed T cells indicate that Nrp1 identifies a sizeable population of antigen-experienced CD44$^+$CD62L$^+$CD8$^+$ T cells in normal mice that was presumably generated from naive T cells by LSECs.

Liver-Primed T Cells Contribute to Anti-infectious Immunity

The characteristics of liver-primed T cells reported here raised the question whether they contribute to anti-infectious immunity. To monitor antiviral activity of liver-primed T cells, we used a model where CTL effector function against luciferase-expressing target cells in the liver is visualized by a decrease of in vivo bioluminescence (Stabenow et al., 2010). We adoptively transferred equal numbers of OT-I-derived liver-primed or (matured) DC-primed effector CTLs into mice that were infected with recombinant adenovirus expressing a fusion protein of OVA and luciferase. Mice supplied with liver-primed OT-I T cells efficiently controlled luciferase expression within 7 days (Figures 7A and 7B), which is consistent with development of OVA-specific effector CTLs from liver-primed T cells that controlled AdOVA infection in the liver. In contrast, control animals that did not receive any liver-primed T cells failed to control luciferase expression. As expected, adoptive transfer of effector OT-I T cells primed by matured DCs in vitro controlled luciferase expression even more rapidly (Figures 7A and 7B). Liver-primed T cells not only controlled viral infection with a replication-defective adenovirus, but also controlled the bacterial load during infection with replication-competent *L.m.*-OVA (Figure 7C). These results demonstrate that liver-primed T cells participate through the generation of effector CTLs in the development of adaptive immunity against infectious pathogens.

Pathogens can escape innate immunity and some viral infections cause systemic dissemination of viral antigens in the absence of concomitant systemic inflammation (Protzer et al., 2012). It is possible that these pathogens also escape adaptive immunity as antigen-presentation of pathogen-derived antigens by immature DCs likely causes deletion of pathogen-specific T cells. LSEC priming did not induce T cell elimination and thereby prevented deletion of T cells by immature DCs (see Figure 1). However, would LSEC priming also render T cells resistant to subsequent deletion by antigen encounter on immature APCs in a situation of systemic antigen dissemination without inflammation? To address this issue, we transferred the same number of naive or liver-primed CD45.1$^+$ OT-I T cells into C57BL/6 mice and injected DEC205-OVA to induce cross-presentation by immature DCs. When naive T cells encountered their antigen on immature DCs, we observed significantly reduced T cell numbers after AdOVA challenge (Figure 7D). In contrast, liver-primed OT-I T cells were not reduced in response to AdOVA after exposure to immature DCs cross-presenting OVA (Figure 7D). Thus, priming by LSECs induced a differentiation state in T cells that protects them from deletion during subsequent antigen encounter on tolerogenic immature DCs.

This led us to further investigate the consequences for antiviral immunity when systemically distributed antigen is presented first under noninflammatory conditions to naive CD8$^+$ T cells by either LSECs or immature DCs. To this end, identical numbers of naive CD45.1$^+$ OT-I T cells (5×10^5) were transferred into mice where either LSECs or immature DCs cross-presented OVA (scheme see Figure S6A). From the spleens of these mice, similar numbers of total endogenous CD8$^+$ T cells (including proliferated CD45.1$^+$ cells; Figure S6B) were transferred into RAG2$^{-/-}$ recipient mice, which allows us to study the potential of very low numbers of CD45.1$^+$ T cells to generate antiinfectious immunity undisturbed from the endogenous TCR repertoire in the recipient

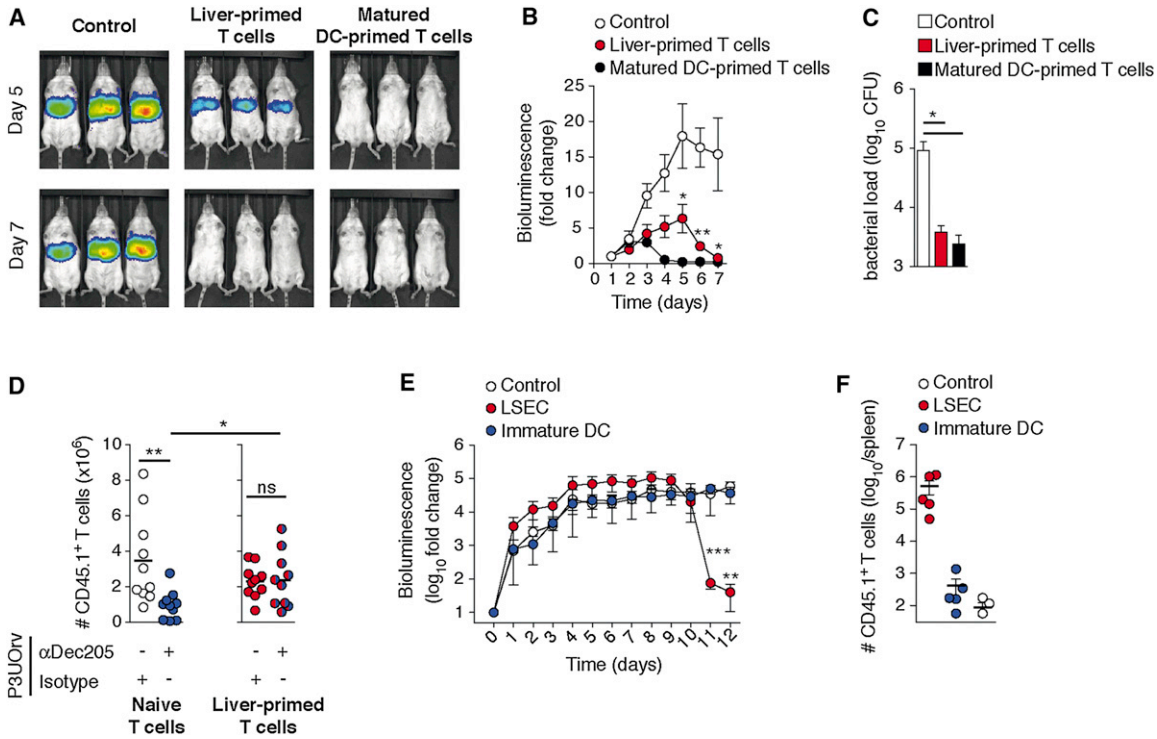

Figure 7. Liver-Primed T Cells Contribute to Anti-infectious Immunity

(A and B) liver-primed OT-I cells or OT-I T cells primed by matured DCs were adoptively transferred into congenic C2J mice that were infected with 5×10^5 AdOVA-LUC 1 day before (5×10^5 T cells/mouse). In vivo bioluminescence was determined as a measure of effector T cell function against virus-infected luciferase-expressing hepatocytes. Representative in vivo bioluminescence images from d5 and d7 postinfection (A) and quantification over time (B).

(C) Effect of 1×10^6 adoptively transferred liver-primed OT-I cells or DC-primed OT-I T cells on bacterial load in liver at d4 after infection with *L.m.*-OVA.

(D) $CD45.1^+$ naive OT-I T cells (1×10^5) or liver-primed OT-I T cells were adoptively transferred ex vivo into congenic $CD45.2^+$ recipients that were subsequently challenged with OVA coupled to anti-DEC205 (DEC205-P3UOrv) or isotype control antibody (ratIgG2a-P3UOrv). Twelve days later, animals were infected with AdOVA and total numbers of $CD45.1^+$ cells were determined in spleen 5 days postinfection.

(E and F) $CD45.1^+$ OT-I T cells primed under noninflammatory conditions by LSECs or immature DCs in vivo (see Figure S6) were transferred into $RAG2^{-/-}$ mice challenged with AdOVA-LUC 2 days before. Effector T cell function was measured by bioluminescence imaging over time (E) and T cell expansion was determined in spleen at d13 (F).

Data are representative of at least two independent experiments with three to five mice per group (A–C, E, and F) or pooled from two experiments (D).

See also Figure S6.

mouse. Under these conditions, T cells initially primed by immature DCs failed to show any effect against viral infection, similar to transfer of T cells from C57BL/6 mice that served as control (Figure 7E). This is consistent with T cell deletion following contact with immature DCs. In contrast, initial priming by LSECs cross-presenting circulating antigen allowed T cells to subsequently expand upon re-encounter of antigen during infection and develop immunity against virus-infected hepatocytes, as shown by decrease in bioluminescence and massive T cell expansion in the liver (Figures 7E and 7F). Taken together, our findings reveal a function of the liver under noninflammatory conditions in complementing $CD8^+$ T cell immunity generated during inflammation by matured DCs (see Figure S7).

DISCUSSION

Our results define a memory-like differentiation state in $CD8^+$ T cells primed in the liver that contributes to antimicrobial immunity after reactivation via combinatorial stimulation through CD28 and IL-12. This distinct T cell differentiation state is induced by a unique APC population in the liver, i.e., LSECs, which cross-present circulating antigen that is systemically distributed under nonimmunogenic conditions. We report that naive T cell stimulation by nonimmune cells can support development of immunity rather than tolerance, which points to an as-yet-unrecognized immune function of peripheral organs such as the liver.

The wide expression of immune sensing receptors (e.g., Toll-like receptors or RIG-I) attributes immune competence also to nonimmune cells to induce innate immunity and inflammation. Similar to professional bone-marrow-derived APCs, nonimmune cells can present antigen to $CD8^+$ T cells, but this is believed to lead to immune tolerance via T cell deletion or anergy (Bertolino et al., 1995; Cohen et al., 2010; Fletcher et al., 2010; Gardner et al., 2008; Redmond et al., 2005). In line with these reports, we had previously shown that naive $CD8^+$ T cell stimulation by LSECs cross-presenting circulating antigens results in T cell nonresponsiveness toward stimulation via the TCR (Diehl et al., 2008; Limmer et al., 2000). Here, we provide evidence, however,

that liver-primed T cells stimulated by cross-presenting LSECs are not terminally committed to this nonresponsive state unlike T cells tolerized toward self-antigens (Schietinger et al., 2012). Instead, liver-primed T cells are reactivated from their nonresponsive state and like TCMs give rise to effector CTLs in response to infectious inflammation. This reveals the existence of a memory-like T cell differentiation state, where cells are nonresponsive toward isolated TCR signaling but have memory-like functions to develop into effector CTLs upon TCR stimulation in combination with costimulatory signals vial CD28 and IL-12.

The conditions during which this memory-like T cell differentiation state is induced are fundamentally different from those required for effector and memory $CD8^+$ T cells generation, which depend on appropriate DC maturation via innate immune receptors and subsequent migration to secondary lymphatic tissue where mature DC cross-prime naive T cells (Zhang and Bevan, 2011). In contrast, liver-primed T cells are generated in the liver by non-bone-marrow-derived organ-resident LSECs cross-presenting circulating antigen under nonimmunogenic conditions. Thus, generation of memory-like T cells by LSECs likely complements effector and memory $CD8^+$ T cell generation during antigen dissemination without inflammation, which does not support induction of conventional immunity.

The developmental programs initiated in T cells by antigen-presenting LSECs or matured DCs differed substantially, which is reflected by the transient (liver-primed) versus long-term (mature DC-primed) acquisition of effector cell function and by their largely different gene expression profiles. However, common features between memory T cells and liver-primed T cells are observed. Reduced inflammatory signaling is associated with memory rather than terminal effector cell differentiation (Badovinac et al., 2004, 2005; Cui et al., 2009; Kalia et al., 2010; Pipkin et al., 2010; Sarkar et al., 2008) and cell-intrinsic regulatory STAT3-signaling in $CD8^+$ T cells shields memory precursor cells from inflammatory signals that induce effector CTL differentiation (Cui et al., 2011). Liver-primed T cells are generated through stimulatory signaling via the TCR together with little costimulatory signaling through CD28 in combination with a balanced and dynamically regulated coinhibitory signaling via PD1 that antagonizes stimulatory TCR signals after 24 hr (Diehl et al., 2008; Lohse et al., 1996; Schurich et al., 2010). This supports the assumption that the short-lived TCR signaling and little costimulation provided by LSECs contributed to the induction of the memory-like T cell phenotype similar to memory T cell generation by short-lived inflammation (Cui and Kaech, 2010). Further similarities exist between liver-primed T cells and memory T cells. Activation of the Wnt-β-catenin signaling pathway during T cell activation by matured DCs promotes memory T cell differentiation and blocks terminal effector cell differentiation (Gattinoni et al., 2009). From our gene profiling data, we found TCF1, a transcription factor downstream of the Wnt-β-catenin signaling pathway that is critical for memory T cell differentiation (Zhou et al., 2010), to be 50-fold upregulated in liver-primed T cells compared to T cells primed by matured DCs. Liver-primed T cells also express molecules known to be associated with development of memory T cells such as CD27, IL-7Rα (CD127), IL-2Rβ (CD122), and Eomes (Hendriks et al., 2000; Intlekofer et al., 2005; Kaech et al., 2002a). Liver-primed T cells are $KLRG1^{low}$, express bcl2 (Diehl et al., 2008), and respond to IL-15-dependent survival signals, features reported for TCMs (Harty and Badovinac, 2008). Interestingly, most of the liver-primed T cells share the same phenotype, indicating that they form a homogenous T cell population. This is in contrast to the diverse cell differentiation states induced by matured DCs during infectious inflammation that give rise to terminal effector T cells, TCMs, and TEMs (Chang et al., 2007). Taken together, the development of the distinct $CD8^+$ T cell differentiation state after priming by LSECs shares many similarities with the development of memory T cells, although it occurs under nonimmunogenic conditions in the liver and is executed by nonprofessional liver-resident APCs.

Our phenotypic and functional analyses together with global gene expression profiling and bioinformatic analysis revealed that liver-primed T cells represent a distinct population of antigen-experienced CD8 T cells. Antigen-experienced T cells are grouped into effector T cells and memory T cells, nonresponsive anergic or exhausted T cells. Liver-primed T cells differ from effector T cells as they do not express KLRG1 and lack sustained effector functions. Liver-primed T cells do not respond to stimulation via the TCR or IL-2 and continuously express identical levels of CD8, which discriminates them from $CD8^+$ T cells that have become refractory to stimulation (Mescher et al., 2006) or detuned following recent activation (Xiao et al., 2007). They further differ at the gene expression level from T cells that were exhausted after priming during chronic viral infection (Wherry et al., 2007) and from those undergoing deletion (Parish et al., 2009). In particular, liver-primed T cells do not express high levels of PD1 or TIM-3, molecules associated with transcriptional programming of exhausted T cells (Quigley et al., 2010; Youngblood et al., 2011). While tolerized T cells remain committed to their tolerant state (Schietinger et al., 2012) or are deleted (Kurts et al., 1997), liver-primed T cells survive and can be reactivated. In summary, liver-primed T cells do not share similarities with tolerized or exhausted T cells.

Although gene expression profiling showed that liver-primed T cells are distinct from TCMs and TEMs, liver-primed T cells share more than 40% of similarity with the core gene expression signature of TCMs and show several memory-like functions. Liver-primed T cells express the lymphoid homing molecule CD62L and respond to chemotactic signals through CCR7. Conversely, liver-primed T cells did not express the molecule CD103 that is found on nonmigratory tissue memory T cells (Jiang et al., 2012; Sheridan and Lefrançois, 2011). Similar to TCMs (Kaech and Wherry, 2007; Williams and Bevan, 2007), liver-primed T cells locate to T cell zones in secondary lymphatic organs in vivo, where they are exposed to antigen-presenting DCs. A key feature of TCMs is their ability to give rise to effector CTLs upon reactivation. Liver-primed T cells also generate recall responses and give rise to fully functional effector CTLs, which requires concomitant signaling via the TCR, CD28, and IL-12. Surprisingly, TCMs require the same combinatorial signaling to generate recall responses, which suggests that inflammation-induced DC maturation is necessary to induce the large numbers of effector CTLs from the pool of TCMs and liver-primed T cells, which is required to provide protection against infecting pathogens.

LSECs do not discriminate between foreign or autoantigens for antigen presentation to CD8 T cells; nevertheless, the memory-like differentiation state in liver-primed T cells is unlikely to cause autoimmunity. The reactivation of liver-primed T cells requires combinatorial signaling via TCR/CD28 and IL-12, which can only be delivered by professional APCs matured during infectious inflammation. Thus, the requirements for induction of immunity against autoantigens are equally high as for naive T cells. Furthermore, liver-primed T cells differ from TCMs by not responding to TCR stimulation with direct cytokine production or cytotoxic effector function. This particular feature of liver-primed T cells can be mistaken for a sign of "tolerance" in situations where the cognate antigen is recognized in the absence of costimulatory signals, but this may provide additional safety against development of autoimmunity.

As conventional T cell activation and memory markers could not discriminate TCMs from liver-primed T cells, we sought to identify a molecular marker to identify these cells in the normal T cell repertoire of wild-type mice. In a detailed biolayout analysis of the global gene expression profiles, we found Neuropilin 1 (Nrp1) as the cell surface molecule with the largest difference in gene expression that separated liver-primed T cells from TCMs also at the protein level. Murine regulatory T cells and IL-17-producing NKT cells also express Nrp1 that mediates cell-cell contact through homotypic interaction (Bruder et al., 2004). Although Nrp1 is expressed on recently activated $CD8^+$ T cells (Kaech et al., 2002a), the expression levels of CD62L allowed us to distinguish recently activated or effector CTLs ($CD62L^{low}$) from liver-primed T cells ($CD62L^{hi}$). Whether Nrp1 expression on liver-primed T cells is functionally relevant for their unique differentiation state requires further investigation, but it is known that Nrp1 increases hedgehog signaling (Hillman et al., 2011) and that hedgehog signaling mitigates activation via the TCR (Crompton et al., 2007). In normal mice, approximately 5% of antigen-experienced $CD44^+CD62L^{hi}CD8^+$ T cells express Nrp1 and fail to respond to TCR stimulation, whereas $CD44^+$ $CD62L^{hi}CD8^+$ $Nrp1^{neg}$ T cells, i.e., TCMs, produce IFN-γ. This suggests that liver-primed T cells constitute a substantial proportion of antigen-experienced T cells under physiological conditions and may serve to support immune responses against infectious microorganisms.

It is an unresolved issue how adaptive immunity is generated when systemic distribution of microbial antigens, such as surface or structural viral antigens that do not trigger innate immunity and inflammation, leads to antigen-presentation by immature DCs, which can cause cross-tolerance of pathogen-specific T cells. Here, we demonstrate that priming by LSECs can rescue naive T cells from deletion by immature DCs and also spares them from cross-tolerance upon re-encounter with antigen on immature DCs. We propose that T cell priming by LSECs in the liver protects from attrition of the T cell repertoire in situations where professional APCs fail to promote T cell survival or differentiation into memory T cells, such as systemic distribution of antigens in the absence of inflammation. This may occur during viral infection of the liver by hepatotropic viruses such as hepatitis B virus or hepatitis C virus, that cause infection in approximately 50% of the world's population (Protzer et al., 2012). In the experiments performed here, effector CTLs generated during recall responses arising from liver-primed T cells during infectious inflammation controlled bacterial and viral infection. Liver-primed T cells may thus contribute to immune control of bacteria colonizing the body surfaces, because immune responses against these bacteria are readily induced once they enter the body (Duerkop et al., 2009). Thus, T cell priming by LSECs may form a "prealert" state where conservation of T cells from deletion or tolerance preempts infections with pathogens that have not entered the body yet or have escaped innate immune sensing.

The survival of liver-primed T cells under noninflammatory conditions and their memory-T-cell-like function to give rise to effector CTLs controlling pathogen infection is evidence that organ-resident APCs, which are not derived from the bone marrow, contribute to antipathogen immunity. Generation of liver-primed T cells may precede or complement the generation of effector and memory T cells during the early phases of infection, which then allows the host to mount specific T cell immunity upon re-encounter with infectious pathogens under inflammatory conditions. The understanding of this T cell differentiation state may be harnessed therapeutically, for instance, to specifically activate liver-primed T cells to overcome chronic viral infection of the liver.

EXPERIMENTAL PROCEDURES

Cell Isolation and Coculture Experiments

Naive $CD8^+$ T cells or dendritic cells were isolated from spleen and purified by autoMACS (Miltenyi Biotec). LSECs were isolated by gradient centrifugation followed by immunomagnetic sorting (CD146) (Diehl et al., 2008). Cocultures of naive T cells with LSECs or matured DCs were conducted as described previously (von Oppen et al., 2009). Briefly, OT-I T cells were added to LSECs or matured DCs previously loaded with 100 μg/ml OVA; OT-I to antigen-presenting cell ratio 1:1. Matured DCs were obtained from spleen of mice injected with 100 μg CpG 1668 4 hr before isolation. In some experiments, neutralizing antibodies for IL-12 (anti-mouse IL-12, clone C17.8) or type I IFN signaling (anti-mouse IFNAR1, clone MAR1-5A3) were added to cocultures at 10 μg/ml. H-$2K^b$-restricted SIINFEKL-specific B3Z cells were used to detect cross-presentation in vitro by determining IL-2 release from T cells by ELISA. Animal experiments performed were approved by the local authorities in Northrhine-Westphalia.

Generation and Analysis of OT-I Memory T Cells

Low, physiological numbers (5×10^2) (Marzo et al., 2005) of $CD45.1^+$ or $CD90.1^+$ OT-I T cells were adoptively transferred into sex-matched $CD45.2^+$ $CD90.2^+$ C57BL/6 recipient mice. One day later, mice were infected with 5×10^3 colony forming units (cfu) *L.m.*-OVA by i.p. injection. The course of the OT-I T cell response identified by the respective congenic marker CD45.1/CD90.1 was followed in blood over time. Memory T cells were obtained at d45–d70 postinfection from spleen if not indicated otherwise. Memory OT-I cells were identified by expression of the respective congenic marker CD45.1 or CD90.1 in conjunction with a $CD8^+CD44^+CD127^+$ phenotype. TCMs ($CD62L^+$) and TEMs ($CD62L^-$) were distinguished by CD62L expression. For functional analysis or adoptive transfer experiments, TCMs and TEMs were isolated from spleen by enrichment with autoMACS (untouched CD8 T cell isolation kit, Miltenyi) followed by FACS sorting for their respective markers. TCMs and TEMs obtained from the same animals were used as controls.

Infection with *Listeria monocytogenes* OVA

Mice were infected i.p. with *Listeria monocytogenes* expressing OVA (*L.m.*-OVA) or with wild-type *Listeria monocytogenes* (wt *L.m.*) acquired from log phase of growth in BHI medium. cfu (5×10^3) were used for analysis of

T cell responses and to generate memory T cells; 5×10^4 cfu for analysis of bacterial load in liver.

Bioluminescence Measurement In Vivo

Measurement of bioluminescence in livers of C2J or RAG2$^{-/-}$ mice was performed each day postinfection with AdOVALUC as described previously (Stabenow et al., 2010). In brief, bioluminescence was analyzed with an IVIS 200 system (Caliper LifeSciences) 5 min. after i.p. injection of Luciferin (50 mM, Caliper LifeSciences) in PBS. Data analysis was performed with Living Image 2.50.1 software (Caliper LifeSciences).

Fluorochrome Labeling of T Cells and Immunofluorescence

T cells were labeled with 1 μM CFSE (Invitrogen) or 5 μM FarRed DDAO-SE (7-hydroxy-9H-[1,3-dichloro-9,9-dimethylacridin-2-one], succinimidyl ester; Invitrogen) for 15 min at 37°C. T cell zones were visualized by staining with fluorochrome-conjugated anti-CD3ε (500A2; BD). Sections were viewed with an Olympus IX71 with a 10× objective. Images were captured, and fluorescently labeled T cells per mm^2 T cell area were counted with Cell F software (Olympus). For quantification of labeled T cell numbers in the T cell zones within spleen and lymph nodes, 10–20 T cell zones were quantified for each animal.

T Cell Migration

Chemotaxis of CD8$^+$ T cells was quantified using transwell migration assays. Liver-primed, DC-primed, or naive OT-I cells (2×10^5 cells in 300 μl RPMI/0.5% FCS) were loaded in triplicates into 5 μm pore-size polycarbonate transwell inserts (Costar, Corning). CCL19 (100 ng/ml; Peprotech) or CCL21 (200 ng/ml; Peprotech) was added to 700 μl RPMI/0.5% FCS in the lower compartment. Control assays were performed without chemokine. Transmigrated cells were counted after 4 hr of incubation at 37°C.

Analysis of T Cell Function

In vitro, T cell stimulation was done with plate-bound αCD3 antibody (145.2C11), αCD3/CD28 microbeads (Invitrogen), or PMA (5 ng/ml; Sigma Aldrich) and ionomycin (200 ng/ml, Sigma). In some experiments, the cytokines IL-2 (5–20 ng/ml), IL-7 (5–20 ng/ml), IL-15 (5–20 ng/ml), IL-12 (0.5–5 ng/ml), IL-23 (5–20 ng/ml), TNF (5–20 ng/ml), IL-6 (5–20 ng/ml), IFN-γ (5−50 ng/ml), or IFN-α (type 4, 500–2,000 U/ml) were added to T cell stimulation assays. Restimulation of OT-I-derived liver-primed T cells or memory T cells after FACSorting was performed in coculture with CD45.2$^+$CD90.2$^+$ splenocytes as feeder cells with PMA/ionomycin or SIINFEKL peptide (0.5 μM) for 4 hr in the presence of Brefeldin A and Monensin (eBioscience).

To analyze T cell proliferation, naive CD8$^+$ T cells were labeled with 1 μM CFSE (Invitrogen) before coculture experiments or adoptive transfer. CFSE dilution was measured by flow cytometry compared to unstimulated controls. Alternatively, EdU (5-ethynyl-2′-deoxyuridine, 10 μM) was added for a 20 hr stimulation period and subsequently detected by Click-iT EdU Alexa Fluor 488 Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's protocol. Gating on EdU$^+$ cells was performed on living T cells in comparison to identical control groups stimulated in the absence of EdU. Specific cytotoxicity was determined in vitro as described (Diehl et al., 2008). LPS-free Ovalbumin (Hyglos) was used for in vivo experiments at a concentration of 500 μg/mouse. For analysis of proliferation by BrdU incorporation in vivo, mice were injected with 1.5 mg BrdU i.p. 20 hr before analysis.

Coupling of DEC205 to P3UOrv

In brief, anti-DEC205 antibody (NLDC-145) or isotype control antibody was coupled to the fusion protein of three protein G domains and ovalbumin (P3UOrv) at 4°C in PBS, as described (Kratzer et al., 2010). Antibody/fusion protein complexes (DEC205-P3UOrv) (1.3 μg) were injected i.p. into mice.

Statistical Analysis

If not indicated otherwise, the unpaired two-tailed Student's t test was used for data comparisons. Data are shown as mean ± SEM or mean ± SD with $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

For further details, please refer to Extended Experimental Procedures.

ACCESSION NUMBERS

Microarray data are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE27139.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.008.

LICENSING INFORMATION

ACKNOWLEDGMENTS

We thank the IMMEI flow cytometry core facility for technical support. We thank Marc Beyer for providing RAG2$^{-/-}$ mice. This work was supported by grants from the DFG (SFB 704, SFB 670, and SFBTR57).

Received: August 30, 2012
Revised: December 20, 2012
Accepted: February 5, 2013
Published: March 14, 2013

REFERENCES

Angelosanto, J.M., and Wherry, E.J. (2010). Transcription factor regulation of CD8+ T-cell memory and exhaustion. Immunol. Rev. *236*, 167–175.

Badovinac, V.P., Porter, B.B., and Harty, J.T. (2004). CD8+ T cell contraction is controlled by early inflammation. Nat. Immunol. *5*, 809–817.

Badovinac, V.P., Messingham, K.A., Jabbari, A., Haring, J.S., and Harty, J.T. (2005). Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nat. Med. *11*, 748–756.

Barry, M., and Bleackley, R.C. (2002). Cytotoxic T lymphocytes: all roads lead to death. Nat. Rev. Immunol. *2*, 401–409.

Bertolino, P., Heath, W.R., Hardy, C.L., Morahan, G., and Miller, J.F. (1995). Peripheral deletion of autoreactive CD8+ T cells in transgenic mice expressing H-2Kb in the liver. Eur. J. Immunol. *25*, 1932–1942.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C., and Steinman, R.M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J. Exp. Med. *196*, 1627–1638.

Bouneaud, C., Garcia, Z., Kourilsky, P., and Pannetier, C. (2005). Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo. J. Exp. Med. *201*, 579–590.

Bruder, D., Probst-Kepper, M., Westendorf, A.M., Geffers, R., Beissert, S., Loser, K., von Boehmer, H., Buer, J., and Hansen, W. (2004). Neuropilin-1: a surface marker of regulatory T cells. Eur. J. Immunol. *34*, 623–630.

Chang, J.T., Palanivel, V.R., Kinjyo, I., Schambach, F., Intlekofer, A.M., Banerjee, A., Longworth, S.A., Vinup, K.E., Mrass, P., Oliaro, J., et al. (2007). Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science *315*, 1687–1691.

Cohen, J.N., Guidi, C.J., Tewalt, E.F., Qiao, H., Rouhani, S.J., Ruddell, A., Farr, A.G., Tung, K.S., and Engelhard, V.H. (2010). Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. J. Exp. Med. *207*, 681–688.

Crompton, T., Outram, S.V., and Hager-Theodorides, A.L. (2007). Sonic hedgehog signalling in T-cell development and activation. Nat. Rev. Immunol. *7*, 726–735.

Cruz-Guilloty, F., Pipkin, M.E., Djuretic, I.M., Levanon, D., Lotem, J., Lichtenheld, M.G., Groner, Y., and Rao, A. (2009). Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. J. Exp. Med. *206*, 51–59.

Cui, W., and Kaech, S.M. (2010). Generation of effector CD8+ T cells and their conversion to memory T cells. Immunol. Rev. *236*, 151–166.

Cui, W., Joshi, N.S., Jiang, A., and Kaech, S.M. (2009). Effects of Signal 3 during CD8 T cell priming: Bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation. Vaccine *27*, 2177–2187.

Cui, W., Liu, Y., Weinstein, J.S., Craft, J., and Kaech, S.M. (2011). An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. Immunity *35*, 792–805.

Diehl, L., Schurich, A., Grochtmann, R., Hegenbarth, S., Chen, L., and Knolle, P.A. (2008). Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. Hepatology *47*, 296–305.

Duerkop, B.A., Vaishnava, S., and Hooper, L.V. (2009). Immune responses to the microbiota at the intestinal mucosal surface. Immunity *31*, 368–376.

Fletcher, A.L., Lukacs-Kornek, V., Reynoso, E.D., Pinner, S.E., Bellemare-Pelletier, A., Curry, M.S., Collier, A.R., Boyd, R.L., and Turley, S.J. (2010). Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. J. Exp. Med. *207*, 689–697.

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell *99*, 23–33.

Gardner, J.M., Devoss, J.J., Friedman, R.S., Wong, D.J., Tan, Y.X., Zhou, X., Johannes, K.P., Su, M.A., Chang, H.Y., Krummel, M.F., and Anderson, M.S. (2008). Deletional tolerance mediated by extrathymic Aire-expressing cells. Science *321*, 843–847.

Gattinoni, L., Zhong, X.S., Palmer, D.C., Ji, Y., Hinrichs, C.S., Yu, Z., Wrzesinski, C., Boni, A., Cassard, L., Garvin, L.M., et al. (2009). Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. Nat. Med. *15*, 808–813.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. *5*, R80.

Harty, J.T., and Badovinac, V.P. (2008). Shaping and reshaping CD8+ T-cell memory. Nat. Rev. Immunol. *8*, 107–119.

Hendriks, J., Gravestein, L.A., Tesselaar, K., van Lier, R.A., Schumacher, T.N., and Borst, J. (2000). CD27 is required for generation and long-term maintenance of T cell immunity. Nat. Immunol. *1*, 433–440.

Hillman, R.T., Feng, B.Y., Ni, J., Woo, W.M., Milenkovic, L., Hayden Gephart, M.G., Teruel, M.N., Oro, A.E., Chen, J.K., and Scott, M.P. (2011). Neuropilins are positive regulators of Hedgehog signal transduction. Genes Dev. *25*, 2333–2346.

Höchst, B., Schildberg, F.A., Bottcher, J., Metzger, C., Huss, S., Turler, A., Overhaus, M., Knoblich, A., Schneider, B., Pantelis, D., Kurts, C., Kalff, J.C., Knolle, P.A., and Diehl, L. (2012). Liver sinusoidal endothelial cells contribute to CD8 T cell tolerance toward circulating carcinoembryonic antigen in mice. Hepatology *56*, 1924–1933.

Huang, W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57.

Ichii, H., Sakamoto, A., Hatano, M., Okada, S., Toyama, H., Taki, S., Arima, M., Kuroda, Y., and Tokuhisa, T. (2002). Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. Nat. Immunol. *3*, 558–563.

Intlekofer, A.M., Takemoto, N., Wherry, E.J., Longworth, S.A., Northrup, J.T., Palanivel, V.R., Mullen, A.C., Gasink, C.R., Kaech, S.M., Miller, J.D., et al. (2005). Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat. Immunol. *6*, 1236–1244.

Intlekofer, A.M., Takemoto, N., Kao, C., Banerjee, A., Schambach, F., Northrop, J.K., Shen, H., Wherry, E.J., and Reiner, S.L. (2007). Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. J. Exp. Med. *204*, 2015–2021.

Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C., and Kupper, T.S. (2012). Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. Nature *483*, 227–231.

Joshi, N.S., Cui, W., Chandele, A., Lee, H.K., Urso, D.R., Hagman, J., Gapin, L., and Kaech, S.M. (2007). Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity *27*, 281–295.

Kaech, S.M., and Wherry, E.J. (2007). Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity *27*, 393–405.

Kaech, S.M., Hemby, S., Kersh, E., and Ahmed, R. (2002a). Molecular and functional profiling of memory CD8 T cell differentiation. Cell *111*, 837–851.

Kaech, S.M., Wherry, E.J., and Ahmed, R. (2002b). Effector and memory T-cell differentiation: implications for vaccine development. Nat. Rev. Immunol. *2*, 251–262.

Kalia, V., Sarkar, S., Subramaniam, S., Haining, W.N., Smith, K.A., and Ahmed, R. (2010). Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. Immunity *32*, 91–103.

Kallies, A., Xin, A., Belz, G.T., and Nutt, S.L. (2009). Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. Immunity *31*, 283–295.

Kratzer, R., Mauvais, F.X., Burgevin, A., Barilleau, E., and van Endert, P. (2010). Fusion proteins for versatile antigen targeting to cell surface receptors reveal differential capacity to prime immune responses. J. Immunol. *184*, 6855–6864.

Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F., and Heath, W.R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J. Exp. Med. *186*, 239–245.

Kurts, C., Robinson, B.W., and Knolle, P.A. (2010). Cross-priming in health and disease. Nat. Rev. Immunol. *10*, 403–414.

Limmer, A., Ohl, J., Kurts, C., Ljunggren, H.G., Reiss, Y., Groettrup, M., Momburg, F., Arnold, B., and Knolle, P.A. (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.

Lohse, A.W., Knolle, P.A., Bilo, K., Uhrig, A., Waldmann, C., Ibe, M., Schmitt, E., Gerken, G., and Meyer Zum Büschenfelde, K.H. (1996). Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. Gastroenterology *110*, 1175–1181.

Marzo, A.L., Klonowski, K.D., Le Bon, A., Borrow, P., Tough, D.F., and Lefrançois, L. (2005). Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat. Immunol. *6*, 793–799.

Mescher, M.F., Curtsinger, J.M., Agarwal, P., Casey, K.A., Gerner, M., Hammerbeck, C.D., Popescu, F., and Xiao, Z. (2006). Signals required for programming effector and memory development by CD8+ T cells. Immunol. Rev. *211*, 81–92.

Mueller, D.L. (2010). Mechanisms maintaining peripheral tolerance. Nat. Immunol. *11*, 21–27.

Parish, I.A., Rao, S., Smyth, G.K., Juelich, T., Denyer, G.S., Davey, G.M., Strasser, A., and Heath, W.R. (2009). The molecular signature of CD8+ T cells undergoing deletional tolerance. Blood *113*, 4575–4585.

Pipkin, M.E., Sacks, J.A., Cruz-Guilloty, F., Lichtenheld, M.G., Bevan, M.J., and Rao, A. (2010). Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. Immunity *32*, 79–90.

Protzer, U., Maini, M.K., and Knolle, P.A. (2012). Living in the liver: hepatic infections. Nat. Rev. Immunol. *12*, 201–213.

Quigley, M., Pereyra, F., Nilsson, B., Porichis, F., Fonseca, C., Eichbaum, Q., Julg, B., Jesneck, J.L., Brosnahan, K., Imam, S., et al. (2010). Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. Nat. Med. *16*, 1147–1151.

Rao, R.R., Li, Q., Odunsi, K., and Shrikant, P.A. (2010). The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. Immunity *32*, 67–78.

Redmond, W.L., and Sherman, L.A. (2005). Peripheral tolerance of CD8 T lymphocytes. Immunity *22*, 275–284.

Redmond, W.L., Marincek, B.C., and Sherman, L.A. (2005). Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance in vivo. J. Immunol. *174*, 2046–2053.

Rutishauser, R.L., and Kaech, S.M. (2010). Generating diversity: transcriptional regulation of effector and memory CD8 T-cell differentiation. Immunol. Rev. *235*, 219–233.

Rutishauser, R.L., Martins, G.A., Kalachikov, S., Chandele, A., Parish, I.A., Meffre, E., Jacob, J., Calame, K., and Kaech, S.M. (2009). Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. Immunity *31*, 296–308.

Sarkar, S., Kalia, V., Haining, W.N., Konieczny, B.T., Subramaniam, S., and Ahmed, R. (2008). Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. J. Exp. Med. *205*, 625–640.

Sarris, M., Andersen, K.G., Randow, F., Mayr, L., and Betz, A.G. (2008). Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. Immunity *28*, 402–413.

Schietinger, A., Delrow, J.J., Basom, R.S., Blattman, J.N., and Greenberg, P.D. (2012). Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. Science *335*, 723–727.

Schurich, A., Berg, M., Stabenow, D., Böttcher, J., Kern, M., Schild, H.J., Kurts, C., Schuette, V., Burgdorf, S., Diehl, L., et al. (2010). Dynamic regulation of CD8 T cell tolerance induction by liver sinusoidal endothelial cells. J. Immunol. *184*, 4107–4114.

Schwartz, R.H. (2003). T cell anergy. Annu. Rev. Immunol. *21*, 305–334.

Sheridan, B.S., and Lefrançois, L. (2011). Regional and mucosal memory T cells. Nat. Immunol. *12*, 485–491.

Shin, H., Blackburn, S.D., Intlekofer, A.M., Kao, C., Angelosanto, J.M., Reiner, S.L., and Wherry, E.J. (2009). A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. Immunity *31*, 309–320.

Stabenow, D., Frings, M., Trück, C., Gärtner, K., Förster, I., Kurts, C., Tüting, T., Odenthal, M., Dienes, H.P., Cederbrant, K., et al. (2010). Bioluminescence imaging allows measuring CD8 T cell function in the liver. Hepatology *51*, 1430–1437.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C. (2003). Tolerogenic dendritic cells. Annu. Rev. Immunol. *21*, 685–711.

Takada, K., and Jameson, S.C. (2009). Naive T cell homeostasis: from awareness of space to a sense of place. Nat. Rev. Immunol. *9*, 823–832.

Takemoto, N., Intlekofer, A.M., Northrup, J.T., Wherry, E.J., and Reiner, S.L. (2006). Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. J. Immunol. *177*, 7515–7519.

Theocharidis, A., van Dongen, S., Enright, A.J., and Freeman, T.C. (2009). Network visualization and analysis of gene expression data using BioLayout Express(3D). Nat. Protoc. *4*, 1535–1550.

von Oppen, N., Schurich, A., Hegenbarth, S., Stabenow, D., Tolba, R., Weiskirchen, R., Geerts, A., Kolanus, W., Knolle, P., and Diehl, L. (2009). Systemic antigen cross-presented by liver sinusoidal endothelial cells induces liver-specific CD8 T-cell retention and tolerization. Hepatology *49*, 1664–1672.

Wakim, L.M., and Bevan, M.J. (2010). From the thymus to longevity in the periphery. Curr. Opin. Immunol. *22*, 274–278.

Wherry, E.J., Teichgräber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H., and Ahmed, R. (2003). Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. *4*, 225–234.

Wherry, E.J., Ha, S.J., Kaech, S.M., Haining, W.N., Sarkar, S., Kalia, V., Subramaniam, S., Blattman, J.N., Barber, D.L., and Ahmed, R. (2007). Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity *27*, 670–684.

Williams, M.A., and Bevan, M.J. (2007). Effector and memory CTL differentiation. Annu. Rev. Immunol. *25*, 171–192.

Wirth, T.C., Xue, H.H., Rai, D., Sabel, J.T., Bair, T., Harty, J.T., and Badovinac, V.P. (2010). Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. Immunity *33*, 128–140.

Xiao, Z., Mescher, M.F., and Jameson, S.C. (2007). Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. J. Exp. Med. *204*, 2667–2677.

Youngblood, B., Oestreich, K.J., Ha, S.J., Duraiswamy, J., Akondy, R.S., West, E.E., Wei, Z., Lu, P., Austin, J.W., Riley, J.L., et al. (2011). Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8(+) T cells. Immunity *35*, 400–412.

Zhang, N., and Bevan, M.J. (2011). CD8(+) T cells: foot soldiers of the immune system. Immunity *35*, 161–168.

Zhou, X., Yu, S., Zhao, D.M., Harty, J.T., Badovinac, V.P., and Xue, H.H. (2010). Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. Immunity *33*, 229–240.